Structure/Function Studies
of
Protein Phosphatases

by

Rachel Natanya Brenner

Submitted to the Department of Chemistry in Partial
Fulfillment of the Requirements for the
Degree of

MASTER OF SCIENCE IN CHEMISTRY
at the

Massachusetts Institute of Technology
June 1995

©1995 Rachel N. Brenner
All Rights Reserved

The author hereby grants to MIT permission to reproduce and to distribute
publicly paper and electronic copies of this thesis document in whole or in
part

Signature of Author .................................................................
Department of Chemistry
May 31, 1995

Certified by .................................................................
Jun Liu
Assistant Professor of Chemistry and Biology

Accepted by .................................................................
Dietmar Seyferth
Departmental Committee on Graduate Studies
ABSTRACT
A high degree of homology exists within the catalytic subunits of many ser/thr phosphatases, notably PP1, PP2A, and PP2B. These enzymes are regulated by regulatory proteins in trans, or by inhibitory elements within the catalytic subunit itself. In order to better understand both the mechanism of catalysis which these phosphatases most likely share, as well as the intricacy of the multi-domained structure of calcineurin, a study was initiated on lambda phosphatase and calcineurin(PP2B) truncation mutants. Calcineurin clones containing only the N-terminal sequence which corresponded to the entire lambda phosphatase sequence (as viewed from sequence alignments) were completely inactive in the pNPP and phosphoRII peptide phosphatase assays, although less severe truncations did display catalytic activity. In keeping with reported results, the Calcineurin B regulatory protein greatly stimulated the activity of one of the truncation mutants which retained a Calcineurin B binding domain.

Thesis Supervisor: Dr. Jun Liu
Title: Assistant Professor of Chemistry and Biology
CHAPTER 1

Kinetic Studies of λ Phosphatase
Protein kinases have long been regarded as essential for regulation of a wide range of biochemical processes; it is only relatively recently that protein phosphatases have gained recognition as important regulatory molecules as well. Protein phosphatases can oppose the transient effects of protein kinase stimulation or stimulate processes themselves which can be likewise antagonized and regulated by kinase action. Phosphatases have been implicated in such diverse functions as transcriptional control, cell cycle, cell differentiation, glycogen metabolism, growth factor responses, development, and many other cellular processes.

There are two main classes of phosphatases, categorized based on substrate specificity: protein tyrosine phosphatases (PTPases) and serine/threonine phosphatases. (The presence of in vivo phosphohistidine has been documented, having gone unnoticed until relatively recently due to the lability of the phosphohistidine bond to standard amino acid sequencing/analysis conditions; some of the tyr/ and ser/thr phosphatases, including PP1, PP2A, and PP2C, have shown some phosphohistidine phosphatase activity in in vitro assays.--Kim et al., 1993) These two classes of enzymes appear to have quite different catalytic mechanisms, as well as different substrate specificities.

Protein tyrosine phosphatases have been studied extensively in the past several years. With the partial amino acid sequence determined for PTP1B in 1988 (Tonks et al., 1988), a new family of protein phosphatases was defined. Some protein tyrosine phosphatases contain sequences homologous to known domains which target the phosphatase to specific subcellular compartments, thereby effectively limiting the amount of possible substrates by lack of availability. They may also contain protein binding domains, such as SH2 and SH3 domains. These binding domains, located outside of the active site of the enzyme, may afford a more stable interaction with particular substrates able to bind these sequences. Some PTPases, such as hLAR, carry cytoskeletal domains which imply a role for PTPases in cell architecture and morphology. Other PTPases include dLAR, which is expressed in the central nervous system in drosophila, and, with its extracellular domain similar to N-CAM (neural cell adhesion molecule), may be involved in axonal development; VH1 from vaccinia virus, with as yet undetermined function; dPTP61F in drosophila with its two isoforms, generated by alternate splicing--one with a hydrophobic C-terminus which localizes to the particulate fraction, and one with a basic C-terminus which is targeted to the nucleus, potentially involved in transcriptional regulation; Cdc25, which activates MPF by dephosphorylation thereby triggering entry into the cell cycle; and many others (Walter and Dixon, 1993).

Mechanistic studies on CD45 and LAR include analysis of Cys→Ser point mutations in the wildtype enzyme and labeling studies with the sulfhydryl modifier iodoacetimide (14C-labeled iodoacetimide labels the enzyme stoichiometrically, with concomitant inactivation of phosphatase activity). These experiments, along with the detection of a “trapped”
phosphoenzyme intermediate, demonstrate that the mechanism of catalysis proceeds via a phosphocysteinyl enzyme intermediate (reviewed in Walton and Dixon, 1993). Catalytically inactive PTPases with this characteristic cysteine mutated to serine have been used as reagents to probe for substrates, as in the case of PTP1B where the catalytically inactive Cys→Ser mutant was shown to bind the epidermal growth factor receptor, a receptor tyrosine kinase, in vivo by immunoprecipitation of the EGFR/PTP1BCS complex (Milarski et al., 1993). The crystal structure of the bovine liver low molecular weight protein tyrosine phosphatase has been determined. This study defines apatentive phosphate binding pocket, occupied by a sulfate anion (the protein was crystallized from an ammonium sulfate solution) which purportedly corresponds to the phosphate of a phosphoprotein substrate. The pocket is composed of a stretch of seven continuous residues, including several basic residues, such as Arg18, which would make contacts to the phosphate. This pocket is also lined with two cysteine residues, one of which has been proposed to initiate a nucleophilic attack on the phosphate, generating a Cys12 phosphoprotein intermediate. Both the low molecular weight PTPase and PTP1B share a CXXXXXR motif.

Protein serine/threonine phosphatases constitute the other major family of protein phosphatases. Ser/thr phosphatases were originally classified according to their substrate specificity (mainly activity toward phosphorylase kinase), metal requirements, and susceptibility toward inhibitor proteins. PP1, PP2A, and PP2B share homology in the amino acid sequences of their catalytic subunits, whereas PP2C seems to be the product of an evolutionarily unrelated gene. PP1 has increased specificity for the β subunit of phosphorylase kinase over the α subunit and its activity is blocked by inhibitor-1 and inhibitor-2, whereas PP2A, PP2B, and PP2C preferentially dephosphorylate phosphorylase α and their activity is not blocked by inhibitors 1 or 2. The phosphatase activity of PP2C is Mg²⁺-dependent, while the activity of PP2B (= calcineurin) is Ca²⁺-dependent and is further activated by Mg²⁺ and Mn²⁺ (reviewed in Cohen, P. et al., 1988).

While it seems likely that the catalytic mechanism for dephosphorylation would be conserved for PP1, PP2A, and PP2B with the striking conservation of tens of residues found in the N-terminal half of these enzymes (see Figure 1), substrate specificity varies widely. Much of this ability for similar catalytic subunits to discriminate between substrates is probably conferred by the association of several different types of regulatory subunits with the catalytic subunits. Both the catalytic and regulatory subunits of each of the four previously mentioned phosphatases (PPases) have several isoforms as well. Taking into account the large number of different possible holoenzymes that can be generated along with the effects of subcellular localization, one can imagine an exquisitely fine-tuned substrate specificity, far beyond what was originally recognized, for ser/thr protein phosphatases.

PP1 has been shown to associate with a wide variety of regulatory subunits in mammalian cells, often in a tissue-specific manner. For example, the G subunit (glycogen-binding subunit) which is expressed only in skeletal muscle, mediates the association of PP1 with glycogen,
thereby bringing PP1 into close proximity of its phosphoenzyme substrates involved in glycogen metabolism (reviewed in Shenolikar, 1994). In muscle tissue, PP1 associates with the M complex (composed of two proteins, 130kD and 25kD) and binds to smooth muscle myosin (Dent et al., 1992). Dephosphorylation of myosin results in muscle relaxation (Gong et al., 1992).

Hagiwara et al. (1992) have implicated PP1 in hormone signalling pathways: they find that the attenuation of the cAMP response pathway is dependent on functional PP1. The cAMP response pathway operates by activating CREB, a transcriptional activator/binding protein which binds to CRE’s (cAMP Response Elements). Dephosphorylation of the activated CREB at Ser13 correlates with a decrease in CRE- specific transcription. Cotransfection of neuronal PC-12 cells with PP1 causes decreases in levels of CRE-reporter gene transcription from a CRE-CAT reporter plasmid, consistent with the deactivation of active CREB by PP1-catalyzed dephosphorylation. PP2A cotransfection, on the other hand, actually increased CAT activity in vitro. In in vitro assays, the presence of inhibitor-2 (a protein inhibitor specific for PP1) causes an increase in CREB phosphorylation in nuclear extracts. Microinjection of inhibitor-1 into the cytoplasm of CRE-CAT transfected REF-52 cells or cotransfection of a plasmid encoding inhibitor-1 likewise caused an increase in CAT activity as compared to control cells microinjected with IgG1. These results support an in vivo role for PP1 in attenuation of hormone stimuli which activate cAMP-dependent pathways.

PP1 has also been shown recently to bind to human Rb (retinoblastoma tumor suppressor gene), by means of the yeast two-hybrid system, immunoprecipitation, and biochemical assays involving phosphatase inhibitors. This association is abrogated in late G1 as cells progress through the cell cycle and Rb becomes hyperphosphorylated and unable to bind to DNA. PP1 activity has been shown, by okadaic acid inhibition studies, to reside mainly in the nucleus during G2-M (5:1 Nuclear PP1: Cytoplasmic PP1), and during all other stages of the cell cycle PP1 activity in nuclear extracts is about twice the amount of activity associated with the cytoplasmic and particulate fractions (Dohadwala et al., 1994). In similar experiments, microinjection of PP1 into either the nucleus or the cytoplasm of REF-52 (Rat Embryo Fibroblast) cells 10-12 hours after serum stimulation of G0 cells corresponded to an increased nuclear localization of Rb and reduced levels of BrdU incorporation, as detected by immunofluorescence staining of normal and in-situ nucleus-extracted cells. Such findings suggest a link between G1 arrest and the phosphorylation state of Rb, as influenced by PP1 activity. Involvement of PP2A is possible as well, as microinjection of PP2A C subunit (but not AC) had similar effects on Rb localization and BrdU incorporation (implying perhaps that a different endogenous A subunit is conferring this substrate specificity/activity on the C subunit of PP2A). (Alberts et al., 1993b).

In addition to the regulatory influences of additional subunits on PP1, the catalytic subunit itself has been proposed to be regulated by covalent modification—specifically, by phosphorylation. A C-terminal sequence of the catalytic subunit of PP1, T-P-P-R, previously
identified as a motif which can be phosphorylated by a cyclin-dependent kinase (Cdc2/Cyclin A), has been shown to be phosphorylated by cdk in vitro. This phosphorylation inactivates PP1 activity toward phosphorylase a substrate. These experiments further support a role for PP1 in regulation of the cell cycle.

PP2A is involved in a wide variety of cellular processes as well. The holoenzyme is composed of three subunits— a catalytic subunit as well as A and B regulatory subunits. Again, there are several different genes encoding these subunits in a given organism, as well as different spliceoforms of each gene, giving rise to a huge number of possible PP2A activities with differing substrate specificities.

In S. cerevisiae, a connection has been found between PP2A and RNA Polymerase III. tpd3 strains (with mutations in the yeast PP2A A subunit homologue, Tpd3) exhibit defective transcription of tRNA genes. Extracts of tpd3 cells grown at 23°C and assayed for tRNA synthesis in in vitro transcription reactions using a tRNA3Glu DNA template were not competent for tRNA transcription as compared to TPD3 extracts. Tpd3 mutant extracts grown at 37°C had undetectable levels of tRNA transcription. Reconstitution experiments in which purified RNA polymerase III or TFIIB (part of the initiation machinery of pol III), but not TFIIC, were added to tpd3 extracts supported tRNA synthesis to levels similar to that of TPD3 extracts treated similarly. These results indicate that the A subunit of PP2A plays some role in pol III regulation. Interestingly, the loss in function is not reversible by addition of okadaic acid to the transcription reactions. This finding suggests that increased phosphatase activity of the uncomplexed C subunit cannot be the cause of the transcription defect. Rather, Van Zyle et al. (1992) suggest, changes in substrate specificity may induce PP2A phosphatase activity toward a protein which then becomes inhibitory to the transcription apparatus by interaction with one or more of its components. Changes in the phosphorylation state of one of the subunits of RNA pol III itself could be affected by the alteration of PP2A substrate specificity as well (Van Zyle et al., 1992; Shenolikar, 1994). Arndt et al. (1989) have similarly discovered a gene which seems to be involved in RNA pol II transcription called SIT4, which encodes a 36 kD protein with 50% homology to the bovine PP2A catalytic subunit. sit4 mutants exhibited increased levels of HIS4 gene transcription (normally transactivated by three other genes which have been knocked out in the S. cerevisiae strain used in this study). It is unknown whether RNA pol II is directly activated by dephosphorylation mediated by the SIT4 gene product, or if the linkage is less direct, perhaps involving the dephosphorylation and activation of another transcription factor.

