Calcineurin: Form and Function

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I. Introduction 1483
II. A Brief History and Overview of Calcineurin 1484
A. Calcineurin: the early years 1484
B. Calcineurin properties 1484
III. Physiological Roles for Calcineurin 1490
A. Lower eukaryotes 1490
B. Higher eukaryotes 1494
C. Inhibitors of calcineurin 1497
IV. Calcineurin Structure 1500
A. A dinuclear metal-binding phosphoesterase motif 1500
B. Three-dimensional structure 1500
C. Active site architecture 1502
D. Metal ion requirements 1503
V. Enzymatic Mechanism 1504
A. Mechanism of phosphoryl group transfer: evidence for direct transfer to water 1504
B. Catalytic role of the dinuclear metal center 1505
C. Conserved active site residues 1505
D. A model for the calcineurin catalytic mechanism 1508
VI. Regulation 1510

Rusnak, Frank, and Pamela Mertz. Calcineurin: Form and Function. Physiol Rev 80: 1483–1521, 2000.—Calcineurin is a eukaryotic Ca\textsuperscript{2+} and calmodulin-dependent serine/threonine protein phosphatase. It is a heterodimeric protein consisting of a catalytic subunit calcineurin A, which contains an active site dinuclear metal center, and a tightly associated, myristoylated, Ca\textsuperscript{2+}-binding subunit, calcineurin B. The primary sequence of both subunits and heterodimeric quaternary structure is highly conserved from yeast to mammals. As a serine/threonine protein phosphatase, calcineurin participates in a number of cellular processes and Ca\textsuperscript{2+}-dependent signal transduction pathways. Calcineurin is potently inhibited by immunosuppressant drugs, cyclosporin A and FK506, in the presence of their respective cytoplasmic immunophilin proteins, cyclophilin and FK506-binding protein. Many studies have used these immunosuppressant drugs and/or modern genetic techniques to disrupt calcineurin in model organisms such as yeast, filamentous fungi, plants, vertebrates, and mammals to explore its biological function. Recent advances regarding calcineurin structure include the determination of its three-dimensional structure. In addition, biochemical and spectroscopic studies are beginning to unravel aspects of the mechanism of phosphate ester hydrolysis including the importance of the dinuclear metal ion cofactor and metal ion redox chemistry, studies which may lead to new calcineurin inhibitors. This review provides a comprehensive examination of the biological roles of calcineurin and reviews aspects related to its structure and catalytic mechanism.

I. INTRODUCTION

The year 1999 marked the 20th anniversary of the isolation of the Ca\textsuperscript{2+}- and calmodulin-dependent protein serine/threonine phosphatase calcineurin (206). During the past 20 years, the biological roles of calcineurin have progressed from a putative inhibitor of the calmodulin-dependent phosphodiesterase (444) to the ground-breaking discovery that it is the target of the immunosuppressant drugs cyclosporin A (CsA) and FK506, pharmacological reagents that have been used to demonstrate it as a major player in Ca\textsuperscript{2+}-dependent eukaryotic signal transduction pathways (238). In recent years, several milestones regarding calcineurin structure have been achieved including the
Calcineurin, Ca²⁺
Calcineurin and NF-AT family of transcription factors 173, 338–340
Role of calcineurin in brain ischemia and injury 285
Neural roles for immunosuppressive drugs, immunophilins, control of adenylyl cyclase 13, 369
General aspects of calcineurin 60, 61, 130, 145, 167, 202–205, 207, 208, 316, 371

TABLE 1. Calcineurin reviews listed according to subject

<table>
<thead>
<tr>
<th>Review Topic</th>
<th>Reference No.</th>
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<tbody>
<tr>
<td>General aspects of calcineurin</td>
<td>60, 61, 130, 145, 167, 202–205, 207, 208, 316, 371</td>
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<tr>
<td>Control of adenylyl cyclase</td>
<td>13, 369</td>
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<td>Role of calcineurin in brain ischemia and injury</td>
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<td>Calcineurin and NF-AT family of transcription factors</td>
<td>173, 338–340</td>
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<tr>
<td>Calcineurin, Ca²⁺ signaling, and the cell cycle</td>
<td>266, 276, 357, 358, 405</td>
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<td>Calcineurin in yeast and other lower eukaryotic organisms</td>
<td>47, 68, 71, 141–143, 303, 366, 387</td>
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<td>Calcineurin, T lymphocyte activation, and the mechanism of action of immunosuppressive agents</td>
<td>30, 46, 55, 133, 134, 155, 170, 189, 192, 222, 236, 237, 249, 255, 274, 311, 361, 363, 370, 378, 412, 413, 439, 455, 458, 472</td>
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<td>Structure of calcineurin and related phosphatases</td>
<td>22–24, 199, 215, 223, 244, 307, 351, 392, 411, 428, 430, 432, 433, 457</td>
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<td>Calcineurin in the mammalian nephron</td>
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<td>Calcineurin localization via AKAP</td>
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<tr>
<td>Calcineurin and ion channel regulation</td>
<td>404, 465</td>
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<td>Calcineurin and apoptosis</td>
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<tr>
<td>Calcineurin regulation of dystrophin</td>
<td>275</td>
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<tr>
<td>Calcineurin and protein phosphatases in plants</td>
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<tr>
<td>Role of calcineurin in hypertension</td>
<td>356</td>
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<tr>
<td>Regulation of microtubules by calcineurin: tau phosphorylation</td>
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NF-AT, nuclear factor of activated T cells; AKAP, protein kinase A anchoring protein.
Cloning efforts have provided evidence that all eukaryotic organisms possess one or more genes for each subunit; Table 3 is a compilation of known calcineurin A and calcineurin B gene sequences to date. Genes for calcineurin A and B subunits have been identified in yeast, filamentous fungi, protozoa, insects, and mammals. The $\alpha\beta$-quaternary structure of calcineurin observed in mammals is conserved in lower eukaryotic organisms. These subunits are tightly associated and can only be dissociated by use of denaturants (271).

1. Calcineurin A

A) CLASSIFICATION. In addition to calcineurin, the serine/threonine protein phosphatase family members include protein phosphatases 1 (PP1), 2A (PP2A), and 2C (PP2C), phosphatases essential for a number of signal transduction pathways in eukaryotic cells (61, 371). The original classification of this family was proposed by Ingebritsen and Cohen (167), separating almost all the serine/threonine phosphatase activity in mammalian tissue extracts into two classes (60, 61, 167, 371). Type 1 protein phosphatases were found to dephosphorylate the $\beta$-subunit of phosphorylase kinase, whereas type 2 protein phosphatases dephosphorylate the $\alpha$-subunit of phosphorylase kinase. Differences between the two types are also found with inhibitors; type 1 is inhibited by phosphopeptide inhibitors 1 and 2, whereas the type 2 class is not affected by these inhibitors.

A difference in divalent metal ion dependence led to the resolution of the type 2 enzymes into PP2A, PP2B (calcineurin), and PP2C (60). PP2A was originally described as having no requirement for divalent metal ion, calcineurin is regulated by Ca$^{2+}$/calmodulin, and PP2C is Mg$^{2+}$ dependent.$^1$ A more detailed discussion regarding the importance of metal ion cofactors of these phosphatases is described in section ivD. Differences among type 2 members are also found with regard to sensitivity to inhibition by macrolide inhibitors (see sect. ivC). PP2A and PP1 are inhibited by okadaic acid, whereas calcineurin is specifically inhibited by the immunosuppressant drugs CsA and FK506, in the presence of cyclophilin and FK506-binding protein (FKBP), respectively (238, 361).

Although different in metal ion dependence, sensitivity to inhibitors, and substrate specificity, PP1, PP2A, and calcineurin have homologous amino acid sequences and are evolutionarily related (26, 61, 371). In fact, the PP1/PP2A/calcineurin superfamily ranks among one of the most highly conserved enzyme families encountered (61). Within the active site domain, PP1 shares 49% amino acid identity with PP2A and 39% identity with calcineurin. Recent work has found homologs of this family in cyanobacteria (373, 473) and the archa (229, 251, 386). With the determination of the amino acid sequences of these phosphatases, it was found that the original classification of PP2C along side PP1, PP2A, and calcineurin does not hold at the primary sequence level. Thus PP2C does not share any homology with PP1/2A/calcineurin and is considered to be in a separate superfamily (33).

B) DOMAIN STRUCTURE. The active site of calcineurin is located on the A subunit which, in mammals, is 57–59 kDa depending on the isoform. The size of the catalytic subunit can be up to $\sim$20% larger in lower eukaryotic species [e.g., Saccharomyces cerevisiae, 63 and 69 kDa (72, 242, 467); Schizosaccharomyces pombe, 64 kDa (327, 468); Drosophila melanogaster, 62 and 65 kDa (38, 132, 158); Cryptococcus neoforms and Dictyostelium discoideum, 71 kDa (75, 310)]. Nevertheless, there is strict conservation throughout all eukaryotic organisms such that all calcineurin A genes encode for a polypeptide consisting of a catalytic domain homologous to other serine/threonine protein phosphatases and three regulatory domains at the COOH terminus that distinguish calcineurin from other family members (Fig. 1). These domains have been identified as the calcineurin B binding

\[\text{TABLE 2. Physical properties of calcineurin}\]

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<td>57–71 kDa (lower eukaryotes)</td>
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<td>Myristoylation of calcineurin B</td>
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$^1$ Although PP2A was originally characterized as having no divalent metal ion dependence (60), more recently it has also been found to be stabilized or reactivated by divalent metal ions (43).
### Table 3. A comprehensive list of published calcineurin A and calcineurin B gene sequences

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domain (56, 132, 164, 379, 451), the calmodulin-binding domain (132, 191), and the "autoinhibitory" domain (137, 326), which binds in the active site cleft in the absence of Ca$^{2+}$/calmodulin (197) and inhibits the enzyme, acting in concert with the calmodulin binding domain to confer calmodulin regulation.

These domains have been identified through standard biochemical mapping procedures including primary sequence comparisons, partial proteolysis experiments, peptide interaction studies, site-directed mutagenesis studies, and cross-linking studies. The X-ray structures of calcineurin (see sect. ivB) confirm the identification of residues involved in these regulatory domains and also indicate that the autoinhibitory domain forms an α-helix that binds to the substrate-binding cleft of the enzyme (197). It has been shown that when Ca$^{2+}$/calmodulin binds to the enzyme, inhibition ceases and likely involves a conformational change that exposes the active site. Interestingly, Perrino (324) has provided recent evidence for additional autoinhibitory elements within residues that are situated between the calmodulin-binding and autoinhibitory domains noted in Figure 1.

The NH$_2$ and COOH termini are highly variable between species as well as between calcineurin A genes within the same organism (130, 188, 208). Particularly striking are unusual amino acid compositions and sequences of particular isoforms. For example, the mammalian calcineurin Aβ-isoform contains the sequence MAPEPARRAPPCLPPPPPPPPGAD... at the NH$_2$ terminus (117, 129, 220). The COOH terminus of the canA gene product from Dictyostelium contains a fourfold repeat of the sequence RXNSX(G/A)(E/D)LX as well as the repetitive sequence .TTNNINPSITTENNNSNEQLOQQQQQQQQQQQPPPSTTTTSTTT... and, along with the NH$_2$ terminus, is enriched in glutamine and asparagine (75). The COOH terminus of calcineurin A from C. neoformans is highly enriched in proline, serine, threonine, and glycine (310). The function of these variable domains is unknown, but they may play a role in substrate recognition and/or localization.

C) Calcineurin Phosphorylation. Purified calcineurin from bovine brain contains up to 0.6 equivalents of phosphate (195), suggesting that it may be phosphorylated in vivo. Calcineurin can be phosphorylated by protein kinase C (138, 420), casein kinase I (382), and casein kinase II (136, 451), and casein kinase II (138, 451). Calcineurin can also be phosphorylated by protein kinase C (138, 420), casein kinase I (382), and casein kinase II (136, 451). The site of phosphorylation by casein kinase II has been determined to be the serine in the sequence -RVFS(p)VLR- near the COOH terminus of the calmodulin-binding domain (Fig. 1) (138, 257). Although phosphorylation could be blocked by Ca$^{2+}$/calmodulin, the kinetic properties of the phosphorylated and dephosphorylated forms are similar (136, 138). Furthermore, phosphorylated calcineurin still binds and is activated by calmodulin. Whether phosphorylation represents a means of regulating calcineurin activity in vivo remains to be demonstrated.