PP2A has also been implicated in drosophila development. Drosophila possesses, in addition to a catalytic subunit with 94% homology to the human PP2A catalytic subunit, an A subunit (Pr65) and a B subunit (Pr55–78% homologous to the human PR55α, human PR55β, and S. cerevisiae Cdc55). B subunit mRNA is present in drosophila imaginal discs at increased levels, and drosophila with a mutatedPr55 gene (i.e. the twins mutation) develop altered wings. The aar mutant (abnormal anaphase resolution) possesses a mutation which is linked to
twins, and whose phenotype can be rescued by either the wild type gene for twins, or the wild type PR55 gene. aar mutants display a range of phenotypes, including overcondensed chromosomes, hyperploidy, and lagging chromosomes which are defective in pole migration (Gomes et al., 1993). The aar mutation is lethal at the late larval-early pupal stage and can be rescued with the wild type PR55 gene (Mayer-Jaekel et al., 1993). In other organisms, though, like S. Cerevisiae, it seems that there is a functional redundancy regarding PR55, as Cdc55 null mutants grow nearly normally and Cdc55 mutants have a different set of phenotypes from the Drosophila aar mutants (Healy et al., 1991).

Another area in which PP2A functions is signal transduction. The A subunit has been demonstrated to be a key regulator of specificity in several studies involving the effect of PP2A activity on c-jun transcriptional activation. In vivo experiments show that F9 cells transfected with a CAT reporter gene downstream of an AP-1 site as well as c-jun under an RSV promoter show three times as much CAT activity when cotransfected with the PP2A C subunit than control levels or levels obtained when the cells are cotransfected with AC complexes of PP2A. Cells not transformed with c-jun show an increase in CAT activity when cotransfected with PP2Ac as well, supporting a role for endogenous c-jun, as well as overexpressed c-jun, in mediating the observable increase in AP-1 transactivation. Similar results are obtained from microinjection studies by the same authors (Alberts et al., 1993a).

Studies on the interaction of PP2A with DNA tumor viruses have suggested roles for PP2A in viral transformation and signal transduction pathways of transformed cells as well. The binding of PP2A to specific viral tumor antigens causes either a change of substrate specificity, or inhibition of phosphatase activity which is compatible with the viral program. For example, the polyoma virus middle T antigen binds the AC complex of PP2A and thereby increases its activity as a protein tyrosine phosphatase over the native holoenzyme. The SV40 small t antigen binds either the AC complex or the A subunit alone, and this binding can be competitively reversed by addition of certain types of B subunit, implying that it acts as a surrogate B subunit which binds the A subunit in a similar manner to that of the B subunit. The ternary complex (i.e. the AC complex incubated with small t) shows reduction or enhancement of activity toward certain substrates, demonstrating increased activity toward histone H1 at 50-100 nM small T as well as decreased activity toward myelin basic protein and myosin light chains in vitro (Yang et al., 1991). One of the substrates which the ternary complex exhibits reduced activity towards in vitro is the SV40 large T antigen, which, when phosphorylated, binds to the SV40 origin of replication and directs viral replication and transcription (Prives, 1990; Farmer et al., 1992). SV40 small t effectively blocks PP2A-mediated dephosphorylation of SV40 large T antigen. In the presence of small t, the AC complex of PP2A exhibits a 3-fold reduction in in vitro phosphatase activity on large T immunoprecipitates containing large T/p53 complexes, as measured by 32P release. Two dimensional peptide maps of large T incubated with PP2A or p53 incubated with PP2A, both in the absence and presence of small t, show specific changes in the phosphopeptide map which indicate blockage of PP2A-catalyzed
dephosphorylation of specific sequences—for AC, ABC, and C forms of PP2A (Scheidtmann et al., 1991), indicating an interaction between PP2A and both p53 and SV40 large T.

PP2A-mediated dephosphorylation of MEK1 and ERK1 is also inhibited by small t in vitro when mono-Q FPLC-purified PP2A AC complexes or C subunit are incubated with purified SV40 small t antigen and ERK1 or MEK and kinase activity measured by $^{32}$P incorporation in a myelin basic protein substrate (Sontag et al., 1993). When monkey kidney CV-1 cells are transfected with small t, increased phosphorylation of MEK and ERK-2 can be detected which correlates with a higher level of basal ERK and MEK activity, implying an inhibition of PP2A activity. Immunoprecipitates of ERK2 in transformed cells which were immunoblotted with anti-small t antibodies were found to contain bound small t antigen, as did a GST fusion protein of the A subunit. The activity of Raf-1 (a proposed substrate of PP1) is unaffected, as assessed by a coupled kinase assay in which the ability of small t to modify the activity of immunoprecipitated Raf-1 from CV-1 cells transformed with small t is tested by incubation with MEK1 and a kinase-deficient ERK2. Immunoprecipitated Raf-1 from transformed and nontransformed cells exhibited similar abilities to activate MEK1-catalyzed phosphorylation of ERK2, both in the absence and presence of EGF (Sontag et al., 1993). These results complement the findings of Kovacina et al. (1990) that the insulin-induced activation of Raf-1 kinase activity, which correlates with its phosphorylation on serine, is reversed by PP1. Raf-1 immunoprecipitates of cellular extracts of HeLa cells treated with exogenous PP1 exhibit Raf-1 kinase activity at control levels.

PP2B, otherwise known as calcineurin, is a ser/thr phosphatase which mediates the effects of transient increases in cytosolic Ca$^{2+}$. In addition to the catalytic subunit (which shares homology with PP1 and PP2A), PP2B is regulated by a calcium-binding B subunit as well as by calmodulin. Both the A (catalytic subunit) and B subunits have several different genes which give rise to different isoforms of calcineurin. These proteins are expressed differentially in several tissues, including brain, hematopoietic cells, and testes. Similar genes encoding both subunits have been found in lower organisms as well, such as Neurospora Crassa (Higuchi et al., 1991) and S. Cerevisiae (Cyert et al., 1991). Calcineurin has been found to be critical in several different cellular processes, in which it acts as a calcium sensor in the cell; upon increases in intracellular free calcium concentrations, calmodulin binds to calcineurin, resulting in an activation of the phosphatase activity associated with calcineurin (to be discussed in depth in Chapter 2). Immunosuppressants cyclosporin A and FK506 were discovered to bind a cyclophilin protein present in cellular extracts. This drug/immunophilin complex was found to bind to calcineurin, hence identifying calcineurin as a protein important for T cell activation in response to antigenic stimuli (Liu et al., 1991-referenced in Chapter 2). Clipstone and Crabtree (1992) demonstrated that overexpression of calcineurin in Jurkat cells transformed with a vector containing a gene encoding calcineurin as well as a vector containing the protein alkaline phosphatase downstream of multiple copies of NF-AT (Nuclear Factor of Activated T cells; a transcription factor which enhances transcription of Interleukin-2. They were able to show
that NF-AT-dependent expression was sensitive to concentrations of CsA or FK506 that were inhibitory to calcineurin. NF-AT itself is a dimer, composed of one subunit which is synthesized after stimulation of PKC activity in the T cell, and another subunit which exists in the cytoplasm before stimulation and translocates to the nucleus, where it forms a heterodimer with the other subunit and activates genes such as IL-2 involved in T cell activation.

Calcineurin may be directly involved in this translocation of NF-AT, which involves dephosphorylation of the cytosolic NF-ATp (McCaffrey et al., 1993 and references therein).

PP2C shares no sequence homology with the other ser/thr phosphatases discussed above. It is classified as a class “2” (PP"2"A) enzyme because it preferentially dephosphorylates the A subunit of phosphorylase kinase over the B subunit. Unlike PP2A and PP1, PP2C is insensitive to the inhibitor okadaic acid, even at 1μM concentrations (reviewed in Cohen et al., 1990). The in vivo activity of PP2C has not yet been studied, although it seems to be expressed in most types of mammalian tissue, most notably in liver, heart, brain, and muscle. It displays in vitro activity against glycogen synthase and myosin light chains, both with a K_m of less than 10 μM. It has a high level of activity toward HMG-CoA reductase and reductase kinase, both enzymes involved in cholesterol metabolism, in vitro (Ballou and Fischer, 1986; Shenolikar, 1994). It is possible that PP2C is the major regulator of cholesterol synthesis in vivo, although experimentation which addresses this question has not yet been performed.

Recently several phosphatases have been discovered which possess dual specificity and thus do not fit neatly into the two proposed categories above; these proteins act both as ser/thr phosphatases and as phosphotyrosine phosphatases. Two examples of such proteins are Cdc25 and 3CH134 (MKP-1). Both of these proteins share the most homology with vaccinia viral protein VH1 (25% amino acid identity between Cdc25 and VH1 in the PTPase domain, and 26% identity between 3CH134 and VH1 in the PTPase domain) (Walter and Dixon, 1993). Cdc25 plays an important role in the cell cycle: it dephosphorylates MPF, thereby activating it and inducing mitosis. MPF (Mitosis Promoting Factor), is a heterodimer of two subunits, namely, a Cdc2 ser/thr kinase subunit complexed with a regulatory Cyclin B subunit. The kinase has been shown to be regulated by phosphorylation at Tyr15, Thr14, and Thr167. The Tyr15 and Thr14 sites represent negative regulatory sites. These residues are phosphorylated in the inactive Cdc2/cyclin B complex, and both can be dephosphorylated by Cdc25 as can be detected by gel mobility shifts as well as MPF activity induced by Cdc25 as opposed to other PTPases. A Cdc25 mutant with a cys→ser mutation in the conserved PTPase active site motif CXXXXXR has been found to be inactive regarding both Thr14 and Tyr15 dephosphorylation, implying that Cdc25 employs the same mechanism to dephosphorylate tyrosine as it does to thr/ser (ser activity was found against ser-phosphorylated casein by another group of investigators).

3CH134 has been similarly characterized as a dual specificity enzyme. It has been suggested that 3CH134 acts as a feedback inhibitor to the MAP kinase pathway in that it is
induced upon serum stimulation and acts to downregulate MAP kinase activity by
dephosphorylating MAP kinase on tyrosine and threonine residues, both in vivo and in vitro. In vitro
dephosphorylation catalyzed by 9E10 immunoprecipitates from Cos cells transfected
with Myc epitope-tagged 3CH134 was detected at Tyr and Thr (by two dimensional
phosphamino acid analysis) against recombinant phosphorylated and purified MAP kinase.
The Cys→Ser mutant (3CH134CS) showed lack of dephosphorylation of both tyrosine and
threonine. In vivo dephosphorylation of tyrosine was detected, by immunoblot analysis with
antiphosphotyrosine antibodies, in PTPase inhibitor-treated lysates of transformed Cos cells
stimulated for five minutes with serum after a period of serum starvation. Again, the CS
mutant showed MAP kinase tyrosine phosphorylation levels to be similar to that of control
levels of untransformed cells. As with other catalytically inactive PTPases, the 3CH134CS
mutant was shown to be capable of binding endogenous MAP kinase in myc-tagged 3CH134CS-
transformed Cos cells, as evidenced by the ability to immunoprecipitate MAP kinase from
cellular extracts using the 9E10 anti-myc epitope antibody. Evidence for the involvement of
3CH134 in feedback control of the MAP kinase in vivo was derived from an experiment
correlating the time course of 3CH134 expression with the kinetics of dephosphorylation and
inactivation of MAP kinase. Starting at one hour after serum stimulation of NIH3T3 serum-
starved cells, 3CH134 protein becomes immunoprecipitable with 9E10 antibodies. Concurrently,
the appearance of faster-migrating species signifying less phosphorylated forms of MAP
kinase was noted on SDS-PAGE gels, as well as a corresponding decrease in the kinase activity
of these new MAP kinase forms, as detected in an in-gel kinase assay using myelin basic protein
as a substrate. This downregulation of MAP kinase activity was completely abrogated by the
inclusion of cycloheximide in the serum used to induce activation of the MAP kinase pathway,
进一步支持了观察到的激酶活性的表达与新3CH134蛋白的表达以及激酶活性的减少
之间的关系受到研究(Sun et al., 1993).

Given the importance of protein phosphatases in so many aspects of cell function—from cell
cycle regulation to hormonal stimulation, from transcription to cell differentiation and
development—we decided to initiate a study on one particular phosphatase in the hopes of
generalizing mechanistic characteristics to other enzymes with catalytic subunit homology
such as PP2B. In 1988, Cohen et al. reported the presence of a putative protein phosphatase in
bacteriophage λ which showed 35% identity to rabbit PP1α and PP2Aα in the N terminal half
of the open reading frame, designated ORF221 (Figure 1)(Cohen et al., 1988). The following
year, a study was published by the same group which demonstrated that the λ gt10 strain of
bacteriophage λ did indeed encode a phosphatase with in vitro activity toward 32P-labeled
casein and phosphorylase. Lysates from bacteria infected with a different strain called λ gt11,
which contains an N-terminal deletion of ORF221 including the first of three sequences
conserved in other ser/thr phosphatases such as PP1, PP2A, and PP2B, did not exhibit any such
phosphatase activity. These results point to a new phosphatase, of low molecular weight (25
kD), which would be a prime candidate for mutational studies in order to identify which
Figure 1. Sequence alignment of Calcineurin, λ phosphatase, PP1, and PP2A. Identical residues across the four enzymes are marked.
residues play a role in catalysis. Knowledge of the active site of ORF221 could be applied to other ser/thr PPases. Identification of residues important for substrate binding could yield $K_m$ mutants which bind more tightly to the substrate, or inhibit the release of the dephosphorylated product after turnover—such a mutant could possibly be used in a fashion similar to the cys$\rightarrow$ser mutant of PTPases, i.e. function as a substrate binding protein and therefore as a reagent to identify in vivo substrates of these PPases.

With these aims in mind, we proceeded to clone and purify ORF221, and define kinetic parameters for the wild type enzyme.

**Materials and methods**

**Cloning of ORF 221/ lambda phosphatase.** The lambda DNA used as a template for PCR cloning was strain $\lambda$cl ind1ts857Sam7, whose genome was initially sequenced by Sanger et al. (1982). Bases 43228-43890 on the right arm of this lambda phage encodes the full length ORF221. Primers were designed to include a 5' EcoRI restriction site and a 3' HindIII restriction site in order to facilitate cloning into a pHNI$^+$ plasmid carrying a tac promoter upstream of the MCS. The sequence of the 5' primer was: 5'-AGCCTAGAATT CCTGAAGGAGAGACGATGCGCTATCAGAAAAAT-3' (LPIT17 primer); the sequence of the 3' primer was: 5'-GCATGCAAGCTTCATGCGCCTTCTTCCCCTG-3' (LP2T18 primer, from Bio-Synthesis). [Italics denote bases added to the clone through PCR. Restriction sites are underlined.] PCR conditions included 4 mM MgCl$_2$, 1 $\mu$M each primer, and 10 ng of template DNA (PCR reagents were from Perkin-Elmer). PCR method was as follows: 96°C, 6'; 96°C, 1'/45°C, 1'/72°C,1.5'--3 cycles; 94°C,1'/45°C,1'/72°C,1.5'--30 cycles; 72°C, 6'/25°C, 30'/4°C,999'. PCR DNA was restriction-digested with the appropriate enzymes and further purified with Gene Clean (Bio-101, Inc.). Miniprep plasmid DNA was similarly digested, and heated to 65°C to inactivate restriction enzymes. Ligations were performed at 16°C overnight with T4 DNA ligase (Promega). The ligation mixture was transformed into competent DH5$\alpha$ bacteria. Clones were selected on LB plates containing ampicillin. After restriction digestion of the plasmid DNA to verify the presence of proper length inserts, the clones were sequenced by dideoxy sequencing methods using Sequenase (USB).

**Expression and purification of recombinant lambda phage.** The pHN1$^+$/ORF221 plasmid was transformed into XA90 bacteria. Overnight cultures inoculated into 10 mls of LB containing 100 $\mu$g/ml ampicillin were inoculated into 1 liter of LB containing 100 $\mu$g/ml ampicillin and induced to a final concentration of 2mM IPTG (isopropyl-$\beta$-D-thio glucopyranoside; Sigma) when the culture reached an OD$596$ of 0.6. The bacterial cultures were harvested after a 2.5-hour induction by centrifugation at 5,000 x g for 10 minutes. The pellet was washed once in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, pH7.5) and resuspended at 4 ml lysis buffer/g pellet. Cells were lysed by two passes at 12,000 psi in a French Press. The cell
debris was removed by centrifugation at 10,000 x g for 20 minutes. DNA was precipitated and removed from the extract by adding 1/5 volume of 2% (w/v) protamine sulfate (Sigma) to the extract at 40°C, stirring, for 20 minutes. After centrifugation, the extract was NH4SO4-precipitated by adding ammonium sulfate to 60% at 40°C, stirring, for 20 minutes and then centrifuged again at 10,000 x g for 20 minutes. The pellet was resuspended in lysis buffer and loaded directly onto a 100 x 3 cm G-75 gel filtration (Pharmacia) column pre-equilibrated in lysis buffer (all at 40°C). The column was developed by gravity and fractions collected were assayed for protein content by A280 and SDS-PAGE electrophoresis, as well as for activity in a pNPP phophatase assay. Active fractions were pooled and concentrated with an Amicon diafiltration system and dialyzed into lysis buffer + 1mM DTT + 30% glycerol. Protein concentrations were measure by Bradford assay using BSA (Bovine Serum Albumin; Boehringer-Mannheim) as a protein standard. Protein was then stored at -20°C.

**Phosphatase assays.** Assays were run at ambient temperature in 1x assay buffer: 50 mM Tris, 1mM EDTA and 1mM EGTA, pH 7.5, including 1 mg/ml BSA. Enzyme dilution buffer was identical to the assay buffer. When present in the assay buffer, the final concentration of Mn²⁺ was 2 mM MnCl₂; when present in the assay buffer, the final concentration of Mg²⁺ was 10 mM MgCl₂. The reaction was started by adding pNPP (para-nitrophenylphosphate; Gibco-BRL) to the assay mixture immediately after addition of enzyme to the assay buffer; the reaction was mixed, and the rate of change in OD₄₁₀ was monitored by spectrophotometer (Hewlett-Packard), corresponding to the rate of appearance of the para-nitrophenol anion. Kinetic parameters and their standard deviations were calculated on Kaleidagraph, using the extinction coefficient of 1.8 x 10⁴ M⁻¹ cm⁻¹ for paranitrophenolate.

**Results and Discussion**

From a one liter culture of bacteria, 24.6 mgs of ORF221 were obtained after ammonium sulfate precipitation and G-75 purification, corresponding to about 50% of the ammonium sulfate pellet and 49% of the initial crude after DNA precipitation (see Figure 1). At this stage of purification, the G-75 eluate corresponded to approximately a two-fold purification over the crude extract (see Figure 1 and Table I). Crude extracts of untransformed XA90 showed levels of activity slightly above background, whether in the presence of Mn²⁺ or Mg²⁺; crude extracts containing ORF221 were stimulated 7- to 8-fold by inclusion of 2 mM Mn²⁺ in the assay buffer over the level of activity obtained with 10 mM Mg²⁺, which was barely above background levels. Assays on protein from a 30-45% ammonium sulfate pellet showed a greater than 10-fold stimulation of Mn²⁺ over Mg²⁺ activity (data not shown) in 20 mM pNPP (a phosphotyrosine analogue). A higher level of activation was obtained if Mn²⁺ was included in the enzyme dilution buffer and the preincubation time lengthened, suggestive of a slow...
Figure 2. Purification of ORF221. Different stages of the purification of λ phosphatase and their protein profiles (12% Acrylimide SDS-PAGE gel). Lane 1, 10 µg crude cellular extracts of XA90 transformed with the pHN1+/ORF221 plasmid. Lane 2, 10 µg 60% ammonium sulfate pellet resuspended in lysis buffer. Lane 3, 5 µg of G-75 gel filtration-purified protein at the expected molecular weight of 25 kD.
transition of the enzyme to a more active conformation, as has been suggested previously for phosphatases such as PP2B (Ballou and Fischer, 1986). The purification process involved precipitated by either addition of protamine sulfate to the extracts, or by DNAse treatment; both G-75 enzyme preparations gave similar activities at 20 mM substrate, and addition of protamine sulfate of up to 60 μg/ml reaction was found to be neither inhibitory nor stimulatory to the rate of reaction. In this, ORF 221 differs from rabbit PP2A0,1, and 2 isoforms which are stimulated by 5 μg/ml protamine (Cohen,P. et al., 1988).

The protein profile of the G-75-purified protein indicates the presence of a contaminating lower band. This band was present whether or not protease inhibitors were included in the lysis buffer. The lower band was not separable from the 25 kD band (migrating near the expected molecular weight for ORF221 of 25, 222 daltons) visible upon IPTG induction by DEAE or Carboxy-methyl sepharose (Pharmacia). Two-dimensional electrophoresis shows that the lower band has a similar pI (around neutral) to the upper band, thus explaining the inability to obtain a better purification with DEAE or CM sepharose. Cibacron blue resins as well as several other dye resins, aminoethyl, polylysine, hydroxyapatite, and phenyl sepharose resins were all tried on a small scale. Polylysine separation, when scaled up, gave irreproducible results. Phenyl sepharose bound both proteins quite well in 0.4 M ammonium sulfate, but elution in a 0.4-->0 M ammonium sulfate gradient (containing 10 mM KH2PO4, pH 7.5) seemed to elute out the contaminating protein at a specific point in the gradient, whereas the 25 kD band eluted out over a broad range of concentrations both before and after the elution of the contaminating band. Heat denaturation experiments demonstrated the temperature sensitivity of ORF221 and the resistance of the lower band to thermal denaturation at >550°C and thus could not be used as a method of purification.

At this stage, kinetic constants for the G-75 purified ORF221 were determined and a GST fusion protein synthesized to try to circumvent the purification problem (the GST-ORF221 protein was cloned by a coworker). In the presence of 2mM Mn2+, the Km was determined to be 2.8 mM +/- 0.20, and Kcat was 16.36 sec-1. λ phosphatase exhibited a high specific activity toward pNPP (38.91 +/- 2.22 μmol/min/mg) (Table II). Similar results were obtained with the GST fusion protein (data not shown).

During the time in which we began working on the coupled assay system for determination of kinetic parameters using a peptide substrate (according to the method of Etzkorn et al., 1994) before beginning mutational studies, several papers were published which addressed precisely this issue as it pertains to pNPP catalysis.