2. Calcineurin B

A) Sequence Diversity and Isoforms. The calcineurin B subunit is also highly conserved throughout evolution, with mammalian calcineurin B showing 86% amino acid sequence identity with insect calcineurin B (i.e., Drosophila) and 54% identity with calcineurin B from S. cerevisiae (Fig. 2). This high degree of conservation allows functional interchange of calcineurin B subunits between mammalian and N. crassa catalytic subunits (423). The gene for mammalian calcineurin B encodes a protein of 170 amino acids containing four Ca$^{2+}$-binding EF-hand motifs (Fig. 2) (2).
In mammals, there are two calcineurin B genes, one which is ubiquitously expressed, while mRNA for the second gene is found only in testes (48, 289, 424).

B) NH$_2$-TERMINAL MYRISTOYLASTION. The mature calcineurin B protein is missing the initiator methionine, and the new α-amino group of glycine at position 2 is acylated with myristic acid (1). This modification has been conserved throughout evolution from yeast to mammals, suggesting a crucial physiological role (71). To explore possible biological roles for calcineurin myristoylation, Heitman and colleagues (483) generated a mutant of calcineurin B in which glycine at position 2 was mutated to alanine, thereby preventing myristoylation. Surprisingly, expression of the wild-type and mutant proteins in $S$. cerevisiae demonstrated that myristoylation was not required for membrane association nor for interaction with immunosuppressant drug complexes. Indeed, the nonmyristoylated protein exhibited full biological function. These results were subsequently confirmed in biochemical experiments with purified myristoylated and nonmyristoylated calcineurin heterodimer which showed equivalent enzymatic activities, inhibition by the CsA/cyclophilin immunosuppressant drug complex, and interactions with a synthetic phospholipid monolayer (182). Interestingly, the myristoylated protein exhibited substantial thermal stability (~12°C) relative to the nonmyristoylated protein (182). At present, it is unknown whether the biological role of calcineurin B myristoylation is to impart increased stability to the protein or whether there is another role yet to be identified.

C) CALCIUM BINDING PROPERTIES. Klee et al. (204) were the first to discover that calcineurin binds Ca$^{2+}$. With the use of flow dialysis, it was demonstrated that four Ca$^{2+}$ bind with high affinity [dissociation constant ($K_d$) <10$^{-6}$ M] and that the Ca$^{2+}$-binding sites were localized to the calcineurin B subunit. The complete primary sequence determination of calcineurin B revealed homology with calmodulin (35% identity) and troponin C (29% identity) (2), most of which was confined to four Ca$^{2+}$-binding “EF-hand” motifs. More detailed thermodynamic aspects of Ca$^{2+}$ binding became possible when the recombinant calcineurin B subunit was obtained via heterologous expression in $Escherichia coli$. Using the purified recombinant protein, Burroughs et al. (40) studied the metal binding properties using Eu$^{3+}$ and Tb$^{3+}$ luminescence spectroscopy. Four Eu$^{3+}$-binding sites were revealed, two
with relatively low affinity ($K_d$ values of $1 \pm 0.2$ and $1.6 \pm 0.5$ μM) and two with relatively high affinity ($K_d$ values of $0.14 \pm 0.002$ and $0.20 \pm 0.010$ μM). $Tb^{3+}$ also bound but with slightly weaker affinities ($K_d$ values of $0.04 \pm 0.01$ and $0.17 \pm 0.02$ μM for the COOH-terminal sites and 1–3 μM for the NH$_2$-terminal sites). Direct Ca$^{2+}$ binding to calcineurin B has also been studied by flow dialysis, which found one high-affinity ($K_d = 0.024$ μM) and three lower affinity sites ($K_d = 15$ μM) (176). The NMR-active isotope $^{113}\text{Cd}$ has been used as a Ca$^{2+}$ surrogate to identify four similar but distinct metal binding sites consisting of all-oxygen coordination of pentagonal bipyramidal geometry as expected for an EF-hand Ca$^{2+}$-binding site (176), later confirmed in the X-ray structure.

**FIG. 2.** Multiple sequence alignment of calcineurin B sequences from diverse organisms. Calcineurin B sequences of *Saccharomyces cerevisiae* (221), *Neurospora crassa* (213), rat brain (48), rat testes (289), human (131), bovine (2, 304), *Drosophila melanogaster* (132, 450), and *Naegleria gruberi* (346) were aligned using the multiple sequence alignment editor of the Wisconsin Package version 9.0, Genetics Computer Group (Madison, WI). The four Ca$^{2+}$-binding EF-hand motifs are indicated. The residues that participate in Ca$^{2+}$ coordination are noted by an asterisk. The consensus sequence is defined in which a residue is conserved in all 8 sequences.
(124, 197). Ca\(^{2+}\) binding to individual sites of calcineurin B has been studied using point mutants of this subunit altered in each of the four EF-hands (104). This study confirmed the higher Ca\(^{2+}\) affinity for COOH-terminal EF-hand sites and also found that Ca\(^{2+}\) binding at these sites is likely to be structural.

**D) CALCINEURIN B HOMOLOGS.** EF-hand proteins have been classified into 39 distinct subfamilies containing anywhere from two to eight EF-hand domains (178). Calcineurin B proteins represent one subfamily of EF-hand proteins based on sequence alignments and congruence of domain and interdomain regions (302). In recent years, a number of Ca\(^{2+}\)-binding proteins containing EF-hand domains have been identified from cloning studies and shown to be homologous to calcineurin B. These include NCS-1, a neuronal calcium sensor that inhibits rhodopsin phosphorylation in a Ca\(^{2+}\)-dependent fashion (81), modulates calmodulin targets (359), and may regulate exocytosis (264); a protein p22/CHP (for calcineurin homologous protein) that is required for constitutive membrane traffic (25) and inhibits serum and GTPase stimulation of the Na\(^{+}/H^+\) exchanger NHE1 (234); CIB (for calcium- and integrin-binding protein), a 22-kDa Ca\(^{2+}\)-binding protein that interacts with the cytoplasmic tail of the integrin \(\alpha_{IIb}\) portion of the GPIIb/IIIa fibrinogen receptor (297); and a number of Ca\(^{2+}\)-binding proteins containing EF-hand domains have been identified from cloning studies and shown to interact with the cytoplasmic tail of the integrin \(\alpha_{IIb}\) portion of the GPIIb/IIIa fibrinogen receptor (297). A protein product of the SOS3 gene of Arabidopsis thaliana involved in tolerance to the ionic component of salt stress in plants (239). Homology to calcineurin B ranges from 27 to 31% for NCS-1, CIB, and SOS3 and to up to 43% for p22/CHP. Like calcineurin B, p22/CHP is myristoylated while NCS-1, CIB, and SOS3 contain the requisite consensus sequences for myristoylation. The fact that a constitutively active form of yeast calcineurin in transgenic tobacco plants resulted in increased salt tolerance provides evidence for a possible functional overlap between SOS3 and calcineurin B (320). On the contrary, biochemical studies with NCS-1 suggested that it could replace calmodulin rather than calcineurin B in activating calcineurin and other calmodulin-dependent enzymes (359). Furthermore, although p22/CHP is completely congruent with the calcineurin B family of proteins, arguments have been forwarded that it is more appropriate to place it in a separate subfamily (25). Undoubtedly, further studies are required to determine whether any or all of these proteins can function in a fashion comparable to calcineurin B.

**III. PHYSIOLOGICAL ROLES FOR CALCINEURIN**

**A. Lower Eukaryotes**

Genetic methods for the selective deletion of one or both calcineurin subunits to assess biological function by noting the phenotype of the mutant strain are now possible in several eukaryotic organisms. In addition, the immunosuppressant drugs CsA and FK506, as specific calcineurin inhibitors, have provided complementary tools for discerning the role of calcineurin in many eukaryotic organisms (71, 142, 303, 387). Some of the most thorough work investigating biological roles for calcineurin have used the yeast *S. cerevisiae* as a model system. There are two genes for the catalytic subunit of calcineurin in *S. cerevisiae* (CNA1/CMP1 and CNA2/CMP2) and only one gene for the B subunit (CNB1). Calcineurin is essential in CsA- and FK506-sensitive yeast strains (36). Recent work has begun to explore the role of calcineurin in slightly more complex organisms such as *N. crassa* (153, 213, 335) and *D. discoideum* (75, 159, 252). Furthermore, calcineurin has either been isolated or detected from the human pathogens *C. neoformans* (310), *Leishmania* species (21, 342), the malarial parasite *Plasmodium falciparum* (87), helminth parasites (347), and schistosomes (186). In some of these, growth can be inhibited by the immunosuppressive agents FK506 and its analogs as well as CsA (16, 87, 186, 309, 342), thus raising the possibility that novel calcineurin inhibitors might be developed as specific antifungal and antiparasitic agents. The following sections detail what has been learned regarding physiological roles for calcineurin in lower eukaryotic organisms and is summarized in Table 4.

1. *Saccharomyces cerevisiae*

A) RECOVERY FROM PHEROMONE-INDUCED GROWTH ARREST. Haploid cells of *S. cerevisiae* produce one of two mating

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**TABLE 4. Physiological roles of calcineurin in lower eukaryotic organisms**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Function</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Recovery from mating factor (\alpha)-induced growth arrest, cation (e.g., Li(^{+}), Na(^{+}), Mn(^{2+})) resistance, Ca(^{2+})-homeostasis, Ca(^{2+})-mediated G(_2) arrest, onset of mitosis</td>
<td>71, 72, 279, 461, 467</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>Cytokinesis, mating, nuclear and spindle pole body positioning, polarized growth, proper septation, chloride homeostasis</td>
<td>327, 399, 468</td>
</tr>
<tr>
<td><em>Dictyostelium discoideum</em></td>
<td>Differentiation, stalk cell/spore formation</td>
<td>150</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>Hyphal growth/condiation, maintenance of apical Ca(^{2+}) gradient, proper septation</td>
<td>213, 335</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Virulence, pH and CO(_2) homeostasis, temperature-sensitive growth, resistance to Li(^{+})</td>
<td>310</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>Cell cycle progression through G(_1)/S, nuclear division, polarized growth, proper septation</td>
<td>303, 343</td>
</tr>
<tr>
<td><em>Paramecium tetraurelia</em></td>
<td>Exocytosis</td>
<td>200, 282</td>
</tr>
</tbody>
</table>
pheromones, α-factor and α-factor. Exposure of haploid strains to the opposite mating pheromone prepares cells for mating by inducing cell cycle arrest in G1. This is mediated by an elaborate signal transduction pathway involving a rise in intracellular Ca2+ and activation of calcineurin (47, 142). Growth arrest can be observed as a zone of clearing surrounding a source of α-factor on a lawn of cells and occurs within 24 h at 30°C. Escape from α-factor-induced cell cycle arrest involves three metabolic processes that have been referred to as recovery, adaptation, and survival (287). Recovery is defined as the ability of cells to resume growth after removal of the pheromone, whereas adaptation is a process in which cells eventually resume growth in the continuous presence of pheromone. Both recovery and adaptation may involve common signaling components and can be observed by a shrinking of the zone of clearing and increasing turbidity within it, usually within ~24 h at 30°C. Survival differs from recovery and adaptation in that it describes whether a cell remains viable after exposure to pheromone.

Coincident with the cloning of genes for the two yeast calcineurin A subunits (CNA1/CMP1 and CNA2/CMP2, Table 3), strains deficient in either subunit were viable but failed to recover from α-factor-induced growth arrest (72, 73, 242). Mata strains containing a single CNA1 or CNA2 mutation were twice as sensitive as wild type to α-factor-induced growth arrest, whereas the double mutant CNA1/CNA2 was four times as sensitive, as assessed by the size of the halo after 24 h at 30°C (72, 73). Furthermore, once arrested, the double mutant failed to resume growth. In contrast, the CNB1 mutant did not show an increased sensitivity compared with wild type, but like the CNA1/CNA2 double mutant, it failed to recover from growth arrest. In wild-type cells, the immunosuppressant drugs CsA and FK506 also inhibited recovery from α-factor-mediated growth arrest, and these required the presence of their respective immunophilins cyclophilin and FKBP (107). In addition, expression of the CNA1/CMP1 gene increased in the presence of α-factor, the result of 5′-noncoding sequences in the CNA1/CMP1 gene matching closely the consensus sequence for the α-factor element (467).