In an earlier paper (missed by our literature searches), Zhuo et al. (1993) had expressed this λ phosphatase under a T7 promoter in BL21-DE3 bacteria, and purified it to apparent homogeneity with gel filtration chromatography and phenyl sepharose hydrophobic interaction chromatography (cell extracts were loaded in 0.5M NaCl, and eluted in 0 M NaCl). They assayed for activity using pNPP, full length serine-phosphorylated casein and tyrosine-
<table>
<thead>
<tr>
<th>Purification step</th>
<th>Amount of protein (mgs)</th>
<th>Approximate specific activity (μmol/min/mg)</th>
<th>Purification (x-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude extract</td>
<td>50.3</td>
<td>19</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>0-60% (NH₄)₂SO₄</td>
<td>49.8</td>
<td>--</td>
<td>--</td>
<td>99</td>
</tr>
<tr>
<td>G-75 gel filtration</td>
<td>24.6</td>
<td>37</td>
<td>1.94</td>
<td>49</td>
</tr>
</tbody>
</table>

Table I. Partial purification of ORF221/λ phosphatase. Activity was assessed at a concentration of 0.2 mg/ml of induction protein and high substrate concentrations (20 mM pNPP) in the presence of 2 mM MnCl₂.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metal</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$K_{cat}$ (sec$^{-1}$)</th>
<th>$K_{cat}/K_m$ (M$^{-1}$sec$^{-1}$)</th>
<th>$V_{max}$ (Msec$^{-1}$)</th>
<th>Sp. act. (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF221</td>
<td>Mn$^{2+}$</td>
<td>pNPP</td>
<td>2.82 ±0.20</td>
<td>16.36</td>
<td>5.8x10$^3$</td>
<td>1.30x10$^{-7}$</td>
<td>38.91±2.22</td>
</tr>
</tbody>
</table>

Table II. Kinetic parameters of ORF221: pNPP phosphatase assay in the presence of 2mM MnCl₂ and 7.9 nM enzyme.
phosphorylated casein and their respective shorter peptides, as well as histidine-phosphorylated proteins and a phosphotyrosyl peptide deriving from the substrate of the insulin receptor kinase as substrates. They report that protamine has no effect on the λPPase; neither do other polylsines, or trifluoperazine (a calmodulin-binding inhibitor). They demonstrate that Mn^{2+} and Ni^{2+} stimulate the activity of the enzyme at 200 μmolar concentrations; among the many divalent and trivalent cationic metals tested, they show that the Mn^{2+} activity is almost completely inhibited by an equimolar amount of Zn^{2+} or Cu^{2+}.

Activity is also inhibited by NaF, a noncompetitive PPase inhibitor (Ingebritsen and Cohen, 1983). As has been reported with other ser/thr phosphatases, phosphoserine and phosphothreonine are not good inhibitors of Mn^{2+}-stimulated pNPPase activity; phosphothreonine displays no inhibition whatsoever at 25 mM, and phosphoserine and phosphotyrosine inhibit this PPase activity at 90 mM or not at all at 20 mM, respectively.

In terms of kinetic characterization of the enzyme using the aforementioned substrates, the authors report a K_{m} of 7.62 mM for pNPP in the presence of Mn^{2+}, a K_{cat} of 930 sec^{-1}, and a K_{cat}/K_{m} of 0.12 μM^{-1}sec^{-1}. These values indicate a lower level of efficiency than the values we obtained for the K_{cat}/K_{m} of OR221 of 0.058 μM^{-1}sec^{-1}; in our study as well, the enzyme seemed to have a higher affinity for pNPP (i.e., a three-fold lower K_{m}) than did that of Zhuo et al. In their further characterization of the wild type enzyme, they found that the enzyme exhibits a preference toward phosphoserine in 32P-labeled casein over phosphotyrosine, reflected in both tighter binding and faster maximal velocity, the opposite preference is established when the kinetic parameters relating to the Ser- and Tyr-phosphopeptides derived from casein. The enzyme binds each peptide equally well but has a 6-fold higher K_{cat} for the phosphotyrosine peptide than the phosphoserine peptide. Dephosphorylation of the phosphohistidyl substrates NRII and CheA were also detected by autoradiograph of SDS PAGE gels containing the products of the dephosphorylation reaction.

Several months later a second paper reporting the purification and characterization of ORF221 appeared which described two mutations as well. The author demonstrates that okadaic acid, a PP1 and PP2A inhibitor, was not inhibitory to the enzyme even to concentrations of 1 μM (i.e. that used to inhibit PP1 activity completely--PP2A is ten-fold more sensitive). Inhibitor 1 and inhibitor 2, proteins which inhibit PP1 activity, also had no effect on λPPase activity. No tyrosine phosphatase activity is detected using Raytide (Oncogene Science) or CAMK II peptide (sequence:RRREEETEEE), as well as phosphorylated EGFR, as substrates. In this paper, the author made two (gross) mutations--one involved deletion of conserved residues 73-77, corresponding to a RGNHE stretch in the wild type protein, while the other involved a Cys138→Phe or Ala mutation. All three types of mutations reduce phosphatase activity toward pNPP to less than 5% of wild type phosphatase activity. These mutations show that the wild type 25-26 kD protein cloned was indeed
responsible for the phosphatase activity attributed to it, but in general these mutations are
gross mutations which probably reflect changes in conformation of the protein away from a
conformation conducive to catalysis rather than necessarily implying a direct role in catalysis
for any of these mutated residues.

In a follow-up study, Zhuo et al. present their findings regarding several point mutations of
residues in \( \lambda \) phosphatase which are conserved in other PP1, PP2A, and PP2B subunits and
determine their effects on metal binding, substrate binding, and catalysis. They mutate two
amino acids in the first homologous region, D20N and H22N; four amino acids in the second
major homologous region, D49N, D52N, R53A, and D59Q; and three in the third major
homologous region, R73A, H76N and E77Q. The \( K_m \) of Mn\(^{2+}\), \( K_m \) of substrate binding, and \( K_{cat} \)
or specific activity were determined for the mutant PPases, using the substrate pNPP.

The study shows that D20, H22, D49, H76, and E77 are all involved in metal binding;
mutations in these residues give rise to \( >10 \)-fold increases in \( K_m \) values for Mn\(^{2+}\) binding. All of
these mutants are characterized by quite low values of \( K_{cat} \) as well, except for the E77Q mutant
which, though its metal binding capacity is greatly perturbed (beyond that of the other
mutants), the \( K_{cat} \) is much higher than that of the other mutants, by at least 100-fold. This
would seem to indicate an important role for E77 in the actual binding of the metal, but a much
less important role in catalysis. Mutants D52N and E59Q induce a 40-fold and 10-fold reduction
in activity, respectively, although the \( K_m \) s for Mn\(^{2+}\) and pNPP are similar to wild type; the
conclusions to be drawn from this are unclear (the mutation could affect the accessibility of the
metal to the substrate binding site by blocking it sterically while not compromising the binding
of each, or may be important for water binding rather than metal binding, for example).

The R53A and R73A were tested in a pNPPase assay in the presence of either Ni\(^{2+}\)
stimulation or Mn\(^{2+}\) stimulation. In the wild type enzyme, Mn\(^{2+}\) binds \( >40 \)-fold more tightly
than Ni\(^{2+}\). In the R53A mutant, the Mn\(^{2+}\) activity shows no major perturbation of either the
metal or substrate binding, and yet the specific activity is reduced drastically; the Ni\(^{2+}\) activity,
however, shows a 20-fold increase in the \( K_m \) of pNPP as well as a drastic reduction in specific
activity. The R73A mutant is characterized by a slightly tighter metal binding capability and
a less drastic reduction in specific activity than that of the R53A mutant; assays in Ni\(^{2+}\)
showed a similar ability to bind Ni\(^{2+}\) as compared with wild type enzyme, a slightly weaker
binding of pNPP, and a less drastic reduction in specific activity as well. IC\(_{50}\) s for inorganic
phosphate inhibiting the pNPP reaction are all raised significantly over wild type values,
indicating along with the pNPP \( K_m \) data for the Ni\(^{2+}\) activity that Arg53 and Arg73 are both
critical for substrate and phosphate binding (these effects seem metal-dependent— the Mn\(^{2+}\)
activity seems to be much less affected by these mutations—which point to possible
conformational differences at the active site based on the stimulatory metal used).

It will be interesting to see continued mutational analysis of the ORF221 to identify
residues important for substrate binding- whether for pNPP binding or peptide substrate binding- and to apply these studies to other protein phosphatases with significant homology to ORF221. Recently the catalytic subunit of human PP1 has been cocrystallized with an inhibitor, microcystin LR, and the crystals obtained diffract to 2.8 angstroms (Barford and Keller, 1994). With a crystal structure in hand, the current understanding of the mechanism of catalysis and the architecture of the substrate-and/or inhibitor- binding cavity will be greatly enhanced.

References


Cohen, P.T.W., Collins, J.F., Coulson, A.F.W., Berndt, N. and da Cruz e Silva, O.B. (1988). Segments of bacteriophage λ (orf221) and φ80 are homologous to genes coding for


CHAPTER 2

Structure/Function Studies of Calcineurin
PP2B, or calcineurin, was originally discovered as an inhibitory protein in mammalian brain which inactivated a Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Sharma and Kalra, 1994). Several years later the enzyme was identified as PP2B, a protein phosphatase which had already been purified previously (Stewart et al., 1982). Calcineurin is a heterodimer of the A (catalytic, 61kD) and B (regulatory, 19kD) subunits (reviewed in Ballou and Fischer, 1986). The regulatory subunit binds four moles of calcium, one in each of four EF-hand domains characteristic of other calcium-binding proteins such as calmodulin (Klee et al., 1979). The A subunit contains a N-terminal catalytic domain which shares a high degree of homology to PP1 and PP2A (see Chapter 1, figure 1), followed by a putative calcineurin B (CNB) binding domain. C-terminal to the CNB binding domain is a calmodulin binding domain, followed by an autoinhibitory domain (Kincaid, R.L., 1993). Calcineurin acts as a calcium sensor in the cell, undergoing a gain in function upon increases in intracellular free calcium and subsequent binding of calcium to the CNB calcium binding sites; at these raised levels of Ca^{2+}, calmodulin binds the AB heterodimer with nanomolar affinity (Klee et al., 1979; Hubbard and Klee, 1987). These subunit changes effect a conformational change which activates the phosphatase activity of calcineurin toward its substrates. Tightly bound Fe^{3+} and Zn^{2+} ions have been detected in native bovine brain enzyme (King and Huang, 1984), suggesting that calcineurin is a metalloenzyme.

Calcineurin has a wide tissue distribution in mammals; it has been detected at high levels in brain and testes, as well as in skeletal muscle, kidney, heart, ovary, spleen, and thymus (reviewed in Ballou and Fischer, 1986; Kincaid et al, 1990). Multiple genes which encode both the A and B subunits have been identified, each in turn giving rise to several different spliceoforms.