As expected, the activator protein of calcineurin, calmodulin, has also been shown to be required for escape from cell cycle arrest after exposure to pheromone (287). Calmodulin mutants did not display increased sensitivity to α-factor, nor did these mutant strains appear to be affected in either recovery or adaptation. Indeed, both calcineurin and calmodulin mutants adapted as well as a wild-type strain to low concentrations of pheromone, and both mutants recovered after pheromone removal with the same kinetics as the wild-type strain. The process that appeared to be affected was survival, a result consistent with previous work indicating that Ca2+ is also essential for survival after exposure to α-factor (166). Interestingly, in addition to calcineurin, the Ca2+, calmodulin-dependent protein kinases (CMK1 and CMK2), yeast protein kinase C (PKC1), and a mitogen-activated protein (MAP) kinase (MPK1) are also required for recovery from growth arrest, thus indicating that enzymes of opposing function are required for surviving exposure to α-factor (287, 301, 461).

One downstream signaling component in S. cerevisiae regulated by calcineurin is the yeast transcription factor Crz1p/Tcn1p. Crz1p/Tcn1p is required for calcineurin-dependent induction of genes for the vacular and secretory Ca2+ pumps, Pmc1p and Pmr1p, respectively; one of two genes encoding β-1,3 glucan synthase, FKS2; and the gene for the plasma membrane Na+ pump, PMR2 (Fig. 3) (263, 388). In addition, calcineurin has been shown to regulate the high/low-affinity state of the plasma membrane K+ channel, Trk1p (269), and inhibit the vacuolar H+/Ca2+ exchanger Vcx1p (69) by posttranslational mechanisms. Some of these are presented below in more detail.

B) ADAPTATION TO SALT STRESS. The search for additional phenotypes found that calcineurin-deficient yeast exhibited decreased tolerance to the monovalent cations Na+ and Li+, but not K+, Ca2+, and Mg2+ (269, 300). The role of calcineurin in Na+ /Li+ tolerance is thought to be mediated by transcriptional and posttranslational mechanisms. Adaptation to high salt stress requires the presence of a plasma membrane Na+ -ATPase involved in Na+ and Li+ efflux, Pmr2p. Cells deficient in calcineurin accumulate Na+ and Li+ due to decreased expression of Pmr2p (269). Although no changes in intracellular Ca2+ have been observed after induction of the high-salt response, evidence indicates that Ca2+ mediates this response. Ca2+, via calmodulin activation of calcineurin, regulates adaptation to high salt stress by induced expression of Pmr2p (76, 154, 268), mediated by the transcription factor Crz1p/Tcn1p (Fig. 3) (263, 388). The activity of Pmr2p is also stimulated by Ca2+/calmodulin, thereby providing both transcriptional and posttranslational regulation of Na+ efflux mediated by Ca2+ (349, 454).

Cells deficient in CNB1 are unable to convert the K+ transport system (Trk1p, a K+ channel) to a high-affinity state. In the high-affinity state, this pump has increased affinity for K+, but the Michaelis constant (Km) for Na+ or Li+ is unaffected, thereby resulting in increased Na+ uptake in calcineurin-deficient cells. The mechanism of this regulation has been hypothesized to be direct or indirect dephosphorylation of Trk1p by calcineurin (269).

Other proteins in addition to calcineurin are required for salt tolerance such as the gene products of PDE1, a low-affinity cAMP-dependent phosphodiesterase (154); URE2, a regulator of nitrogen catabolite repression (462); PMA1, the plasma membrane H+ -ATPase (462); HAL3, a protein involved in cell cycle control and ion homeostasis.
STD1, a protein that interacts with the SNF1 protein kinase in two-hybrid and in vitro binding studies (113). Thus multiple parallel pathways are necessary for full induction of this response.

C) CALCIUM HOMEOSTASIS. Calcineurin is involved in the regulation of Ca$^{2+}$ pumps and exchangers responsible for Ca$^{2+}$ homeostasis in yeast (Fig. 3). These maintain cytoplasmic [Ca$^{2+}$] in the range of 100–300 nM (68, 78). In addition, other ion transporters indirectly influence intracellular [Ca$^{2+}$]. One of these is the vacuolar H$^+$/Ca$^{2+}$ exchanger (Vcx1p), which provides the driving force for Ca$^{2+}$ sequestration by the Ca$^{2+}$/H$^+$ exchanger encoded for by the VCX1 gene (114, 144, 408). Two Ca$^{2+}$-ATPases, Pmc1p and Pmr1p, are responsible for depleting the cytosol of Ca$^{2+}$. The former is localized to the vacuole (70), while the latter is important in the secretory pathway and localizes to the Golgi (349). Mutants deleted in either Pmc1p or Pmr1p cannot grow in media containing high Ca$^{2+}$. Deletion of the gene for either calcineurin subunit, or treatment of cells with CsA or FK506, restores growth to either single PMC1 or double PMC1/PMR1 mutants in high Ca$^{2+}$ media (70), indicating that calcineurin activation can have a negative effect on growth. As noted above, activation of calcineurin leads to transcriptional induction of the PMC1 and PMR1 genes via Crz1p/Tcn1p (263, 388).

Calcineurin mutants are also sensitive to extracellular Mn$^{2+}$. Wild-type strains are able to prevent Mn$^{2+}$ entry, whereas mutants exhibit an increased uptake phenotype (100), therefore indicating that the regulation of Mn$^{2+}$ homeostasis by calcineurin follows a different
mechanism than monovalent cation transport, in which export is regulated by a P-type ATPase (269, 300). An alternative hypothesis has been proposed in which Pmr1p, the Golgi-localized Ca\(^{2+}\) pump, plays a role in Mn\(^{2+}\) tolerance by sequestering Mn\(^{2+}\) to late compartments in the secretory pathway (263). Mn\(^{2+}\) may also be transported into the vacuole via the Ca\(^{2+}/H^+\) exchanger Vcx1p (332).

D) \(\beta\)-GLUCAN SYNTHASE AND CELL WALL SYNTHESIS. Calcineurin is responsible for transcriptional regulation of \(FKS2\), one of two genes encoding \(\beta\)-1,3-glucan synthase (Fig. 3) (95, 480). Calcineurin-dependent regulation occurs through Crz1p/Tcn1p (263, 388). The Fks1p protein is the predominant synthase expressed during optimum growth, but expression of Fks2p is induced upon treatment of cells with mating pheromone, high Ca\(^{2+}\), or growth on poor carbon sources. Deletion of the \(FKS1\) and \(CNB1\) genes results in lethality due to the inability to induce \(FKS2\) (114). In fact, \(FKS1\) mutants are hypersensitive to FK506 (95). These results suggest that calcineurin plays a role in regulating cell wall structure.

2. Schizosaccharomyces pombe

Like the budding yeast, treatment of \(S.\) \(pombe\) with FK506 or deletion of the \(ppb1^+\) gene, encoding for the calcineurin A subunit (Table 3), is not lethal. However, calcineurin in fission yeast appears to have distinct functions. Calcineurin-deficient \(S.\) \(pombe\) cells exhibit drastic \(Cl^-\)-sensitive growth (399) and are defective in cytokinesis, transport, nuclear and spindle pole body positioning, cell shape (468), and sporulation (327). One function for calcineurin in \(S.\) \(pombe\) that appears to overlap with \(S.\) \(cerevisiae\) is the mating process, although the roles for calcineurin in mating appear to be distinct in these two organisms. In \(S.\) \(cerevisiae\), calcineurin is required for the cell to recover from or survive growth arrest after exposure to pheromone. It thus may function to assist cells to reenter the cell cycle if they respond to \(\alpha\)-factor but fail to mate (see sect. \(\text{III}\) \(\text{A}\)). In \(S.\) \(pombe\), calcineurin is required for the mating response, and calcineurin mutants in this organism are sterile (327, 468). Northern analysis indicates that the transcript for calcineurin varies during the cell cycle and can be induced by nitrogen limitation, a condition that favors mating in \(S.\) \(pombe\) (327). The latter effect was dependent on the transcription factor \(ste1\).

3. Neurospora crassa

\(N.\) \(crassa\) has been widely used as a model system for studying eukaryotic gene expression. In this fungus, calcineurin is thought to play a major role in hyphal extension during mycelial growth and in determining apical orientation. Thus calcineurin mRNA exhibited the highest expression during early mycelial logarithmic growth but was repressed before conidulation upon entry into station-ary phase (153). A similar pattern of protein expression was observed but with about a 12-h lag behind message expression. Expression of antisense mRNA to the catalytic subunit, treatment with CsA and FK506, or disruption of the calcineurin B gene caused growth arrest precede by aberrant and increased hyphal branching and entry into conidiation, consistent with a role in apical growth (213, 335). Growth on two different carbon sources, glutamate and sucrose, did not influence the level of expression, indicating that calcineurin is not involved in mechanisms related to catabolite repression (153).

4. Aspergillus nidulans

Similar to \(N.\) \(crassa\), calcineurin A mRNA levels vary during the cell cycle in \(A.\) \(nidulans\), and disruption of the \(cnaA^+\) gene resulted in growth arrest (303, 343). After growth arrest in metaphase upon treatment with nocodazole followed by resuspension in fresh media to allow for synchronous growth, it was shown that calcineurin A message levels peak after the end of mitosis before DNA replication, with the highest expression appearing at the \(G_7/S\) boundary. Inducible gene disruption by homologous recombination revealed that the calcineurin A gene was essential for normal growth, and disruption was lethal. Further results indicated that the \(cnaA^+\) gene was required for early cell cycle events before DNA replication. Taken together with other data, this study suggested that calcineurin as well as other calmodulin targets may be required during different periods of the cell cycle.

5. Cryptococcus neoformans

Fungal diseases are becoming an increasing health problem, most notably in individuals infected with human immunodeficiency virus (HIV); \(C.\) \(neoformans\) represents one of these life-threatening infectious agents (6). Heitman and colleagues (5, 67, 309, 310) have begun to explore the role of calcineurin in this pathogen with the goal of using novel CsA and FK506 analogs that have increased specificity toward the fungal calcineurin compared with the host (human) enzyme. Such compounds may eventually prove useful as antifungal agents with reduced toxicity and immunosuppressive effect toward the host. One such candidate is the FK506 analog L-685,818 (18-hydroxy, 21-ethyl-FK506). L-685,818 is toxic to \(C.\) \(neoformans\), mediated by binding and inhibiting the fungal calcineurin, but has reduced immunosuppressive activity in humans (309). Growth studies in the presence of CsA and FK506 indicate that these drugs are growth inhibitory at 37°C but not at 24°C and that they inhibit a common target. Disruption of the calcineurin A gene resulted in mutant strains that are viable at 24°C but do not survive under conditions that mimic the host environment including elevated temperature, 5% \(CO_2\), or alkaline pH (310).
These mutant strains are no longer pathogenic, thus indicating that calcineurin is necessary for virulence in this organism.

6. Dictyostelium discoideum

Calcineurin in the slime mold D. discoideum exhibited the familiar developmental pattern of expression as noted above for the filamentous fungi, with the highest level of expression during vegetative growth and decreasing expression during multicellular development (75). CsA and FK506 had no effect on growth, a process that can be separated from development in this organism (159). However, these drugs do inhibit developmental processes such as stalk cell spore formation and expression of prestalk and prespore developmental markers.

7. Other lower eukaryotic organisms

A gene for calcineurin B has been isolated in the amoeboflagellate N. gruberi (346). mRNA levels are detectable in the amoebae and are cyclic, with peak abundance during flagellar formation, followed by a gradual decline. In the unicellular organism Paramecium tetraurelia, calcineurin localization was investigated by use of a specific antibody and immunocytochemical methods (209). Calcineurin was largely localized to the cilia and cell membrane, with only a diffuse staining pattern observed within the cell body. Further staining indicated that there was no difference in either localization or abundance in cells prepared either in logarithmic or stationary phase. Thus calcineurin abundance does not appear to change during the cell cycle as it does in the simple fungi. To further explore calcineurin’s role in P. tetraurelia, anticalcineurin antibody or Ca\(^{2+}\)/calmodulin-calcineurin was microinjected into cells. Anticalcineurin antibody blocked exocytosis after treatment with the exocytosis trigger agent, aminoethyldextran, while microinjection of a complex of Ca\(^{2+}\)/calmodulin-calcineurin was microinjected into cells. Anticalcineurin antibody blocked exocytosis after treatment with the exocytosis trigger agent, aminoethyldextran, while microinjection of a complex of Ca\(^{2+}\)/calmodulin-calcineurin induced exocytosis. These results implicate calcineurin as the phosphatase previously shown to dephosphorylate a 63-kDa protein hypothesized to be involved in trichocyst exocytosis (198).

Recently, calcineurin has been isolated from Leishmania major (342) and Leishmania donovani (21). Calcineurin was isolated by chromatographic separation of cytosol from promastigotes where it was hypothesized to be a key regulatory component in the life cycle of this parasite. Interestingly, in L. major, extracellular growth is not inhibited by CsA, and in fact, a high-affinity complex of CsA with L. major cyclophilin forms [inhibitory constant (K\(_i\)) = 5.2 nM] but does not inhibit or form a tight complex with calcineurin from that organism, suggesting a possible mechanism for this organism’s resistance to CsA (342). Interestingly, a complex between CsA, recombinant human cyclophilin, and L. major calcineurin was formed indicating that the parasitic calcineurin is functionally and structurally equivalent to mammalian calcineurin. A similar phenomenon was observed with calcineurin from the tapeworms Hymenolepsis microstoma and Hymenolepsis diminuta such that calcineurin from both organisms was inhibited by CsA complexed with mammalian cyclophilin but not H. microstoma cyclophilin (347). This was not the case with calcineurin from Schistoma mansoni (186) and Plasmodium falciparum (87). One hypothesis to explain the lack of complex formation with calcineurin is that parasitic cyclophilins are structurally different from mammalian cyclophilins, such that cyclophilin residues surrounding the CsA binding site that interact with calcineurin are not conserved in parasitic cyclophilins. Further studies are necessary to resolve these interesting findings.

B. Higher Eukaryotes

1. Calcineurin in plants

Evidence for a plant homolog of calcineurin was first obtained by Luan et al. (246) who demonstrated, using patch-clamp techniques, that CsA and FK506 blocked Ca\(^{2+}\)-dependent inactivation of K\(^+\) channels in Vicia faba. A partially proteolyzed and constitutively active form of calcineurin also inhibited K\(^+\) channel activity. Furthermore, both CsA and FK506 inhibited a Ca\(^{2+}\)-dependent phosphatase activity in cellular extract. Subsequent studies have provided additional evidence for calcineurin function in plants (reviewed in Ref. 245).

To date, however, calcineurin has not been successfully purified to homogeneity from plant tissue nor have bona fide genes for either subunit been cloned. The closest contenders are two EF-hand Ca\(^{2+}\)-binding proteins encoded for by the SOS3 and AtCBL genes that are homologous to the calcineurin B subunit (218, 239). The protein encoded by the SOS3 gene is 30% identical to calcineurin B from various organisms, and mutations in SOS3 render A. thaliana sensitive to Na\(^+\) (239). The SOS3 protein is also homologous to NCS-1 (30% identity), a neuronal Ca\(^{2+}\) sensor in the recoverin family of EF-hand proteins (see sect. uB2o). The AtCBL proteins are most homologous to calcineurin B (32% identity to rat calcineurin B) and can complement a yeast calcineurin B mutation, indicating a calcineurin B-like physiological function (218). Recent work, however, indicates that the AtCBL proteins interact with a novel group of protein kinases in a Ca\(^{2+}\)-dependent fashion (372). A. thaliana contains at least six AtCBL genes. The AtCBL and SOS3 proteins clearly play different roles since they are unable to complement each other (218). It is intriguing that SOS3 and AtCBL encode for Ca\(^{2+}\)-binding proteins, indicating that salt stress in plants may be regulated by Ca\(^{2+}\)-dependent signaling pathways (possibly via calcineurin) as has
been found in *S. cerevisiae* (see sect. μA1n). Further evidence for this hypothesis was obtained in a study demonstrating that overexpression of an activated form of yeast calcineurin conferred salt tolerance in transgenic tobacco plants (320). Similarly, genes for three of the AtCBL isoforms appear to be stress regulated. Whether SOS3 or the AtCBL proteins represent plant calcineurin B homologs or just close relatives will hopefully be resolved if a protein corresponding to plant calcineurin can be isolated and/or cloned and shown to be a functional phosphatase.

2. Calcineurin in mammals

**A) Tissue Distribution.** Calcineurin is widely distributed in mammalian tissues, with the highest levels found in brain (168, 175, 216, 437). In addition, calcineurin A and B subunits have been observed in adipose tissue, adrenal cells (318, 319), bone osteoclasts (19), heart, hindbrain and spinal cord (394), kidney (42, 418, 419), liver (135), B and T lymphocytes (4, 50, 193), lung, medulla, olfactory bulb, pancreas (112), placenta (314), platelets (406, 438), retina (66), skeletal muscle (168), smooth muscle, spleen, testis and sperm (278, 286, 396, 409), thymus (42, 193), and thyroid (121).

Distinct tissue distribution is observed for the various isoforms of each subunit (42, 175, 219). An isoform of the catalytic subunit encoded for by the *PPP3CC* gene (γ-isoform, see Table 3) is testes specific (291, 292), as is the product of the *PPP3R2* gene encoding an isoform of the regulatory subunit (48, 424). With the use of polyclonal antibodies that distinguish between the α- and β-isoforms of calcineurin A (encoded by the *PPP3CA* and *PPP3CB* genes, respectively; Table 3), it was found that calcineurin Aα was more abundant than Aβ in the rat brain and heart, but the relative abundance is reversed in spleen, thymus, and lymphocytes (175, 219). These results partly explain the recent finding that *PPP3CA*-knock-out mice produce T and B cells that mature normally, respond to mitogenic stimulation, and remain sensitive to both CsA and FK506, but are defective in in vivo antigen-to mitogenic stimulation, and remain sensitive to both.

**B) Subcellular Distribution.** Using a radioimmunoassay, Cheung and colleagues (10) measured the subcellular distribution of calcineurin in chick forebrain homogenate. In that study, calcineurin was highly enriched in the cytoplasmic and microsomal fractions as well as synaptosomes. Subsequent studies have confirmed its predominance in the cytoplasm and synaptosomal cytosol (219, 306). Politino and King (329) explored the physical association of calcineurin with synthetic phospholipid vesicles and showed that calcineurin binds small, acidic, unilamellar vesicles in a Ca²⁺-dependent fashion. Furthermore, the phosphatase activity of calcineurin was profoundly affected by phosphatidyglycerol or phosphatidylyserine, with up to a 23-fold increase in activity toward phosphorylated histone, but inhibition using p-nitrophenyolphosphate (p-NPP) or tyrosine phosphate. In a subsequent study it was hypothesized that the phospholipid-binding site was located on the calcineurin B subunit (330). Using synthetic phospholipid monolayers, Kennedy et al. (183) investigated the factors that contributed to calcineurin-phospholipid interactions and found that calcineurin binding is myristoyl independent, mediated by anionic phospholipids and/or diacylglycerol, and also affected by the presence of calmodulin.

There is overwhelming evidence for calcineurin in the nucleus along with other calmodulin-binding proteins such as casein kinase-2 and myosin light-chain kinase (34, 286, 336, 376, 426). In spermatids, calcineurin was localized to the nucleus, and its levels were most abundant during the initial stage of nuclear elongation, with almost no signal present in the cytoplasm (286). In the context of signaling pathways that activate nuclear factor of activated T cells (NF-AT), Shibasaki et al. (376) have shown that calcium induces an association between calcineurin and NF-AT that results in colocalization of both molecules to the nucleus.

Calcineurin has also been shown to be associated with the cytoskeleton (106). The latter finding is of interest given that several substrates of calcineurin are colocalized to the cytoskeleton including tau (115, 122, 181), microtubule-associated protein 2 (122, 284), tubulin (122), dystrophin (275, 440), and dyamin (90, 240).

**C) Calcineurin Function.** Numerous functions have been identified for calcineurin in higher eukaryotic organisms, and it is beyond the scope of this review to cover all of them comprehensively. Klee et al. (208) have provided a recent update and cited a number of specific reviews regarding calcineurin function (208). Table 5 is an attempt to summarize a number of tissues, systems, and specific substrates that are implicated to be regulated by calcineurin. In sections μB2a and μB2e, we provide a short review of the role of calcineurin on two key systems of importance in modern biology, apoptosis and cardiac hypertrophy.

**D) Calcineurin and Apoptosis.** It has been recognized for some time that calcineurin plays a role in programmed cell death of T and B lymphocytes (32, 110, 116, 481). Recently, it has also been shown that calcineurin plays a role in apoptosis in neuronal cells via the cytochrome c/caspase-3 pathway (17). In T-cell hybridomas, apoptosis can be stimulated by ligation of the T-cell receptor/CD3 complex and has provided a useful in vitro model to

October 2000

CALCINEURIN: FORM AND FUNCTION 1495
investigate signaling pathways responsible for this biological phenomenon. Both CsA and FK506 inhibit this process, implicating calcineurin in the signaling pathway of apoptosis which is known to involve a rise in intracellular Ca\textsuperscript{2+} (110). Similarly, in the B-cell lymphoma cell lines WEHI-231, B104, and BL60, apoptosis induced by cross-linking of surface immunoglobulin receptors was inhibited by these immunosuppressant drugs (32, 116).

In lymphocytes, calcineurin and NF-AT appear to participate in apoptosis, in part by mediating the induction of Fas and Fas ligand which then interact and transduce the apoptotic signal after T-cell receptor ligation (157, 226, 243, 421). Using a constitutive (Ca\textsuperscript{2+} - and calmodulin-independent) form of calcineurin, Shibasaki and McKeon (375) demonstrated that calcineurin functions in calcium-induced apoptosis in mammalian cells deprived of growth factors and that this was a direct consequence of calcineurin’s phosphatase activity. Interestingly, coexpression of Bcl-2 blocked calcineurin-induced apoptosis. At least one mechanism for how this occurs was provided.
subsequently by experiments which showed that Bcl-2 forms a complex with calcineurin that targets it to the cytoplasmic membrane (374). Although still maintaining phosphatase activity, calcineurin bound to Bcl-2 is unable to promote nuclear translocation of NF-AT. Furthermore, BAD, a proapoptotic member of the Bcl-2 family, is a substrate of calcineurin. Dephosphorylation of BAD by calcineurin enhances BAD heterodimerization with Bcl-xL and apoptosis (443).

However, another intriguing hypothesis is that apoptosis is linked to cellular redox homeostasis. Wovletang et al. (463) showed that inhibitors of the plasma membrane NADH-oxidoreductase (PMOR) activity induce apoptosis through a signaling pathway involving calcineurin (463). It was proposed that PMOR serves as a redox sensor that can regulate the signals required for apoptosis (227). The finding that calcineurin activity is sensitive to redox state changes (45, 111, 345, 447, 470, 471) provides support for this hypothesis and a means by which apoptosis could be regulated by the cellular redox potential.

E} Importance of Calcineurin in Cardiovascular Function.

Recently, calcineurin and NF-AT have been implicated in transducing signals responsible for cardiac morphogenesis and inducing cardiac hypertrophy (82, 232, 233, 281, 337, 377, 401, 402, 417). Thus disruption of the NF-ATc gene in mice results in failure to develop normal cardiac valves and septa, and the transgenic mice die from congestive heart failure in utero (82, 337). Overexpression of calcineurin has also shown to induce cardiac hypertrophy and heart failure in transgenic mice that could be blocked by the immunosuppressant drug CsA (281). Furthermore, a transgenic mouse model for hypertrophy in which tropomodulin-overexpressing transgenic mice develop progressive dilated cardiomyopathy has provided evidence for increased calcineurin protein levels before the onset of the hypertrophic phenotype, suggesting that calcineurin may play an early regulatory role in this process (402). Similar results were found in skeletal muscle from mice subject to overload (88) and confirmed later in skeletal muscle cells virally transfected with insulin-like growth factor I (295, 367). Some of these studies have even proposed that immunosuppressant drugs such as CsA and FK506 might be used to treat hypertrophy (312, 401). Indeed, in a subsequent study, Sussman et al. (401) utilized an aortic banding model to induce hypertrophy and showed that treatment with CsA, albeit an excessive dose, resulted in significantly less hypertrophy. However, although a few studies have confirmed this finding (267, 402), several other groups examining calcineurin’s role in this process have failed to demonstrate any efficacy of CsA (86, 247, 290, 476) and, in fact, Molkentin (280) has responded by reporting that CsA protected against pressure-overload hypertrophy after 7 days but not after 21 days. Although the reason for some of these discrepancies may be due to the dose of immunosuppressant drug used, current hypotheses suggest that multiple signaling pathways might be recruited to participate in the hypertrophic response and that inhibition of one parallel pathway (i.e., calcineurin) might delay but not prevent hypertrophy (108, 397, 441). Nevertheless, they implicate a possible role for calcineurin and NF-AT in cardiac function.