Proteolytic digestion studies aimed at mapping the different functionalities implicit in native bovine brain calcineurin have contributed much to our understanding of the domain substructure of the catalytic subunit. Proteolysis studies led Hubbard and Klee (1989) to identify an autoinhibitory domain distinct from the inhibitory effects of the calmodulin domain. Digestion of the CNA/B heterodimer with clostripain (which hydrolyzes proteins at arginine residues) yields different molecular weight fragments, depending on the absence or presence of calmodulin. Aliquots of the proteolysis reaction taken at several time points were characterized by SDS-PAGE gels after gel filtration and assayed for their ability to bind calmodulin, as well as for their pNPP phosphatase activity. Clostripain proteolysis in the absence of CAM quickly resulted in the disappearance of full length CN and concomitant loss
of CAM binding by any of the larger proteolytic fragments present. Simultaneously, a 43 kD proteolytic fragment was generated which still contained bound CNB, and which was characterized by Ca\(^{2+}\)-dependent, CAM-independent pNPPase activity (measured in the presence of Mg\(^{2+}\)) at levels 30-40-fold higher than the activity of the native enzyme tested under similar conditions. The Ca\(^{2+}\)-independent activity of the 43 kD fragment was slightly less than 10-fold higher than the activity of the full length enzyme in the absence of Ca\(^{2+}\). Several minutes later, a 40 kD fragment, still containing bound CNB, appeared which was characterized by lack of phosphatase activity toward pNPP. In similar proteolysis experiments performed in the presence of calmodulin, a different set of proteolytic digestion species were generated. At first, a 57 kD fragment appeared during the first 10 minutes of digestion which was competent to bind calmodulin, and which contained a virtually Ca\(^{2+}\)-CAM independent activity, corresponding to a 10-fold increase in pNPPase activity over that of the native enzyme. Further proteolytic digestion yielded 55 kD, 42 kD, and 14 kD fragments which proved to be catalytically inactive against pNPP as a substrate whether or not Ca\(^{2+}\) was present. The 55 kD and 14 kD species did retain the ability to bind to calmodulin. N-terminal sequencing of the discreet proteolytic forms generated show that like the 61 kD native enzyme, the 57 kD and 43 kD enzymes were blocked toward Edman degradation, unlike the 55, 42, and 14 kD fragments which yielded some sequence information. The authors propose, on the basis of these experiments and others done previously by other colleagues, a functional mapping of native calcineurin. They assert that the protein contains an N-terminal catalytic domain, followed by a CNB binding domain, a calmodulin binding domain, and an autoinhibitory domain at the extreme C-terminus of CNA (see Hubbard and Klee, 1989, and references therein). The 43 kD protein, a form generated by proteolysis in the presence of CAM but which itself no longer binds CAM, exhibits a 3-4-fold increase in Ca\(^{2+}\)-dependent activity compared to the 57 kD fragment and Ca\(^{2+}\)-independent activity which is similar to the Ca\(^{2+}\)/CAM stimulated activity of the 57 kD fragment. Given this extra stimulation of the 43 kD fragment toward pNPP when compared with the 57 kD enzyme, as well as the 30-40 fold increase in pNPPase activity relative to the native bovine brain enzyme, the authors predict the existence of an autoinhibitory domain which is separate from the calmodulin binding domain. The activity of the 57 kD fragment represents the level of stimulation achieved upon ablation of the autoinhibitory domain. The activity of the 43 kD protein, corresponding to a protein lacking both part or most of the calmodulin binding domain and the autoinhibitory domain C-terminal to it, displays an even higher level of activity than would be attributable to merely the lack of a functional autoinhibitory domain. These findings suggest that the calmodulin binding domain itself mediates a detectable level of inhibition distinct from that of the autoinhibitory domain, and that the Ca\(^{2+}\) stimulation of the 43 kD fragment most probably results from effects mediated by the CNB protein which remains bound to the 43 kD protein (Hubbard and Klee, 1989).
Currently three mammalian genes have been cloned which encode calcineurin, with most of the heterogeneity occurring in the N- and C-termini of these genes. The α (Muramatsu et al., 1993) and β (Muramatsu et al., 1992) genes have been cloned from brain tissue (displaying >80% identity among the three proteins), while the γ gene has been cloned from testes (Muramatsu and Kincaid, 1992). Each of these genes generate several different isoforms, purportedly arising from alternate splicing of mRNA. Differences in substrate specificity and tissue distribution of these isoforms and spliceoforms will be key to understanding the role of calcineurin in very different cellular processes which are potentially occurring within any one given cell. The human full length cDNA for CNA \(_{\alpha 1}\) has been cloned (Muramatsu and Kincaid, 1993) from a hippocampal cDNA library, encoding a protein which differs from the mouse CNA \(_{\alpha 1}\) by only two amino acids, and which shows 90% identity in the upstream 5' untranslated region to that of the mouse cDNA. Comparison of the α1, β2, and γ2 sequences show 100% homology in the putative CNB binding domain, only one or two conservative changes in the calmodulin-binding domain, and 1-2 conservative replacements plus one variant residue in the putative autoinhibitory domain (Muramatsu and Kincaid, 1993). In an earlier paper, Kincaid et al. (1990) compare murine and human CNA clones they obtained from cerebellum libraries of the respective organisms. In several of the clones, an in-frame deletion of 30 base pairs was detected, located between the calmodulin (CAM) binding domain and the autoinhibitory domain. This sequence corresponds to the CNA \(_{\alpha 2}\) sequence, which is similar to the γ2 isoform in that both isoforms are characterized by this 10-amino acid deletion. The β isoform is distinct in its N-terminal polyproline sequence, suggestive of possible interaction with SH3 domains (which bind proline-rich sequences) as a mode of targeting or regulation of activity (Saksela et al., 1995; Viguera et al., 1994). Differences in the primary sequence such as these, as well as the many others at the N- and C-termini of the α, β, and γ isoforms, could mediate changes in substrate specificity, targeting, or regulatory control of phosphatase activity.

Multiple genes encoding CNA and CNB have been identified in other organisms as well, including and \(S.\ Ceruisiae, Neospora\ Crassa,\) and Drosophila. The PP2B genes discovered in \(S.\ Ceruisiae\) were discovered by \(^{125}\text{I}\)-labeled calmodulin screening of a yeast expression library followed by CNA hybridization experiments with CAM-binding positive clones. Sequencing of these two clones show some differences between the yeast and human genes upon inspection of sequence alignments of yeast CNA1 and CNA2 alongside human CNA\(_{\alpha 1}\). The yeast CNA1 clone contains a 55-bp N-terminal extension, while the CNA2 clone contains a shorter 29-bp N-terminal extension. The CNA1 and CNA2 sequences contain only about 54% identity with human CNA\(_{\alpha}\) (as opposed to the murine >99% identity), indicating a separate evolutionary origin for the two yeast genes. There are several nonconservative changes within the putative CNB and CAM binding domains and autoinhibitory domains, as well one deletion (relative to CNA\(_{\alpha 1}\)) directly N-terminal to the autoinhibitory domain (which encompasses
but is also twice as large as the deletion in a similar area found for the human γ2 and α2 isoforms). There are also several insertions present in the yeast genes. One of them intervenes at a point in the protein N-terminal to regions where lambda phosphatase, PP1, PP2A, and CNAα exhibit several identical or conserved residues; the other insertion is located between the CNB- and CAM-binding domains. Phenotypically, cna1 and cna2 single mutants and cna1/cna2 double mutants seemed to possess no other irregularities other than a two-fold, or four-fold, reduction in ability of such mutants which are Mata at the mating locus to overcome α factor-induced growth arrest, as determined by halo size surrounding an α factor-treated disc placed on a lawn of yeast cells (Cyert et al., 1991).

A sequence homologous to the human calcineurin A subunit was likewise found in Neurospora Crassa (Higuchi et al., 1991), which seems more closely related to mammalian CNA sequences than to the yeast CNA1 and CNA2. Using a mouse CNAα clone to identify N. Crassa clones from a cDNA library by plaque hybridization techniques, a homologous open reading frame, “Norf1,” was found which displays 62% identity to the mouse CNAα gene. The Norf1 gene contains inserts (relative to the human sequence) between the CNB and CAM binding domain, as well as both before and after the autoinhibitory domain. The autoinhibitory domain itself contains quite a few nonconservative residue changes (unlike the well-conserved CNB- and CAM-binding domains of Norf1). Recombinant Norf1 protein was purified by CAM-sepharose chromatography, and pNPP phosphatase activity was assayed for in both the absence and presence of Mn2+, CAM, and/or CNB which had been purified from bovine brain. Norf1 displays twice as much Mn2+/Ca2+-stimulated activity as the CAM-sepharose purified bovine brain CN used in their assay, although the opposite results are obtained in Mg2+/Ca2+ basal activity measurements. Regarding dephosphorylation of protein substrates in the presence of Mn2+, Mg2+, and Ca2+, bovine CN was 10-fold more active in inhibitor-1 dephosphorylation, whereas levels of casein and histone H1 dephosphorylation were found to be comparable for Norf1 and bovine brain CN. Reconstitution of Norf1 with bovine brain CNB caused no change in pNPP hydrolysis, seeming to imply that the role of CNB in Norf1 activity was greatly reduced from that of mammalian calcineurin. The effects of CNB on Norf1-catalyzed dephosphorylation of protein or peptide substrates was not examined in this study (Higuchi et al., 1991).[Studies undertaken in a different lab comparing the effects of Norf1 reconstitution with CNBβ1 from brain vs. CNBβ2 from testes actually show that while both β1 and β2 (each with his-tags for purification purposes) form heterodimers with Norf1, β1 is much more effective at stimulating the activity of Norf1 toward pNPP than β2 is. Such results support the notion of the existence of conformational differences and perhaps subtle changes in substrate specificity depending on the combination of A and B subunits present. (Ueki and Kincaid, 1992)].

Consistently, the regions between the putative CNB binding domain and the CAM binding domain have a much higher degree of heterogeneity than other parts of calcineurin, both
within species, as in comparing isoforms and spliceoforms, as well as across other species. The second CNA subunit found in drosophila is no different: it contains a 27-amino acid insert, including an acidic set of residues followed by a stretch of 7 asparagines in a row, 13 amino acids later. This drosophila gene has a deletion of several residues immediately N-terminal of the autoinhibitory domain, which itself has several non-conservative changes relative to mammalian (rat) CNA α and β isoforms. While the drosophila enzyme exhibits 73% identity with mammalian α and β isoforms at the protein level overall and about 65% at the nucleotide level, the drosophila calcineurin also contains a 34-amino acid C-terminal extension, continuing a sequence which contains a noticeably large amount of serine and threonine residues.

Studies on the interaction between CNA, CNB, and CAM using recombinant subunits, either coexpressed or reconstituted, have contributed a great deal toward our understanding of the C-terminal functions of the calcineurin A. Perrino et al. (1992) assessed the single contributions of CNB and CAM toward the pNPPase and RII peptide phosphatase activity of a recombinant CNAα2 expressed in Sf9 cells in a baculoviral system. Upon reconstitution of the recombinant CNAα2 with bovine brain CNB (β1 isoform) in the presence of Mn2+ and absence of Ca2+, the activity of the enzyme against 32P-labeled RII peptide in the presence of Mn2+/CAM was found to be similar to that of bovine brain-purified CNAα1/CNBβ1 holoenzyme (667 nmol/min/mg vs. 410 nmol/min/mg). Both Mn2+/CAM and Mn2+/CNB stimulated the basal CNA RII-peptide phosphatase by 4-fold and 6-fold, respectively (Mn2+/CAM stimulated native bovine brain CN by 5-fold). A synergistic 86-fold activation of CNAα2 to 667 nmol/min/mg was obtained after reconstitution with CNB and incubation with calmodulin. Analysis of the kinetic parameters of these enzymes revealed that the calmodulin stimulation of the RII phosphatase activity of both the CNA or CNA/B enzymes was mediated by a change in Vmax rather than Km. Interestingly, the effects of CNB upon the activity of the catalytic subunit manifested themselves by a lowering of Km in the A/B dimer relative to the Km of the A subunit, as well as a small change in Vmax. Regarding pNPP hydrolysis, reconstitution of the recombinant CNA with bovine brain CNB had no effect on specific activity, although subsequent CAM stimulation was much higher for the calcineurin A/B heterodimer than for the single CNA subunit. These results indicate that while calmodulin binding may function mainly by relieving the enzyme of autoinhibition and not by changing the conformation of the active site itself, the B subunit exerts its influence mainly by effecting such a conformational change as would allow the enzyme to bind its RII peptide substrate with higher affinity.

The predicted CNB binding domain of the catalytic subunit has been extremely well conserved from yeast to human (and perfectly conserved from drosophila to human). In a recent paper, residues 328-390 from the CNAα2 isoform, expressed as a GST fusion protein, were competent to bind to rat brain myristoylated CNB when incubated in the presence of Ca2+ or
Mn\(^2+\). The fusion protein was also shown to reduce the activity of wild type CNA/CNB reconstitution, presumably by competing for CNB, in a concentration-dependent manner. In several point mutations where hydrophobic residues were mutated to either acidic or uncharged residues (at Val349, Phe350, Phe356, and Val357). These mutations greatly reduced the hydrophobicity of the CNB binding domain greatly and the mutant fusion proteins were found to be unable to bind CNB. (The interaction between wild type CNA and CNB, once the heterodimer has been formed, has been known to rely on hydrophobic contacts which are disrupted only in conditions as strong as 6M urea—Merat et al, 1985). The mutants showed slightly higher Mn\(^2+\)/CAM -stimulated phosphatase levels against phosphorylated RII substrate than did the wild type (pointing to relative conformational integrity). Yet, upon the addition of CNB, these mutants exhibited less than 10% of the fully stimulated wild type activity treated similarly. These studies further establish the importance of calcineurin B interactions with and regulation of the catalytic subunit to yield a conformationally efficient phosphatase (Watanabe et al., 1995).

In a recent study, investigators characterized the phosphatase activity of two C-terminal truncation mutants of the CNA\(_{C2}\) (or CNA\(_{Q}\)) enzyme: CNA\(_{A457}\) (denoting a C-terminal truncation starting after residue 457) and CNA\(_{A420}\). CNA/B heterodimers obtained by coexpression in Sf9 cells using the baculovirus system and containing a mixture of non-myristoylated and myristoylated CNB subunits, were assayed for Mg\(^2+\)-stimulated \(^{32}\)P-RII activity in the absence and presence of Ca\(^2+\)/CAM. This study found the \(_{A457}\) mutant to be partially Ca\(^2+\)-independent, and the \(_{A420}\) mutant to be "completely" Ca\(^2+\)-independent. They find that the wild type, \(_{A457}\), and \(_{A420}\) mutant heterodimers display a similar sensitivity of the \(K_m\) of RII phosphopeptide toward Ca\(^2+\). All three enzymes show a 4-5-fold reduction in \(K_m\) upon the inclusion of Ca\(^2+\) in the assay buffer, from about 110 \(\mu\)molar to around 26 \(\mu\)molar for all three. When the \(_{A420}\) enzyme was expressed alone and assayed in the absence of CNB and presence of Ca\(^2+\) and/or CAM, the measured \(K_m\) is close to 110 \(\mu\)molar—implying that the Ca\(^2+\)-induced tighter binding of the RII peptide substrate is mediated by a conformational change in the A subunit effected by CNB calcium binding. Without CNB, the wild type and \(_{A420}\) enzymes are characterized by very low phosphatase activity. However, in the presence of CNB, Ca\(^2+\), and CAM, the \(_{A420}\) mutant and \(_{A457}\) mutant (which both still possess calmodulin binding domains) display even greater specific activity toward phospo-RII peptide than the wild type enzyme. This high specific activity is mostly Ca\(^2+\)- and CAM -independent for the \(_{A420}\) mutant, and partially Ca\(^2+\)/CAM-independent for the \(_{A457}\) mutant. According to this study, then, the specific activity (and thus the \(K_{cat}\)) of the \(_{A420}\) mutant is independent of Ca\(^2+\), while the \(K_m\) is Ca\(^2+\)-dependent, (Perrino et al., 1995).
Aside from the internal regulatory functions of the CNB subunit, the catalytic subunit has been shown to undergo phosphorylation at a residue located within the CAM-binding domain. Native calcineurin has been found to contain up to 0.6 mol phosphate/mol calcineurin (King and Huang, 1984), so the suggestion that phosphorylation of PP2B regulates its function in vivo may be entertained as a distinct possibility. \textit{In vitro} phosphorylation of calcineurin by a Ca$^{2+}$/CAM independent form of CAM kinase II (CAMKII), an enzyme abundant in neural tissue, results in rapid incorporation of 0.8 mol 32P/mol calcineurin (Hashimoto et al., 1988).

Phosphorylation catalyzed by protein kinase C (PKC) has been observed to take place rapidly as well, to a stoichiometry of 1 mol 32P/mol calcineurin (Hashimoto and Soderling, 1989). In two independently confirmed reports (Hashimoto et al., 1989; Martensen et al., 1989), the phosphorylation site for both CAMKII and PKC was found to correspond to residue 411 in the CNA$\alpha_1$ sequence, which lies just within the C-terminal edge of the putative CAM binding domain. Ca$^{2+}$ was required for phosphorylation of calcineurin but not for phosphorylation of other substrates by PKC or CAMKII, implying that the Ca$^{2+}$ played an essentially substrate-directed role, i.e. phosphorylation could only occur upon realization of a calcium-induced conformational change, most probably due to CNB calcium binding. Addition of CAM to the kinase buffer inhibited the phosphorylation, although once phosphorylated, calmodulin was still able to stimulate phosphatase activity toward pNPP or myosin light chain substrates. Hashimoto et al. (1988) found, additionally, that the phosphorylation affected both $V_{\text{max}}$ and $K_{\text{m}}$ values of the enzyme, depending on the substrate. For the phosphorylated calcineurin, the $K_{\text{m}}$ for myosin light chain was two-fold higher than the value of the unphosphorylated form, although the $V_{\text{max}}$ remained similar for both forms. Regarding the kinetic parameters of pNPP hydrolysis, although the $K_{\text{m}}$ values for the two forms were similar, the unphosphorylated enzyme's specific activity was two-fold higher than that of phosphorylated calcineurin. In any case, it seems that this phosphorylation causes a downregulation of phosphatase activity. There is evidence for \textit{in vitro} Ca$^{2+}$-dependent dephosphorylation of the CAMKII/PKC phosphorylation site by both PP1 and, more efficiently, PP2A, which provides the possibility of a mechanism for the return of calcineurin to the unphosphorylated state as well (Hashimoto et al., 1988). \textit{In vivo} data regarding this phosphorylation/regulation issue and/or identification of the actual phosphorylation site(s) corresponding to the detected levels of native phosphorylation have not yet been presented.

The immunosuppressants Cyclosporin A (CsA) and FK506 (Tacrolimus) have been found to bind and inhibit calcineurin's protein phosphatase activity by first binding to endogenous cyclophilins (CyP A, B, or C; in the case of CsA) or FKBP's (FK506 binding proteins) in the cell and then presenting a complex inhibitory surface to calcineurin (Liu et al., 1991). This interaction has been probed with multiple techniques, ranging from affinity chromatography (Liu et al., 1991) to chemical crosslinking experiments (Li and Handschumacher, 1993; Husi et al., 1994), photoaffinity labeling (Husi et al., 1994), proteolysis experiments (Swanson et al., 1994),
recombinant calcineurin truncations and point mutant experiments (Etzkorn et al., 1994; Parsons et al., 1994; Clipstone et al., 1994), and the yeast two-hybrid system (Clipstone et al., 1994). Drug-inhibited complexes were originally detected by calcium-dependent binding of CNA/CNB/CAM to a GST-FKBP fusion protein immobilized on glutathione Sepharose 4B resin charged with FK506 drug. This complex could be competed off of the resin only with cotreatment of FK506/FKBP or CsA/CyPA but not with treatment of rapamycin/FKBP (rapamycin is an immunosuppressant which binds FKBP as well, but has been found to inhibit the G1-S transition rather than the CsA/CyP-sensitive G0-G1 transition-reviewed in Schreiber et al., 1993; Schreiber et al., 1992; and Sigal and Dumont, 1992). CNA/CNB heterodimers treated with the drug-immunophilin complexes (300nM FK506 and 2.3 μM FKBP) in the presence of Ca2+ and calmodulin showed inhibited phospho-RII dephosphorylation (Liu et al., 1991), while possessing, at the same time, a 4-fold increase in pNPP phosphatase activity (at concentrations of 10 μM CsA + 10 μM CyPA [Swanson et al., 1992], i.e. well above the Ki of 270 nM drug/imunophilin complex; Etzkorn et al., 1994).

Further experiments dissecting this drug/imunophilin/calcineurin interaction have attempted to pinpoint the minimal residues and domains of the holoenzyme required for inhibition by the drug/imunophilin complexes. Reciprocal plots of 1/v vs.1/s have shown that the mode of inhibition with the wild type calcineurin and CsA/wild type CyPA is noncompetitive. In the same study, several point mutations in CyPA had been made, in efforts to better understand which residues are important for creating this interaction. The R69E mutant was found to exhibit 13-fold lower inhibition of phosphatase activity with an apparent Ki of 3400 nM, while the intrinsic peptidyl-prolyl cis-trans isomerase activity of this CyPA mutant was comparable to wild type (Etzkorn et al., 1994). Li and Handschumacher (1993), in chemical crosslinking experiments, detected crosslinking of CsA to CNB in the presence of 1 mM CaCl2/1 mM MgCl2/1 mM MnSO4. They found higher levels of crosslinking upon the addition of calmodulin. In the presence of 2mM EGTA no such complex is detected, in agreement with previous findings as to the Ca2+-dependency of the drug/imunophilin interaction with calcineurin (Liu et al., 1991). Limited proteolysis of native calcineurin yielded a C-terminal truncation enzyme which was characterized by a Ca2+-independent crosslinking of CNB to cyclosporin/cyclophilin complexes (Swanson et al., 1992; Li and Handschumacher, 1993). Husi et al. (1994) reproduced earlier results showing the essential role that CNA plays in the crosslinking of CNB to CsA/CyP and FK506/FKBP complexes, as well as the essential role of Ca2+(but not CAM) in creating the wild type complex between calcineurin and the drug/imunophilin. Importantly, they were able to show, by covalent attachment of a photoaffinity label to CsA, the Ca2+-dependent labelling of both the CNB subunit as well as the CNA subunit in the presence of CAM. These results implied, for the first time, that the CNA subunit makes direct contacts with the drug/imunophilin complex as well as the CNB subunit (Husi et al., 1994). Purified reconstituted CNA/CNB heterodimers of
CNAα1 C-terminal deletion mutants showed different abilities to bind drug/immunophilin in the presence and absence of calcium. While a recombinant CN containing a truncation just C-terminal to the putative autoinhibitory domain bound FK506/FKBP/GST beads in a Ca2+-dependent fashion, the autoinhibitory deletion mutant and the mutant with a C-terminal deletion up to and including the CAM domain bound the drug complex just as well as the native enzyme in the presence of Ca2+, clarifying the results of the proteolysis experiments mentioned earlier. Clipstone et al. (1994) found that a truncation of calcineurin corresponding to residues 99-394 of CNAα1 was competent to bind CNB in vivo as judged by immunoprecipitation with anti-CN antibodies (as was a C-terminal truncation to residue 376), yet was unable to bind drug/immunophilin complexes immobilized on a GST column as described above. A 32-394 mutant was able to bind CNB and both CsA/CyPA and FK506/FKBP. These results indicate possible involvement of CNA in making contacts to the drug/immunophilin complex (although the possibility exists that the 99-394 mutant is improperly folded and therefore unable to bind the complex). This group also used yeast two-hybrid technology to probe the requirement for CNB in drug/immunophilin binding in vivo. A his3 mutant yeast strain carrying a plasmid containing a C-terminal truncation to residue 394 fused to a GAL4 activation domain and a HIS3 gene under the control of a GAL4 promoter, transformed with a plasmid carrying FKBP fused to the GAL4 DNA binding domain was able to grow on plates containing FK506 and lacking his. No FK506-dependent growth was observed for the 394 mutant with the endogenous yeast CNB gene disrupted. Similarly, a 332 mutant (with C-terminal truncation up to residue 332, i.e. a mutant which does not contain the proposed CNB binding domain) transformed with GAL4-FKBP also does not grow on FK506-containing selective media, pointing toward the important contribution of the B subunit toward in vivo inhibition of calcineurin by FK506/FKBP.

In the current study, we created several GST fusion-deletion mutants of calcineurin A, Δ311, Δ332, and Δ376. Each of these has a fully intact catalytic domain, as determined from homology to λ phosphatase and PP1/PP2A; only Δ376 still contains a CNB binding domain. Activity of these clones was assessed using pNPP and RII phosphopeptide as substrates.