Oxidative stress is also thought to play a role in cardiomyopathy and heart failure (381). The possibility that calcineurin may be regulated by oxidative stress indicates that signaling pathways in which it is involved may be important in mediating processes that lead to cardiac dysfunction.

C. Inhibitors of Calcineurin

1. Natural product and synthetic inhibitors

A number of natural products have been isolated that are potent inhibitors of calcineurin and other serine/threonine protein phosphatases. The most potent, specific, and well-known inhibitors of calcineurin are the immunosuppressant drugs CsA and FK506 (Fig. 4), which inhibit calcineurin when complexed with their respective cytoplasmic receptors cyclophilin and FKBP (see Table 1 for an entry for a number of reviews on these drugs). Interestingly, in vitro calcineurin inhibition by these immunosuppressant drug complexes only occurs when a physiological substrate is used to assay the enzyme such as phosphocasein or phospho-R11 peptide, a peptide whose sequence represents the phosphorylation site of the regulatory subunit of cAMP-dependent protein kinase, a well-characterized and more physiological phosphopeptide substrate (31). The use of p-NPP as substrate results in activation of calcineurin by these immunosuppressant drug complexes (238, 403).

A number of other compounds have demonstrated inhibitory activity against calcineurin and other serine/threonine protein phosphatases. Okadaic acid, often used as a potent and specific inhibitor of PP2A, can also inhibit PP1 and calcineurin at higher concentrations. The ID50 of okadaic acid for PP2A has been measured to be ~1 nM, while the ID50 values for PP1 and calcineurin are ~300 nM and ~4 μM, respectively (29). The cyclic peptide microcystin LR is a potent inhibitor of PP1 and PP2A, with a Ki value <0.1 nM. Although the inhibition of calcineurin by microcystin LR occurs at over 1,000-fold higher concentrations, microcystin LR still is a relatively potent inhibitor of calcineurin (IC50 = 0.2 μM) (248). Dibefurin, a novel fungal metabolite, also has modest inhibitory activity against calcineurin (37).

Since the discovery of these natural product inhibitors, several new synthetic dockers have been found to be reasonable inhibitors of calcineurin and other phosphatases. Tatlock et al. (410) utilized computational docking experiments and synthetic derivatives of the exo,exo-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid ring
system of endothall (Fig. 4) to search for enhanced ligand binding to calcineurin (410). Endothall is structurally related to the natural defensive toxin of blister beetles, cantharidin (210), a potent inhibitor of PP1 and PP2A (210) and a weak inhibitor of calcineurin (98). Substitution at the 5-endo position was hypothesized to provide reasonable binding interactions that mimicked the interaction between the active site of calcineurin and its autoinhibitory domain. Incorporation of a trans-cyclopropylphenyl group at this position afforded the most potent inhibitor, with an apparent $K_i$ of 0.5 µM. Interestingly, the tethered dicarboxylic acid moiety and bridgehead oxygen atom of endothall and cantharidin derivatives were modeled to interact with the active site dinuclear metal center (see sect. nC) (410).

A similar approach to inhibitor design incorporating pendant metal-coordinating groups that could anchor the inhibitor to the active site metal ions has been introduced by Widlanski and colleagues (296). A variety of alkylphosphonic acid derivatives containing an additional thiol or carboxylate group (Fig. 4) were explored as inhibitors of alkaline phosphatase and purple acid phosphatase. Assuringly, nearly all bound more tightly than substrate $p$-nitrophenol and up to 55-fold tighter than ethanylphosphonic acid, indicating that these additional function groups could improve binding affinity. Whether binding occurs via direct metal ligation for endothall and/or alkylphosphonic acid derivatives remains to be demonstrated by spectroscopic means. If correct, these compounds could provide a route to the design of more potent and selective metallophosphatase inhibitors.

Peptide inhibitors of calcineurin have also been introduced. One of these, a 25-residue peptide based on the sequence of the autoinhibitory domain of the calcineurin A subunit from residues 457–481 (Fig. 1), is a
relatively potent inhibitor of calcineurin phosphatase activity (137, 325). Recently, a high-affinity calcineurin-binding peptide was selected from a combinatorial peptide library based on the calcineurin docking motif of NF-AT (15). The peptide inhibited NF-AT activation and expression of NF-AT-dependent cytokine genes in T cells, but did not inhibit calcineurin phosphatase activity toward phospho-R_{II} peptide, and thus did not affect the expression of other cytokines that require calcineurin but not NF-AT. The latter point is significant because compounds such as this peptide that selectively interfere with calcineurin-NF-AT interaction without disrupting calcineurin phosphatase activity may prove to be less toxic immunosuppressants compared with CsA and FK506.

At least one other synthetic calcineurin inhibitor has been reported, PD 144795, a benzothiophene derivative shown to have anti-inflammatory and anti-HIV effects (126). Transcriptional activity mediated by p53 and NF-κB were inhibited by both CsA and PD 144795. An in vitro assay of calcineurin activity from Jurkat cell lysate also indicates that PD 144795 led to dose-dependent inhibition of calcineurin.

It was previously concluded by Enan and Matsumura (93) that class II pyrethroid insecticides were potent inhibitors of bovine brain calcineurin, with IC_{50} values of 10^{-9} to 10^{-11} M. In that study, p-NPP and O-phospho-DL-tyrosine were used as substrates in the inhibition assay. However, in an independent study, none of the class II pyrethroids was able to inhibit purified bovine calcineurin using phospho-R_{II} peptide (97). Calcineurin activity in rat brain homogenate and in IMR-32 neuroblastoma cells in culture was also not affected by pyrethroids, indicating that these insecticides are not effective inhibitors of calcineurin (99).

The tyrphostins A8, A23, and A48, members of a family of tyrosine kinase inhibitors, inhibited calcineurin with IC_{50} values of \sim 10^{-5} M (258). However, the use of p-NPP as substrate in these studies should be questioned given the inhibition pattern of calcineurin noted above for CsA, FK506, and the pyrethroid insecticides. A follow-up study using phospho-R_{II} peptide or other suitable phosphoprotein substrate may confirm yet another class of calcineurin inhibitors.

2. Endogenous regulators

In addition to synthetic and natural product inhibitors of calcineurin, a number of endogenous cellular proteins have emerged as inhibitors of calcineurin protein phosphatase activity and thus potential regulators of its in vivo function. One of the first to be identified was a 79-kDa protein kinase A anchoring protein (AKAP79) (58). AKAP79 associates with the regulatory subunit of the cAMP-dependent protein kinase and localizes it to postsynaptic densities. Using a yeast two-hybrid approach to search for proteins that interacted with AKAP79, Scott and colleagues (58) identified a positive clone encoding the calcineurin A subunit. Immunofluorescence studies demonstrated that calcineurin and the regulatory subunit of protein kinase A were colocalized in rat hippocampal neurons via AKAP79. Interestingly, AKAP79 contained a domain homologous to FKBP, hypothesized to be the calcineurin binding domain. A synthetic peptide based on this sequence was a noncompetitive inhibitor of calcineurin activity (58). A subsequent study, however, suggests that AKAP79 interacts with calcineurin through a site distinct from the FKBP-homologous region (177).

Another potential calcineurin regulatory protein is cain/cabin 1, a 2,220-residue phosphoprotein isolated by yeast two-hybrid screens of either rat hippocampal or mouse T-cell cDNA libraries (224, 400). Cain/cabin 1 binds to calcineurin and inhibits it in a noncompetitive fashion. The interaction between cain/cabin 1 and calcineurin was dependent on protein kinase C activation, and overexpression inhibited the transcriptional activation of the interleukin-2 gene and prevented dephosphorylation of the transcription factor NF-AT. Recently, cain/cabin 1 was found to regulate the transcription factor MEF2, itself regulated via calcineurin-dependent pathways, by binding to MEF2 and sequestering it in an inactive state (469).

In an expression library screen searching for proteins that interact with the ubiquitously expressed Na^{+}-H^{+} exchanger NHE1, Lin and Barber (234) identified a novel protein, CHP (see sect. II.D), that specifically bound NHE1 and was critical for growth factor stimulation of exchange activity. Overexpression of CHP in Jurkat and HeLa cells resulted in inhibition of NF-AT nuclear translocation and transcriptional activity that was hypothesized to be the result of calcineurin inhibition (235). Indeed, the phosphatase activity of immunoprecipitated calcineurin was inhibited 50% in cells overexpressing CHP, whereas in a reconstitution assay, the activity of purified calcineurin was inhibited nearly quantitatively in a dose-dependent fashion. These results indicate that CHP could represent yet another member of this emerging class of endogenous calcineurin regulators.

Recently, a protein of the African swine fever virus, A238L, was found to display immunosuppressive activity by inhibiting NF-AT-regulated gene transcription in vivo (277). A238L coimmunoprecipitated with calcineurin after viral infection of Vero cells, and calcineurin phosphatase activity was inhibited in cellular extracts from viral-infected cells. It was hypothesized that A238L may enable the virus to evade host defense systems by preventing transcriptional activation of genes important for host immunity.

Although the classical mechanism for regulating calcineurin activity is via Ca^{2+}/calmodulin, it is intriguing to
speculate that these and possibly other proteins can interact with calcineurin to regulate subcellular targeting and/or activity toward specific substrates in novel yet undefined ways.

IV. CALCINEURIN STRUCTURE

A. A Dinuclear Metal-Binding Phosphoesterase Motif

Before the availability of any structural data, Averill and colleagues (431, 432) predicted that the serine/threonine protein phosphatasas were homologous to purple acid phosphatases and therefore might contain an active site dinuclear metal center. Their hypothesis was based on a comparison of the primary sequences of serine/threonine protein phosphatasas with human, porcine, and bovine purple acid phosphatases, enzymes which were already well characterized and known to contain dinuclear iron centers. Their prediction was correct, and the authors were able to identify three of the metal ligands.

With increasing sequence data available, several groups have completed comprehensive sequence alignments of serine/threonine protein phosphatasas and have identified a number of residues that are conserved in all members of this family (26, 132, 212, 244, 371, 486). These studies identified a “phosphoesterase motif” (Fig. 5) that is conserved not only in PP1, PP2A, and calcineurin, but in many other enzymes involved in the cleavage of phosphoester bonds, including acid and alkaline phosphatasas, bacterial exonucleases, diadenosine tetraphosphatase, 5’-nucleotidase, phosphodiesterase, sphingomyelin phosphodiesterase, an enzyme involved in RNA debranching, and a phosphatase in the bacteriophage λ genome, λ protein phosphatase (244).

Four of the residues in the phosphoesterase motif (bold letters, Fig. 5) are ligands to a dinuclear metal cofactor in PP1 and calcineurin. Thus it has been hypothesized that this motif provides a scaffold for an active site dinuclear metal center in every member of the phosphoesterase family (244, 351). Support for this hypothesis was provided in studies of λ protein phosphatase which demonstrated a spin-coupled dinuclear metal binding site by spectroscopic means (272, 352), and in the recent determination of the three-dimensional structure of E. coli 5’-nucleotidase, a distant member of the metallophosphatase superfamily that has an active site containing two Zn2+ separated by 3.3 Å (211). The conserved phosphoesterase motif suggests a common catalytic mechanism for enzymes involved in phosphotransfer reactions, a hypothesis that seems to be the case for at least two of these, calcineurin and λ protein phosphatase (see below).

The phosphoesterase motif is also found in purple acid phosphatasas, albeit in a slightly modified form (Fig. 5). Despite these differences, the phosphoesterase motif in purple acid phosphatase has a similar β-α-β-α-β fold accommodating the dinuclear metal center (199, 201, 395).

B. Three-Dimensional Structure

The three-dimensional structures of several enzymes in the metallophosphatase family have been solved. X-ray structures (with highest resolution noted in parentheses) of PP1 (2.1 Å) (91, 120), calcineurin (2.1 Å) (124, 197), kidney bean purple acid phosphatase (2.65 Å) (199, 201, 395), mammalian purple acid phosphatase (1.55 Å) (128, 425), and the periplasmic 5’-nucleotidase from E. coli (1.7 Å) (211) have been solved. In these structures, the phosphoesterase motif described in the previous section is represented as a β-α-β-α-β scaffold for an active site dinuclear metal center. The three β-strands of this motif form a parallel pleated sheet that is capped by intervening α-helices. Two metal ions are positioned at the apex of this fold forming a dinuclear metal center with 3.0–4.0 Å between metal ions, with four of the metal ligands provided by residues in loops between β-sheets and α-helices.

A ribbon diagram representing the X-ray structure of phosphate-inhibited calcineurin, complexed with the immunosuppressant drug complex FK506-FKB, is shown in Figure 6. The overall structure of the catalytic subunit (shown in gray) is ellipsoidal and consists of a mixture of α-helices and β-sheets. The metal ions of the dinuclear metal center are obscured in this diagram by the orange van der Waals spheres of phosphate that form a bridge between the two metal ions (see Fig. 10, below). The calcineurin B-binding domain (cf. Fig. 1) is evident in this structure as an α-helix that protrudes from the core of the
molecule, forming the binding site for the calcineurin B subunit (Fig. 6, yellow). Absent in this structure are the calmodulin-binding and autoinhibitory domains, since a truncated form of calcineurin missing these domains was the source of protein for crystallization studies (124). In a subsequent study that determined the structure of the holoenzyme, it was found that the autoinhibitory domain folds into an α-helix that binds to the substrate-binding cleft of the catalytic domain, with one of the glutamate residues forming hydrogen bonds to metal-coordinated water molecules (197). Interestingly, the calmodulin domain was disordered in the X-ray structure, and therefore, our knowledge of how this domain interacts with the active site and autoinhibitory domains to confer calmodulin-regulation remains rudimentary.

The calcineurin B subunit in Figure 6 is colored in yellow and is seen forming a complex with calcineurin A via the calcineurin B-binding helix. Four Ca\(^{2+}\)s, shown as blue spheres, are bound in the EF-hand domains of the B subunit. The NH\(_2\)-terminal myristoyl group of the B subunit is colored as red van der Waals spheres. The phosphate molecule bound to the active site metal ions of the A subunit is represented as orange van der Waals spheres.
C. Active Site Architecture

The dinuclear metal cofactor of calcineurin has been modeled as an Fe$^{3+}$-Zn$^{2+}$ cluster based on the presence of near-stoichiometric quantities of Fe$^{3+}$ and Zn$^{2+}$ (195, 471), electron paramagnetic resonance (EPR) spectroscopic experiments (471), and X-ray diffraction studies which show a dinuclear metal center separated by 3.14 Å in a coordination environment shown in Figure 7A (197). A similar coordination environment and metal-metal distance is also found in PP1 (120), although in that enzyme, the metal ions could not be identified with certainty and were therefore referred to as M1 and M2. In the X-ray structure of calcineurin, the Fe$^{3+}$ was modeled in the M1 site largely based on a comparison to the Fe$^{3+}$-Zn$^{2+}$ active site metal cofactor of kidney bean purple acid phosphatase. In purple acid phosphatase, a tyrosine residue coordinates to the Fe$^{3+}$ (M1 site) and gives rise to a tyrosine-to-iron charge transfer band at 510–550 nm, responsible for the purple color of these enzymes (Fig. 7B) (223, 433). As can be seen in Figure 7, this tyrosine ligand is missing in calcineurin and is replaced by a histidine, thus explaining why calcineurin does not exhibit any appreciable absorbance in the visible region of the optical spectrum (471). The additional substitution of a histidine ligand with a water molecule results in a net water-for-tyrosine substitution at the Fe$^{3+}$ site in calcineurin compared with purple acid phosphatase. The coordination of the Zn$^{2+}$ (M2) provided by amino acid side chains in these enzymes is identical, with ligands provided by two histidines, an aspartic acid that bridges to the Fe$^{3+}$ and an asparagine residue (compare Fig. 7, A and B). Figure 8 shows a comparison of the active sites of kidney bean purple acid phosphatase and calcineurin that illustrates the remarkable superposition of both metal ions and protein ligands in these two enzymes.

In addition to coordination provided by protein side chains, the metal ions in calcineurin and purple acid phosphatase have one or more solvent molecules as additional ligands. All enzymes show bridging and terminal solvent molecules, but at least in one X-ray structure of calcineurin, an additional terminal solvent molecule was modeled into the coordination sphere of the M1 site as shown in Figure 7A (197). From the iron-oxygen bond length (2.1 Å in purple acid phosphatase, Ref. 128), the bridging solvent molecule is most likely a hydroxo group based on distances obtained from model compounds (223).

**FIG. 7.** A: schematic of the active site of human calcineurin based on the 2.1-Å resolution structure described by Kissinger et al. (197). B: schematic of the active site of kidney bean purple acid phosphatase based on the 2.65-Å resolution structure described by Klabunde et al. (201).
D. Metal Ion Requirements

It has been known for some time that divalent cations in assay buffers are necessary to achieve the high activities of purified calcineurin, with the best activators being Mn$^{2+}$ and Ni$^{2+}$ (194, 230, 315). Mn$^{2+}$ and Ni$^{2+}$ have been shown to increase the activity of calcineurin in the absence of Ca$^{2+}$/calmodulin (315), to prevent inactivation, or to restore activity following inactivation by exposure to Ca$^{2+}$/calmodulin (194). Similar divalent metal ion effects have been observed with PP1 and PP2A (3, 28, 43, 53, 94, 478) and with $\lambda$-protein phosphatase (486). It is unclear why these divalent metal ions are potent activators. One possibility is that Mn$^{2+}$ or Ni$^{2+}$ are native metal ions that become lost during purification. Pallen and Wang (317) incubated calcineurin with Ni$^{2+}$ or Mn$^{2+}$ and showed that these metal ions are not dissociable by extensive dialysis or gel filtration but can be released after prolonged exposure to Ca$^{2+}$/calmodulin or by the use of chelating reagents, both of which occur during a typical purification protocol. To address this issue, Rao and Wang (341) used an anticalcineurin immunoaffinity matrix to rapidly purify calcineurin from crude bovine brain extract in the absence of calmodulin. Analysis showed that the immunoprecipitated calcineurin did not contain significant amounts of Ni$^{2+}$ and Mn$^{2+}$. Although no mention was made of the Fe content, ~1 equivalent of Zn$^{2+}$ was found in all samples, suggesting that Zn$^{2+}$ is an intrinsic metal ion but not Ni$^{2+}$ or Mn$^{2+}$.

An alternative hypothesis to explain the mechanism of divalent metal ion activation is that prolonged exposure of calcineurin to Ca$^{2+}$/calmodulin promotes the release of the intrinsic Fe and Zn metal ions and subsequent replacement by Mn$^{2+}$ or Ni$^{2+}$. The most efficient method to purify calcineurin utilizes Ca$^{2+}$/calmodulin affinity chromatography. It is possible that calmodulin binding exposes the active site and thereby promotes loss of Fe and/or Zn while calcineurin is adsorbed to the matrix. Alternatively, because elution of calcineurin from calmodulin-Sepharose requires the use of a metal chelator (e.g., EDTA), it is possible that the Fe$^{3+}$ and/or Zn$^{2+}$ may be removed during elution. Thus far, neither hypothesis has

FIG. 8. Comparison of the active sites of calcineurin and kidney bean purple acid phosphatase. An alignment of the active sites of calcineurin and purple acid phosphatase was performed based on the conserved His-Asp (calcineurin His-151/Asp-121) pair using the program Quanta (Quanta 97 Molecular Modeling Software Package from Molecular Simulations, 1997). The X-ray crystal structures used were Protein Data Bank entries 1AU1 and 1KBP for human calcineurin (197) and kidney bean purple acid phosphatase (201), respectively. The A subunit of calcineurin and a monomer form of purple acid phosphatase were used to generate the alignments. Only the metal ions and the active site residues shown in the figure were used for the alignment. Calcineurin and purple acid phosphatase residues are indicated in red and yellow, respectively. The Zn atom is green, and the Fe atom is purple. The numbers denote the following residues: 1) His-92 in calcineurin, Tyr-167 in purple acid phosphatase; 2) Asp-121 in calcineurin, Asp-169 in purple acid phosphatase; 3) His-151 in calcineurin, His-202 in purple acid phosphatase; 4) Asn-150 in calcineurin, Asn-201 in purple acid phosphatase; 5) His-199 in calcineurin, His-286 in purple acid phosphatase; 6) His-281 in calcineurin, His-323 in purple acid phosphatase; 7) His-325 in purple acid phosphatase; 8) Asp-90 in calcineurin, Asp-135 in purple acid phosphatase; 9) Asp-118 in calcineurin, Asp-164 in purple acid phosphatase.
been rigorously tested. However, King and Huang (195) demonstrated that there was no correlation between the loss of enzymatic activity after calmodulin-dependent inactivation and the iron and zinc content. Both metal ions remained tightly bound during prolonged exposure to calmodulin. Nevertheless, additional studies have demonstrated that up to two equivalents each of Mn$^{2+}$ and Ni$^{2+}$ could bind to calcineurin (317, 482), a result consistent with these metal ions occupying the sites of the dinuclear metal cluster. In support of this was an EPR study following Mn$^{2+}$ binding to λ-protein phosphatase which found that a dinuclear Mn$^{2+}$-Mn$^{2+}$ cluster was formed upon addition of two equivalents of Mn$^{2+}$ to the apoenzyme (352). The situation with calcineurin is not as straightforward due to the presence of calmodulin and the calcineurin B subunit, each of which can provide additional divalent metal ion binding sites. Indeed, an EPR study following Mn$^{2+}$ binding to calcineurin in the presence of calmodulin demonstrated 10 Mn$^{2+}$ sites and attributed 4 each to calmodulin and calcineurin B and 2 to the catalytic subunit (453).

Clearly further work is needed to understand the mechanism whereby exogenous Ni$^{2+}$ and Mn$^{2+}$ activate calcineurin after prolonged exposure to Ca$^{2+}$/calmodulin in vitro and whether or not this activation also occurs in vivo.

V. ENZYMATIC MECHANISM

A. Mechanism of Phosphoryl Group Transfer:
Evidence for Direct Transfer to Water

Much of the initial work on the mechanism of calcineurin focused on determining whether a phosphoenzyme intermediate was formed during catalysis, since other phosphatases are known to proceed by this mechanism (Fig. 9). For example, E. coli alkaline phosphatase catalyzes phosphate ester hydrolysis by first transferring the phosphoryl group to an active site serine residue to form a transient phosphoenzyme intermediate (63, 64). In the next step, the enzyme is regenerated for another round of catalysis by hydrolysis of the phosphoenzyme intermediate. Alkaline phosphatase is a metalloenzyme containing a Mg$^{2+}$ and a Zn-Zn dinuclear center reminiscent of the dinuclear metal sites of the serine/threonine phosphatases and purple acid phosphatases (187). A phosphoenzyme intermediate has also been demonstrated in the protein tyrosine phosphatases (52, 127, 479), enzymes that function without active site metal ions.

Steady-state experiments of calcineurin carried out by Graves and colleagues (262) suggested that a phosphoenzyme intermediate was not formed during catalysis. A linear relationship between log ($V/K$) and the p$K_a$ of the leaving group was observed for a set of four substrates, with a trend toward increasing velocity as the p$K_a$ of the leaving group decreased (262). This result is consistent with a direct transfer to water without formation of a phosphoenzyme intermediate (Fig. 9). Additional experiments failed to demonstrate phosphotransferase activity in the presence of alternate nucleophiles, which also is consistent with a direct transfer mechanism (262). In a subsequent study using p-NPP as substrate, product inhibition studies showed that both phosphate and the phenol were competitive inhibitors of calcineurin (259). These inhibition patterns are consistent with a random uni-bi mechanism. In contrast, an ordered uni-bi mechanism is expected for a phosphoenzyme intermediate, since the product alcohol would be released before the phosphoenzyme intermediate is hydrolyzed. Although these results are consistent with a direct transfer mechanism, they are not conclusive, since they can also be interpreted to indicate a mechanism involving a transient phosphoenzyme intermediate whose breakdown is not rate limiting.