Materials and methods

Cloning of Calcineurin truncation mutants. Human Calcineurin A α (CNAα) template was a generous gift of Dr. Randall Kincaid; this template had one silent mutation. Three truncation mutants were studied: Δ311 (amino acids 1–311 of the full length calcineurin), Δ332, and Δ378, all as fusion proteins to glutathione S-transferase. PCR primers (Bio-Synthesis) were designed such that the 5' primer contains a BamH1 site immediately 5' of the CNAα coding sequence, while the 3' primers contain a HindIII site immediately downstream of a stop codon which follows the coding sequence. The 3' primer for the Δ378 clone contains an XhoI site between the HindIII site and the stop codon to facilitate the cloning of a tandemly expressed
CNB subunit (with 5' XhoI and 3' HindIII restriction sites) in future studies of coexpressed Δ378 and CNB. The primer sequences were as follows: 3' primer for Δ311 (CN37T51): 5'-AGCTACTACGGCTTCGGCTGGCTC-3'; 3' primer for Δ332 (CN38T52): 5'-AGTCACGGCTTCGGCTGGCTC-3'; 3' primer for Δ378 (CN39T53): 5'-AGCTACAGCTCTCTCCGAGTCACGGCTTCGGCTGGCTC-3'; 5' primer for all three clones (CN34T48): 5'-ATCGCGGATCCCATATGTCCGAGCCCAAGGCA-3'. Truncation mutants were created and amplified by PCR. PCR reactions for all three clones included 2mM MgCl2, 1μM each of 5' and 3' primers, and 20 ng of CNAα template per 100 μl reaction (PCR reagents were from Perkin-Elmer). Δ332 and Δ378 were amplified by the following method: 960C, 6'—one cycle; 960C, 1'/ 450C, 1'/ 720C, 1.5'—three cycles; 940C, 1'/ 450C, 1'/ 720C, 1.5'—thirty cycles; 720C, 6'/ 250C, 30'/ 40C, 999'—one cycle. Δ311 was amplified similarly except that the annealing temperature was raised to 550C in all thermal cycles. PCR products were restriction digested with BamHI/HindIII as was the pGEX-KG plasmid. Restriction products were purified by electrophoresis in a low-melt agarose gel at 4°C (NuSieve GTG agarose, FMC Bioproducts, Rockland, ME) and an in-gel ligation was conducted according to standard procedures using T4 DNA ligase, at 16°C, overnight. Ligation products were transformed into competent DH5α cells. DNA from transformed bacteria were restriction-digested with the appropriate enzymes to confirm presence of the clones; dideoxy sequencing was partially completed for the Δ332 and Δ378 mutants. Δ378 was further transformed into competent XA90 E. Coli for expression.

Induction and Purification. Bacteria carrying the GSTfusion protein plasmid were inoculated into 10-12 mls of LB broth containing 100 μg/ml ampicillin and grown at 37°C overnight with shaking. The next day, secondary cultures were grown by diluting the overnight cultures 100-fold into 1 liter of LB containing ampicillin as before. Due to solubility problems/inclusion bodies, the Δ378 mutant secondary culture was grown at 30°C, while the Δ332 and Δ311 cultures were grown at 37°C. Δ311 mutants were induced to 0.5mM IPTG (Isopropyl β-D-thiogalactopyranoside; Sigma) when the OD596 reached 0.6. Δ332 and Δ378 mutants were induced to 0.5 mM IPTG when the OD596 reached 0.9. Δ378 cultures were grown for 4 hours after induction; Δ311 and Δ332 cultures were grown for 5-6 hours. The bacteria were harvested by centrifugation at 8,000 x g for 10 minutes and washed in buffer A(75mM Tris, 120mM NaCl, 1mM EDTA, 1mM EGTA/ pH7.5). Pellets were weighed and resuspended in buffer A+protease inhibitors (1μg/ml leupeptin, 1μg/ml pepstatin, 2 μg/ml aprotinin, and 1mM PMSF) at 5 mls buffer/mg pellet. The cells were lysed by two passages in a French Press at 12,000 psi. Cell debris was removed by a further centrifugation at 10,000 x g for 15 minutes. The crude lysate was incubated with Glutathione-sepharose 4B beads (Pharmacia) which had been preequilibrated with buffer A for 6 minutes at 4°C, rotating. After binding and centrifugation to retrieve the pellet, the pellet was washed four times with 12 volumes of buffer A and then eluted for three hours at 4°C, rotating, in buffer A containing protease.
inhibitors as before, with the addition of 0.75 mM DTT (Malincloud) and 10 mM glutathione (Sigma). Protein concentration of the eluted protein was measured by Bradford assay using bovine serum albumin (Boehringer Mannheim) as a standard. Purification was followed by SDS-PAGE according to the method of Laemmeli. Due to the low concentration of protein in the eluate, BSA dissolved in buffer A was added to a final concentration of 2-3mg/ml and glycerol added to 30%. Proteins were stored at -20°C. Typical yield of purified Δ378 was 0.45 mg of protein from one liter of cultures. The Δ332 mutant yielded 0.7 mgs, and the Δ311 mutant yielded 0.3 mgs, from one liter of bacterial cultures.

Reconstitution of CNAα Δ378 mutant and CNB. Calcineurin B, cloned into a pAED4 vector with a T7promotor and engineered to include a 5' Ndel site and3' Pstl site, was transformed into DH5α and sequenced by coworkers using the dideoxy DNA sequencing method. BL21-DE3 competent cells were transformed with pAED4-CNB. Transformant colonies were inoculated into 10 mls of LB containing 100 μg/ml and grown overnight at 37°C. Secondary cultures were started by a 1/100 dilution of the over night culture into 1 liter of broth as before and grown to an OD596 of 0.6, at which point the cultures were induced with 0.7 mM IPTG (final concentration) for 5-6 hours. Cultures were harvested, washed, resuspended in buffer B (75 mM Tris, 50mM NaCl, 1mM EDTA, pH 7.5) containing protease inhibitors as before, and lysed. After centrifugation at 10,000 x g for 15 minutes to remove cell debris, the crude lysate was precleared by Glutathione-sepharose 4B beads preequilibrated in buffer B. The precleared CNB lysate was next incubated with GST beads which had been freshly incubated with Δ378 crude lysate (also made using buffer B) and which had been washed once with buffer B after Δ378 binding. The reconstitution buffer (B) included either 2mM Ca2+ or 2 mM Mn2+. The incubation was carried out for 3 hours on a rotator at 4°C; the beads were then washed three times in buffer B containing 300 mM NaCl. After one wash with buffer A, the proteins were eluted for three hours in buffer A containing protease inhibitors, 10 mM glutathione, and 0.75 mM DTT. As before, purification was followed by SDS-PAGE and protein concentration measured by Bradford assay, using BSA as the standard. To detect the presence of CNB, Western blotting of the reconstituted protein was performed using a 1 μg/ml dilution of CNB monoclonal primary antibody.

Partial purification of purine ribonucleoside phosphorylase (PRPase). The bacterial purine ribonucleoside phosphorylase preparation purchased from Sigma was approximately 30% pure judging by SDS-PAGE analysis of the crude enzyme preparation. The 29 kDa enzyme was further purified by Q sepharose chromatography (Pharmacia), essentially according to the method of Etzkorn et al. (1994). Six milligrams of crude PRPase were loaded to a 10 ml bed volume of Q sepharose in a 1.5x10cm Econo-Column (BioRad) equilibrated with 50mM Tris, pH 7.5 (at 4°C). The column was developed with a linear gradient from 50mM Tris pH 7.5 (60 mls) to 50 mM MES (2-[N-Morpholino]ethanesulfonic acid, Sigma), pH 5.8 containing 0.5M KCl (60 mls) at a flow rate of 1.1 ml/min. Fractions of 3.3 ml were collected; protein elution was
followed by OD280 and SDS-PAGE. Fractions containing a 29 kD species were tested for PRPase activity using first crude, then purified MESG (methyl thioguanosine), and inorganic phosphate as substrates. Active fractions were pooled and concentrated in a centricon-30 (Amicon); glycerol was added to 30% and the protein was stored at -20°C. The spectrophotometric assay for PRPase activity was performed in 1x buffer C (100 mM Tris, pH 7.6, 1 mg/ml BSA, 0.5 mM DTT) according to the method of Webb (1992). Briefly, in this assay, conducted at ambient temperature, Pi concentrations ranged from 5→25 µM KH2PO4 and the MESG concentration was kept constant at 50 µM. 5 µg of PRPase per ml reaction was added to 1x buffer and preincubated for 2 minutes, at which point MESG was added to 50 µM and the change in absorbance at 360 nm monitored for 1 minute, or as long as the reaction remained linear. The Km was found to be 15.6 µM for inorganic phosphate, similar to the reported 26 µMolar Km; Kcat= 4.44 sec-1; Kcat/Km=5.79 x 106 M-1; specific activity= 9.2 µmol/min/mg purified PRPase.

**MESG Synthesis and Purification.** MESG (methylthioguanosine) was synthesized essentially according to the method of Webb and Broom et al from 2-amino-6-chloropurine nucleoside (Sigma) by a coworker. In a modified purification procedure, crude MESG was dissolved in 10 mgs/ml H20 and further purified by preparative HPLC on a Vydac C18 reverse-phase employing an isocratic elution in 20 mM KOAc, pH5.5 containing 10% (v/v) methanol, with peak detection set at 342 nm and a flow rate of 6.0 ml/min. Individual peaks were collected and lyophilized, then resuspended in H2O to assess activity in the PRPase assay. The active peaks from several runs were combined and lyophilized, and stored at -80°C until resuspended in H2O immediately prior to usage (and stored at -20°C.) Since the λmax was found to be sensitive to pH, the OD at the λmax for a range of pH's was measured in order to correlate the extinction coefficient reported at pH 1 by Broom et.al. (1964) with the apparent extinction coefficient and absorbance at pH 5.5. e 344 at pH 5.5 was found to be 2.18 x 104 M-1 cm⁻¹. An estimated 40% (by weight) of the crude MESG was recovered after HPLC purification which was active in the PRPase assay.

**Phosphorylation and purification of phosphoserine RII peptide.** The 19-amino acid RII peptide was synthesized by the M.I.T. Biopolymers Lab. The kinase reaction and purification were performed according to the method of Etzkorn et. al. (1994). Briefly, in order to phosphorylate 15 mgs of RII in a total reaction volume of 12 mls, cAMP-dependent protein kinase (PKA, 1.3 nM units/mg; Sigma) was first activated by preincubation with cAMP. Specifically, 2 mgs of PKA were preincubated at room temperature for 10 minutes in 537.5 µl H2O, 10.75 µl of 1 mM 3'5'cAMP stock (Sigma), 26.9 µl of 50 mg/ml BSA stock, and 215 µl of 1 M DTT stock. The following reagents were then added in order: 2.69 mls of 4 x buffer (1 x = 50 mM Mops, 10 mM MgCl₂, pH 7.1), 6.02 mls of H2O, 215 µl of 0.1 M ATP stock (Sigma), and 15 mgs of RII peptide which was dissolved in 1.075 mls of H2O. The kinase reaction was incubated at 30°C for 8 hours. The reaction was driven to completion by the further addition of PKA and
ATP to the reaction mixture in the following manner: 0.4 mgs of PKA were preincubated for 10 minutes at room temperature in 270 µl of buffer containing 270 mM DTT, 0.013 mM 3'5'cAMP, and .085 mg/ml BSA. The enzyme was then added to the kinase reaction. After addition of a further 72.9 µl of 0.1 mM ATP to the reaction mixture, the kinase reaction was incubated a further 14 hours at 30°C. Protein was removed from the kinase reaction by filtration in a Centricon-30 (Amicon) prior to HPLC purification. 1-ml injections of the filtered kinase reaction were loaded to a Vdack C18 reverse-phase column preequilibrated with 9:1 of 20 mM NH4OAc:Acetonitrile (=buffer D). The column was developed in a gradient of 100% D-- >75%D/25%E over 15 minutes, where buffer E= a 1:9 mixture of 20mM NH4OAc: Acetonitrile. Phosphorylated RII peaks were combined, lyophilized, and stored as a powder until immediately prior to usage when they were diluted in H2O, with the molarity of the resulting solution calculated from the absorbance at 258 nm as compared to an HPLC-purified RII standard.

Phosphatase Activity assays: (1): PNPP assay. para-Nitrophenyl phosphate (Pharmacia) was dissolved to a 0.5 M stock solution in ddH2O and kept at -20°C. Phosphatase assays (1 ml assays) were carried out at ambient temperature in assay buffer containing 100 mM Tris, pH 7.6, 50mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mg/ml BSA. Reactions were started upon addition of the pNPP substrate to enzyme which had been preequilibrated for four minutes in assay buffer containing stimulatory metals. When present, the final concentration of MgCl2 was 6 mM, CaCl2 was 2 mM, and MnCl2 was 2 mM. Enzyme concentrations varied and are discussed in the legend to figures 4 and 5. The reaction was monitored by a spectrophotometer measuring the change in absorbance at 410 nm and an extinction coefficient of 1.8 x 10^4 M^-1 cm^-1. (2) RII phosphopeptide coupled assay. The coupled assay was carried out in the same buffer as that used for pNPP assays. All coupled assays contained 2 mM MnCl2 in the assay buffer. Enzyme (again, enzyme concentrations are specified in the legend to figures 4 and 5) was preequilibrated for four minutes in a cuvette containing assay buffer, 2 mM MnCl2, .6 µg of Q-sepharose purified PRPase, and 200 µM purified MESG. The reaction was started by the addition of RII phosphopeptide to the cuvette, to a final volume of 0.5 mls per reaction. The reaction was monitored by the change in OD360 over time as the MESG base was liberated from the glycosidic bond to ribose. Employing a change in extinction coefficient of 1.1 x 10^4 at 360 nm, the data were analyzed further on Kaleidagaph.