The most definitive work that addressed whether a phosphoenzyme intermediate was formed during catalysis for the metallophosphatases was performed with bo-
vase spleen purple acid phosphatase. Using a chiral \([^{18}O,^{17}O]\)phosphorothioate ester and carrying out the hydrolysis in \([^{16}O\)H\(_2\)O, Knowles and co-workers (288) demonstrated that purple acid phosphatase carries out hydrolysis with net inversion of configuration at the phosphorus center, thus indicating that the phosphoryl group is transferred directly to water. Because of the similarity of the active sites of calcineurin and PP1 to purple acid phosphatases, it is thought that the catalytic mechanism of the serine/threonine phosphatases also proceeds by a similar mechanism.

B. Catalytic Role of the Dinuclear Metal Center

Several pieces of data indicate that the dinuclear metal center is a key component of the active site of calcineurin. 1) As already mentioned, the dinuclear metal center has a ligand environment similar to purple acid phosphatases, enzymes which contain dinuclear Fe-Fe or Fe-Zn centers previously demonstrated to be essential for catalytic activity (223, 433). 2) Crystallographic (91, 124, 201) and spectroscopic (80, 334, 416, 448, 449, 470) studies indicate that the product of the reaction, phosphate, and product analogs such as tungstate, molybdate, and arsenate, coordinate the metal ions. 3) Redox titrations of either the Fe\(^{3+}\)-Zn\(^{2+}\) or Fe\(^{3+}\)-Fe\(^{2+}\) forms of calcineurin and purple acid phosphatase indicate a correlation between enzyme activity and the oxidation state of the bound metal ions (8, 9, 18, 77, 470, 471).

The metal ions of the dinuclear center could function in numerous ways to catalyze phosphate ester hydrolysis. The Lewis acidity of the metal ion(s) could serve to activate a solvent molecule, a well-known mechanism in several metalloenzymes such as carbonic anhydrase (132a) and adenosine deaminase (459). A metal-activated water molecule has been proposed for purple acid phosphatases (85, 432). Alternatively, a metal-coordinated solvent molecule could serve as a general acid to donate a proton to the leaving group, as has been proposed for inorganic pyrophosphatase (140, 355). In addition to a role in activation of the solvent nucleophile, the metal ions in the serine/threonine phosphatases could be involved in other aspects of the catalytic mechanism. Metal coordination of the phosphate ester could have several positive effects acting to accelerate hydrolysis. Neutralization of the negative charge on the oxygen atoms of the phosphate ester oxygen atoms would increase the electrophilicity of the phosphorus atom, making it more prone to nucleophilic attack. During P-O bond scission, the metal ions could stabilize the developing charge on the leaving group. However, another possible role for the metal ions could be to orient the substrate for in-line attack. These are discussed in section V C in the context of specific active site residues and the effect that mutagenesis of these residues has on catalytic efficiency.

C. Conserved Active Site Residues

In addition to the metal ions and their cognate protein ligands, several conserved residues within 4–9 Å of the dinuclear metal cofactor are involved in catalysis. One of these is a histidine residue that is not a metal ligand but is within 5 Å of either metal ion. The conserved histidine in calcineurin, His-151, is also conserved in PP1 (His-125), purple acid phosphatases (His-202, kidney bean enzyme numbering), and \(\lambda\)-protein phosphatase (His-76). It is represented in the phosphoesterase motif as the underlined residue of Figure 5 (244). This histidine is hydrogen bonded to an aspartic acid that is also part of the phosphoesterase motif, Asp-121 in calcineurin, Asp-95 in PP1, Asp-169 in kidney bean purple acid phosphatases, and Asp-52 in \(\lambda\)-protein phosphatase. Two arginines, Arg-122 and Arg-254, are also present in the active site of calcineurin. These arginines are also conserved in other metallophosphatases and correspond to Arg-96 and Arg-221 in PP1 (120) and Arg-53 and possibly Arg-162 in \(\lambda\)-protein phosphatase. Figure 10 depicts the active site of calcineurin in the phosphate-inhibited form showing the conserved nonligand residues. The corresponding residues in kidney bean purple acid phosphatases are depicted in Figure 11. In purple acid phosphatase, two histidines (His-295 and His-296) are substituted for two arginines in the serine/threonine phosphatase family.

The importance of these residues and their potential role in catalysis has in most cases been addressed by
enzymatic and spectroscopic studies of enzymes in which these residues have been altered by site-directed mutagenesis. The interpretation of these data assumes that mutagenesis alters only the physical-chemical features of the residue of interest and does not alter some other requisite structural feature, (e.g., tertiary structure, metal binding, etc). A review of the primary literature indicates that this assumption has not always been defended by rigorous structural studies. The reader is therefore cautioned that the roles ascribed to some of these active site residues are still tenuous.

1. Role of the histidine/aspartate pair in the metallophosphatases

Studies have shown that mutation of His-151 in calcineurin, or its analog in $\lambda$-protein phosphatase, His-76, leads to significant reductions in enzyme activity (272, 487). When its hydrogen-bonding partner (corresponding to Asp-121 in calcineurin) is mutated, a $10^1$- to $10^2$-fold decrease in the rate constant for catalysis ($k_{cat}$) occurs with little effect on $K_m$ (161, 475, 487). One role considered for this histidine has been as an active site nucleophile. As discussed above, the data argue against this since a phosphoenzyme intermediate probably does not form during the catalytic cycle. Alternatively, this histidine may have a role in binding substrate, orienting the nucleophilic water molecule, and/or in acid/base catalysis. These possibilities are discussed in the following sections.

A) Acid/Base Catalysis. A series of kinetic studies were undertaken to study the effect of mutating the conserved histidine in calcineurin (His-151) and $\lambda$-protein phosphatase (His-76) (272, 283). In both mutant enzymes there were significant reductions in enzyme activity but only small effects on $K_m$ (272, 283). This was the case using $p$-NPP and the [P]-R$_{II}$ peptide as substrates for calcineurin, and $p$-NPP and phenyl phosphate for $\lambda$-protein phosphatase. These substrates were chosen to explore the role of this histidine as a proton donor. A phosphate ester such as $p$-NPP has a better leaving group ($pK_a$ of $p$-nitrophenol = 7.2) compared with either phenyl phosphate or a phosphoseryl peptide ($pK_a$ of phenol = 10, $pK_a$ serine side chain -OH ~ 14). Therefore, if protonation of the leaving group occurs in the transition state, hydrolysis of $p$-NPP will require less catalytic assistance than either phenyl phosphate or a phosphoseryl peptide such as [P]-R$_{II}$ Peptide. As a result, one would expect larger effects on relative $k_{cat}$ (wild-type $k_{cat}$/mutant $k_{cat}$) for substrates with poorer leaving groups. The results showed less than a threefold difference in relative $k_{cat}$ for calcineurin using $p$-NPP versus the [P]-R$_{II}$ Peptide, despite a difference in $pK_a$ for the respective leaving groups of $>10^6$ (272). With $\lambda$-protein phosphatase, mutagenesis of His-76 to Asn (H76N) resulted in the same 500- to 600-fold reduction in $k_{cat}$ using either $p$-NPP or phenyl phosphate as substrates (leaving group $pK_a$ difference $>10^3$) (272). Ideally, more than two substrates with varying $pK_a$ values should be used in this type of Brønsted analysis.

A useful method to determine if a residue is acting as an acid or base in catalysis is to follow the pH dependence of the rate for wild-type and mutant enzymes. Often one arm of the usual bell-shaped dependence curve is missing for the mutant if the residue is acting as a general acid or base. Using $p$-NPP as a substrate, pH dependence studies for wild-type and H76N $\lambda$-protein phosphatase were performed (156). The mutant enzyme had a lower catalytic rate at every pH but still exhibited a bell-shaped pH curve. However, kinetic data were difficult to obtain at low pH due to substrate inhibition that necessitated higher metal ion concentrations. In summary, the difficulties encountered were such that it was not possible to conclude whether this histidine serves as a general acid in catalysis. It was concluded that this residue may function more decisively as a general acid in reactions of phosphoprotein substrates that have a much poorer leaving group. It is noteworthy that the pH optimum for the mutant appeared to be shifted to pH 7.0, compared with 7.8 for the wild-type enzyme. Also the $K_m$ for substrate increased at high pH for the native enzyme to values that were similar to those of the mutant enzyme at all pH values, indicating that protonation of this residue may assist in substrate binding.

B) Kinetic Isotope Effects. Kinetic isotope effect studies have been performed with both calcineurin (150, 260, 261)
and λ-protein phosphatase (156) to study the chemistry of the transition state. In the case of calcineurin, the use of D$_2$O to measure a solvent isotope effect found no effect on $k_{cat}$ but a modest isotope effect (1.35) on $k_{cat}/K_m$ (260). The lack of a solvent isotope effect on $k_{cat}$ may be due to having some other step in the reaction mechanism other than the proton transfer step be rate limiting, a situation that was confirmed in a subsequent study that used heavy atom isotopes of p-NPP (150). In the latter study it was shown that P-O bond cleavage was partially rate limiting at the pH optimum, and therefore, the isotope effects were suppressed, a situation which could be improved upon raising the pH from 7.0 to 8.5. For the λ-protein phosphatase reaction, P-O bond cleavage was fully rate limiting, and the measured isotope effects represented the intrinsic isotope effects on the bond-breaking step of the catalytic mechanism. With both enzymes, the data indicate that the substrate of the reaction is the p-NPP dianion, the predominant form at neutral pH ($pK_a = 4.96$). The isotope effect studies also provide evidence for a dissociative mechanism, in which the transition state has substantial P-O bond cleavage before bond formation to the nucleophilic water molecule. A dissociative mechanism has also been observed for the protein tyrosine phosphatases (148, 151, 152) and for uncatalyzed reactions in solution (149). This result is important because it demonstrates that the metal ions do not change the transition state of the phosphoryl transfer reaction to become more associative, a hypothesis that was previously forwarded based on the idea that metal ions could stabilize the extra negative charge in the transition state that results from bond formation to the nucleophile (139). Instead, the transition state of the phosphoryl group looks very much the same as in the solution reaction.

The $^{15}$N kinetic isotope effects indicate that in calcineurin and wild-type λ-protein phosphatase, there is substantial charge neutralization of the leaving group in the transition state (150, 156). In contrast, the magnitude of this charge increases when His-76 of λ-protein phosphatase is mutated to asparagine. This result suggests that this histidine may be protonating the leaving group in the transition state; its absence would lead to a greater charge accumulation on the leaving group compared with the wild-type enzyme. Interestingly, the magnitude of negative charge on the leaving group is smaller than expected for the mutant enzyme compared with comparable studies with protein tyrosine phosphatases that have the active site general acid removed by mutagenesis. Indeed, the magnitude of the isotope effect in the mutant is significantly less than would result from a full negative charge on the departing p-nitrophenol product. One explanation that was forwarded is that the metal ions may participate in stabilizing the transition state of the reaction by neutralizing the developing negative charge on the leaving group.

C) POTENTIAL ROLE IN SUBSTRATE BINDING. Another possible role of His-76/His-151 could be to assist in substrate binding. With λ-protein phosphatase, the $K_m$ for p-NPP increased from 1 to 70 mM with increasing pH (156). In contrast, the $K_m$ for p-NPP in the λ-PP(H76N) mutant was higher at low pH compared with wild type. As the pH was increased to neutral pH and greater, the $K_m$ values for both enzymes became similar. It is possible that at acidic and neutral pH, where the histidine would be protonated, this residue assists in substrate binding via electrostatic interactions. For example, a proton on His-76 may form a hydrogen bond with one of the substrate oxygen atoms of the phosphorylated substrate. In X-ray structures of other serine/threonine phosphatases with bound inhibitors, phosphate or tungstate, this histidine is within hydrogen bonding distance of the most solvent exposed oxygen atom of the inhibitor (91, 124). A similar orientation is observed with purple acid phosphatases with phosphate or tungstate bound (128, 201, 425). This type of electrostatic interaction will not affect the isotope effect on $^{18}$V/K$_{nonbridge}$. However, if the substrate were actually protonated in the transition state, this would have been revealed in the $^{18}$(V/K)$_{nonbridge}$ isotope effect; this was not observed (156). It is not clear if His-76 really does participate in substrate binding, since at basic pH it would be deprotonated. The pH optimum for the wild-type enzyme is ~8.0, where both the wild-type enzyme and mutant have similar $K_m$ values for p-NPP.

D) GENERAL BASE CATALYSIS. Another possibility for His-151/His-76 would be to act as a general base to deprotonate the metal-bound water molecule that is the nucleophile in the mechanism. In one X-ray structure of calcineurin, the $N_e$ of His-151 is hydrogen-bonded to one of two terminal solvent molecules coordinated to the Fe ion. Considering also Asp-121, which is hydrogen bonded to His-151, the interaction of this histidine/aspartate pair with the metal-coordinated solvent molecule is analogous to the Asp-His-Ser catalytic triad of serine proteases, where an Asp/His pair is important for interacting with the nucleophilic serine residue. The “Asp-His-HO-metal” motif in the serine/threonine phosphatases can be thought of as a catalytic tetrad, with the metal ion serving to lower the $pK_a$ of the nucleophilic water molecule while the Asp-His functions as a catalytic base to assist in hydroxide formation. Histidines in other metalloproteins are thought to perform a similar role by acting as a base to deprotonate a metal-bound water molecule or by assisting in stabilizing a metal-bound hydroxyl group. For example, His-372 in E. coli alkaline phosphatase forms a hydrogen bond with Asp-327 (a bidentate Zn ligand) and is thought to lower the $pK_a$ of a zinc-bound water molecule (464). Mutagenesis studies of Asp-327 to asparagine result in increased enzyme activity, since the negative charge of the hydroxyl group is more stable due to the loss of the aspartate side chain and replacement with a neutral res-
idue. However, this comes at the expense of Zn binding since a carboxylate is a better metal ligand than a carboxamide, and thus a higher concentration of Zn in the assay is needed to achieve this increased activity. Examples of other enzymes where a histidine is postulated to deprotonate a metal-coordinated water molecule include His-320 in Klebsiella aerogenes urease (172, 321), His-231 in thermolysin (27), and His-89 in Serratia nuclease (109).

E) ORIENTATION OF THE NUCLEOPHILIC HYDROXIDE SOLVENT MOLECULE. Another potential role of His-76/His-151 could be to position the metal-coordinated hydroxide for optimal in-line attack of substrate, a mechanism analogous to the concept of “orbital steering” proposed by Storm and Koshland (393) almost 30 years ago to provide an explanation for the great rate accelerations seen in enzyme catalysis. The theory of orbital steering postulates that a major factor in catalytic enhancement is that an enzyme arranges the reaction trajectory to optimize the overlap of attractive (bonding) orbitals and minimize the overlap of repulsive (nonbonding) orbitals. This concept has been debated and contested by numerous groups (39, 174, 270). Nevertheless, in a recent study of isocitrate dehydrogenase by Koshland and co-workers (273), small structural perturbations in isocitrate dehydrogenase were created to evaluate the contribution of precise substrate alignment to the catalytic rate of an enzyme. They found that small changes in the orientation of substrates had large effects on reaction velocity (10^3- to 10^5-fold decreases). Their main conclusion was that orbital steering is an important contribution to the catalytic power of enzymes.

A role in orientation of metal-bound water was proposed for His-238 of murine adenosine deaminase (459) and is possible for the conserved histidine in the serine/threonine phosphatases. Interestingly, small perturbations in the EPR spectra of the dinuclear metal center in CN(H151Q) and λ-PP(H76N) enzymes were observed compared with the wild-type enzymes (272), indicating subtle perturbations to the geometry of the dinuclear metal center. X-ray structures of these mutants would be valuable to obtain better information on whether substrate orientation may be affected.

2. Role of arginines in the active site

As shown in Figure 10, there are two arginine residues in the active site of calcineurin, Arg-122 and Arg-254. These arginine residues are conserved in other phosphatases. Mutagenesis of Arg-122 in calcineurin or its homologs in other phosphatases (Arg-96 in PP1 and Arg-53 in λ-protein phosphatase) resulted in 10^2- to 10^3-fold decreases in k_{cat} and only slight changes in K_m (161, 283, 475, 487). An exception to this was a 20-fold increase in K_m when Arg-53 in λ-protein phosphatase was mutated to an alanine and assayed in the presence of Ni^{2+} (487). However, when this mutant was assayed in the presence of Mn^{2+}, there were no significant changes in K_m. Site-directed mutagenesis studies of Arg-254 in calcineurin or Arg-221 in PP1 resulted in an 200-fold reduction in k_{cat} while values for K_m increased 2- to 10-fold (161, 283). In the X-ray structures of wild-type calcineurin and PP1, the guanidine groups of these arginine residues form salt bridges with the oxygen atoms of bound phosphate or tungstate and stabilize the anion inhibitor (91, 120, 124, 197). The purpose of these arginine residues may be to provide electrostatic stabilization for binding the negatively charged phosphate ester. In addition, they may help neutralize developing negative charge in the transition state. Because the mutation of Arg-122 led to greater decreases in k_{cat} than mutation of Arg-254, it may be that Arg-122 has a more important role in catalysis, perhaps involving stabilization of the transition state. To note, in the active site of kidney bean purple acid phosphatase (Fig. 11), two histidine residues, His-295 and His-296, appear in place of these arginine residues, and it has been suggested that these substitutions might explain the lower pH optimum of purple acid phosphatases (201). Krebs and colleagues (201) have hypothesized that one of these histidines may be the active site residue with an apparent pK_a of 6.9 that produces the basic pH portion of the bell-shaped pH/kinetic profile. In an analogous manner, it is possible that Arg-53 in λ-protein phosphatase produces the basic pH arm observed in the pH dependence studies (156). In mammalian purple acid phosphatases, there is a histidine residue (His-195) corresponding to His-296 of kidney bean purple acid phosphatase, but Glu-194 substitutes for His-295 in the mammalian enzyme and is oriented away from the phosphate in the oxidized enzyme-phosphate complex.

D. A Model for the Calcineurin Catalytic Mechanism

Taking into account the available biochemical and chemical data, we propose a model for the catalytic mechanism of calcineurin and other members of this family (Fig. 12). The first step of the mechanism involves association of the phosphate monoester, as the dianion, with the enzyme (Fig. 12A). In this step, neutralization of negative charge by the metal ions may occur. X-ray structures of calcineurin and PP1 show phosphate and other anionic product inhibitors coordinated to both metal ions, inferring that the substrate phosphoryl group might also coordinate to one or both metal ions at some point during catalysis. In addition, the conserved arginines, Arg-122 and Arg-254, may also play a role in binding substrate and neutralizing charge by forming hydrogen bonds with the oxygen atoms of the phosphoryl group. The intermediate in Figure 12A shows the Zn atom and the two conserved arginines assisting in substrate binding/neutralization of charge.
This neutralization of charge would make the substrate more electrophilic and ready for attack by a nucleophile.

The interaction between the metal-bound water molecule and the conserved histidine His-151 in calcineurin is also depicted in Figure 12. In the first step of this mechanism (Fig. 12A), His-151 is functioning as a general base to remove the proton from the metal-bound water molecule. Alternatively, it may orient the solvent nucleophile for optimal nucleophilic attack of the phosphate ester.

Kinetic isotope studies of calcineurin and λ-protein phosphatase show that the transition state of the reaction is dissociative. A dissociative transition state is represented in Figure 12B, where bond cleavage to the leaving group has occurred before bond formation to the nucleophile. The phosphoryl group in a dissociative mechanism resembles the metaphosphate anion (147).

A metal-bound water hydroxide coordinated to Fe$^{3+}$ is shown as the attacking nucleophile in Figure 12B, with the Fe$^{3+}$ functioning as a Lewis acid to lower the pK$_a$ of the water molecule. Extensive redox studies by Yu and co-workers (470, 471) have demonstrated a requirement for Fe$^{3+}$ by calcineurin and a loss of activity upon reduction to Fe$^{2+}$, a result consistent with a decreased Lewis acidity of Fe$^{3+}$ versus Fe$^{2+}$.

P-O bond scission in the transition state results in a significant negative charge on the leaving group. Neutralization of the charge by protonation (general acid catalysis) or coordination to a metal ion would lower the energy of the transition state and increase the rate of the reaction. Kinetic isotope studies indicate that considerable charge is neutralized in the transition states of calcineurin and λ-protein phosphatase. His-151 may play a role in this charge neutralization (Fig. 12B). It is also possible that one of the metal ions (e.g., Zn$^{2+}$ in Fig. 12B) neutralizes...
the charge to the leaving group by coordination. Another possibility is that a metal-bound solvent molecule acts as a general acid as has been proposed for the mechanism of inorganic pyrophosphatase (140, 355). In addition, the two conserved arginines in the active site may also be important for charge neutralization and transition state stabilization. After bond cleavage and proton transfer to the leaving group occur, the result is the product-inhibited state that has a molecule of orthophosphate bridging the two metal ions of the dinuclear center (Fig. 12C). Evidence for this intermediate is obtained in the X-ray structure of the product-inhibited enzyme which shows phosphate coordinated to both metal ions as depicted (124). An identical coordination is observed in phosphate and tungstate complexes of PP1 (91, 395) and the phosphate complex of purple acid phosphatase (128, 201). Phosphate release, perhaps by exchange with a solvent molecule, regenerates the enzyme for another turnover.

VI. REGULATION

The classical mechanism by which calcineurin is regulated in vivo is via changes in intracellular \( \text{Ca}^{2+} \). Thus, in a resting cell where \([\text{Ca}^{2+}]\) is low, calcineurin is unable to bind calmodulin, and the enzyme exists in an inactive form. In signaling pathways that lead to a rise in intracellular \( \text{Ca}^{2+} \), \( \text{Ca}^{2+} \) binding to calmodulin results in a conformational change, thereby allowing it to bind to calcineurin and activate its phosphatase activity (203). \( \text{Ca}^{2+} \) binding to calcineurin B also appears to play a role (389).

Calcineurin activity has also been shown to be affected by phospholipids, with either activation or inhibition resulting depending on the phospholipid and substrate investigated (163, 329, 330). Recently, recombinant Dictyostelium calcineurin has been shown to be activated by arachidonic acid and unsaturated, long-chain fatty acids (184). These effects may be physiologically significant given the fact that calcineurin is found associated with membranes during fractionation.

Recently, an additional mechanism for regulating calcineurin involving redox reactions of active site metal ions has been considered (350, 447). Previous studies demonstrated that calcineurin is susceptible to redox regulation in vitro (470, 471), a process that may also occur in vivo. Wang et al. (447) found that superoxide dismutase protects calcineurin from inactivation and hypothesized that this might occur by preventing oxidation of active site metals ions. Recently, a number of groups have begun testing this hypothesis and have provided evidence that calcineurin activity can be affected by extracellular oxidants, in particular, \( \text{H}_2\text{O}_2 \) (45, 111, 345). Thus exposure of cells to micromolar concentrations of \( \text{H}_2\text{O}_2 \) results in inhibition of NF-AT (111, 345) or NFkB-mediated processes (45) and appears to be mediated by calcineurin.

The mechanism for this regulation may reside in the redox-active \( \text{Fe}^{3+} \) of the active site dinuclear metal center, which can toggle between reduced (\( \text{Fe}^{2+} \)) and oxidized (\( \text{Fe}^{3+} \)) states. Indeed, redox titrations by Yu and co-workers (470, 471) previously demonstrated that the mixed-valence oxidation state, either \( \text{Fe}^{3+}-\text{Zn}^{2+} \) or \( \text{Fe}^{3+}-\text{Fe}^{2+} \), is required for enzyme activity; reduction to the \( \text{Fe}^{2+}-\text{M}^{2+} (\text{M} = \text{Zn}, \text{Fe}) \) state led to loss of activity (470, 471). At first glance, the in vivo results, which suggest that oxidation (i.e., treatment with \( \text{H}_2\text{O}_2 \)) results in inactivation, appear contrary to the in vitro results. One hypothesis that has been forwarded to reconcile this discrepancy is that calcineurin may exist in different forms containing either Fe-Zn or Fe-Fe dinuclear metal centers. Because the \( \text{Fe}^{3+}-\text{Fe}^{2+} \) form of calcineurin can lose activity by oxidation to the \( \text{Fe}^{3+}-\text{Fe}^{3+} \) state, it may be that calcineurin exists in oxidation-sensitive (\( \text{Fe}^{3+}-\text{Fe}^{2+} \)) and oxidation-inert (\( \text{Fe}^{3+}-\text{Zn}^{2+} \)) states in vivo.

Further work is obviously necessary to determine whether calcineurin is a specific mediator for changes in the redox state of the cytosol. If the dinuclear center of calcineurin has a standard redox potential near the physiological state of the cytosol, a mechanism whereby small alterations in the redox state could mediate changes in enzyme activity would be highly tenable.

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CALCINEURIN: FORM AND FUNCTION


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