Results and Discussion

Three calcineurin (CNA(1)) deletion mutants were constructed: Δ311, Δ332, and Δ378. All three were expressed as GST fusion proteins which were quite insoluble, resulting in the formation of inclusion bodies over the range of temperatures, induction times, and IPTG levels
Figure 1. GST fusion proteins were purified as described in the Materials and Methods section. Their purity was assessed by SDS PAGE on a 12% polyacrylamide gel. Lane1, Δ378 mutant protein, 4 μg loaded; lane2, Δ311 mutant protein, 4 μg loaded; lane 3, Δ332 mutant protein, 4 μg loaded; lane 4, Δ376 mutant protein reconstituted with CNB in the presence of Mn²⁺, 5 μg loaded.
Figure 2. Western blot analysis was performed on a 12% SDS PAGE gel containing purified calcineurin truncation mutants as described in the Materials and Methods section. Anti-calcineurin B monoclonal primary antibody was used to detect the presence of CNB in the CNB-reconstituted and purified Δ378/CNB enzyme. Lane 1, 0.3 μg of non-reconstituted Δ378; lane 2, 0.3 μg of Δ378/CNB reconstituted enzyme (reconstituted in buffer containing Ca$^{2+}$); lane 3, 0.3 μg of Δ378/CNB reconstituted enzyme (reconstituted in buffer containing Mn$^{2+}$).
tested. Low yields of purified protein were obtained, with all three clones yielding less than 1 mg of protein per liter of bacterial culture (discussed in Materials and Methods section). By SDS-PAGE, it seems that the Δ332 protein is much more stably expressed than the Δ311 protein, which seems to be degraded proteolytically in the cell (Figure 1). The Δ311 protein, which contained all of regions of homology with λ phage phosphatase (a small, 221-amino acid, discussed in Chapter 1) and a truncation corresponding to the last residue of λ PPase in sequence alignments of λ PPase and CN, possessed no detectable PNPPase activity, even at high concentrations of both enzyme and substrate (800 nM enzyme, 80 mM pNPP—compared to the Km of full length bovine brain calcineurin heterodimer of 10 mM in the presence of calmodulin-Martin et al., 1985). These results were found with two different Δ311 clones with DNA from separate PCR reactions. Although the clones were only partially sequenced, the assumption is that this truncation mutant is catalytically inactive with pNPP as the substrate (RII activity was not assessed). The same was found to be true of Δ332, which contains all of CNA catalytic domain up to and not including the beginning of the putative CNB binding domain. Two separate clones were found to be similarly catalytically inactive at 800 nM enzyme and 80 mM pNPP.

The only one out of the three proteins studied here to demonstrate catalytic activity toward both pNPP and RII phosphopeptide was the Δ378 truncation mutant. This protein lacks the autoinhibitory domain and calmodulin binding domains, while retaining a functional CNB binding domain (Clipstone et al., 1994). Reconstitution of Δ376 with human CNB was achieved by incubation of GST-bound Δ376 with recombinant CNB extracts from BL21 (DE3) bacteria in the presence of either Ca2+ or Mn2+ for three hours (Wanatabe et al., 1994) washed, and eluted with glutathione as described above (Figures 1 and 2). As can be seen from Figures 3 and 4 and Table 1, the Δ378 pNPPase activity is completely Ca2+-independent, with the Mg2+- and Mg2+/Ca2+-stimulated Km and Vmax (i.e., specific activity) activities yielding comparable values for both parameters. As has been previously reported for the native enzyme (Ballou and Fischer, 1986), Mn2+ stimulated the activity of the enzyme, resulting in an increase in the Vmax by about 6-fold. Reconstitution of Δ378 with CNB in the presence of Mn2+ yielded a Mn2+-stimulated enzyme characterized by a 14-fold increase in Vmax and no change in Km. The Km obtained for the Δ378 and Δ378/CNB enzymes, approximately 65 mM pNPP, is more than 6-fold higher than the Km of 10 mM reported for bovine brain calcineurin in the presence of calmodulin and Mn2+ (Martin et al., 1985), implying a >6-fold decrease in the mutant’s affinity for pNPP.

Martin et al. find Vmax=3.7 μmol/min/mg for calcineurin, which is four-fold higher than the reconstituted Δ378/CNB protein. This difference could potentially reside in the incomplete reconstitution of Δ378/CNB, or the decrease in catalytic efficiency of the mutant enzyme due to non-ideal folding. In any case, one strong implication of our studies regarding pNPPase activity
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metal</th>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>K&lt;sub&gt;cat&lt;/sub&gt;</th>
<th>K&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Sp.act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN(Sigma)</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>205+/−25 μM</td>
<td>5.05</td>
<td>2.47×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3800+/−424</td>
</tr>
<tr>
<td>CNA378</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>688+/−146 μM</td>
<td>.12</td>
<td>1.76×10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>107+/−21</td>
</tr>
<tr>
<td>CNA378/CNB/Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>88+/−6 μM</td>
<td>2.05</td>
<td>2.33×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1415+/−94</td>
</tr>
<tr>
<td>CNA378/CNB/Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>117+/−13 μM</td>
<td>1.25</td>
<td>1.07×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>906+/−95</td>
</tr>
<tr>
<td>CNA378</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>pNPP</td>
<td>69+/−7 mM</td>
<td>.41</td>
<td>5.97</td>
<td>361+/−31</td>
</tr>
<tr>
<td>CNA378/Mg&lt;sup&gt;2+&lt;/sup&gt;/Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>pNPP</td>
<td>70+/−27 mM</td>
<td>.08</td>
<td>.82</td>
<td>.82</td>
<td>66+/−22</td>
</tr>
<tr>
<td>CNA378/Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>pNPP</td>
<td>58+/−5 mM</td>
<td>.06</td>
<td>1.08</td>
<td>56+/−4</td>
<td></td>
</tr>
<tr>
<td>CNA378/CNB/Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>pNPP</td>
<td>74+/−15 mM</td>
<td>1.28</td>
<td>17.39</td>
<td>883+/−164</td>
</tr>
</tbody>
</table>

Table I. Kinetic parameters of deletion mutant enzymes in different conditions. Enzymes were incubated in the presence of Mg<sup>2+</sup>, Ca<sup>2+</sup>, and/or Mn<sup>2+</sup> as noted and enzyme assays were performed as discussed in the Materials and Methods section. Reconstitution of the Δ378 mutant in Ca<sup>2+</sup> or Mn<sup>2+</sup> is specified in the first column of the table. Bovine brain calcineurin (Sigma) was reconstituted in the absence of added metal ions.
Km comparison: pNPPase activity

Km, mM units

Enzyme

Km comparison: RII dephosphorylation

Km, micromolar units

Enzyme

Figure 3
Figure 3. $K_m$ determination and comparison. pNPP and coupled RII phosphopeptide assays were performed on the Δ378 truncation mutant as well as the reconstituted Δ378/CNB enzyme. Activity was measured in assay buffer containing 0.1 mM EDTA and 0.1 mM EGTA, as well as containing Mn$^{2+}$, Mg$^{2+}$, and/or Ca$^{2+}$, as follows. All RII experiments were performed in the presence of Mn$^{2+}$. The RII activity of bovine brain calcineurin (Sigma) was measured at an enzyme concentration of 33.75 nM and in the presence of 2 mM Mn$^{2+}$ (denoted “Sigma CN/Mn”). The RII activity of the Δ378 enzyme alone was tested at an enzyme concentration of 1 μM (denoted “378/Mn”). The RII activity of the Δ378 enzyme which had been reconstituted in the presence of Ca$^{2+}$ (but assayed in the presence of Mn$^{2+}$) was measured at 240 nM reconstituted enzyme (denoted “378/B/Ca/Mn”). The Δ378 enzyme reconstituted with CNB in the presence of Mn$^{2+}$ and assayed for activity in the presence of Mn$^{2+}$ was present at a concentration of 118 nM (denoted “378/B/Mn”). Label identification of the reconstituted and non-reconstituted enzymes, assayed for activity in buffer containing different metal ions, is the same for the pNPP assay as for the coupled assay. Hence, the activity of “378/Mg/Ca” corresponds to that of the unreconstituted Δ378 mutant, in the presence of Mg$^{2+}$ as well as Ca$^{2+}$. Enzyme concentrations for the pNPP assays were as follows: 378/Mg/Ca, 1 μM; 378/Mg$^{2+}$, 1 μM; 378/Mn$^{2+}$, 430nM; 378/B/Mn, 118nM.
specific activity: pNPPase activity

Enzyme

specific activity: RII dephosphorylation

Enzyme

Figure 4
Figure 4. Specific Activity determination and comparison. See legend to Figure 3 for code used to label the x-axis, describing a particular enzyme assayed under specific conditions.
is the importance of the CNB subunit to the catalytic efficiency of the enzyme. The apparent increase in \(V_{\text{max}}\) of the reconstituted protein implies that CNB is not merely regulating substrate specificity, but catalytic efficiency as well. These results concur with previous reports that CNB reconstitution with full length calcineurin, assayed in the presence of \(\text{Mn}^{2+}\), yields a large increase in \(V_{\text{max}}\) relative to the full length enzyme incubated in the presence of calmodulin and the absence of CNB (Perrino et al., 1992). The lack of change in activity of the \(N. \text{Crassa}\) enzyme in response to CNB stimulation in a pNPPase assay described by Higuchi et al. (1990) was later found to be dependent on the CNB isoform used to stimulate Norf1 activity. Consistent with our findings regarding CNB stimulation Ueki et al. (1993) found that CNB\(_{\beta 1}\) reconstitution with full length Norf1 and assayed for in the presence of calmodulin, \(\text{Ca}^{2+}\), \(\text{Mg}^{2+}\), and \(\text{Mn}^{2+}\) doubled the specific activity of Norf1 to levels slightly lower than those obtained in our study (480-720 nmol/min/mg).

RII phosphopeptide phosphatase activity was monitored in a spectrophotometric assay described previously (Etzkorn et al., 1994; Webb, 1992). Briefly, the inorganic phosphate generated by calcineurin-catalyzed dephosphorylation of the RII peptide substrate serves as a substrate for a second enzyme, purine ribosyl phosphorylase (PRP). PRP then catalyzes the cleavage of the glycosidic bond between ribose and MESG (methyl thioguanosine), yielding ribose-1-phosphate and the MESG base, the evolution of which can be monitored by a change in \(\text{OD}_{360}\) (Figure 5). Purification of crude MESG and crude RII phosphopeptide by preparatory HPLC were as described in Materials and Methods (Figures 6 and 7, respectively). Results of the coupled assay are reported in Table I and Figures 3 and 4 (an example of a representative set of the kinetics reaction data used to characterize the kinetic parameters of the enzyme can be found in Figure 8.)

The \(\text{Mg}^{2+}\) and \(\text{Mg}^{2+}/\text{Ca}^{2+}\)-stimulated activity of the \(\Delta 378\) deletion mutant was too low to detect using the coupled assay. Comparison of the \(\text{Mn}^{2+}\)-stimulated activity of the \(\Delta 378\) and \(\Delta 378/\text{CNB}\) enzymes showed a 7-fold decrease in \(K_{\text{m}}\) upon reconstitution of mutant with CNB as well as a >8-fold increase in \(V_{\text{max}}\). These results point to a role for CNB in both regulation of substrate affinity as well as catalytic efficiency of the enzyme. The CNB-reconstituted \(\Delta 378\) mutant appears to bind RII phosphopeptide twice as tightly as reconstituted bovine brain CN under similar conditions although its specific activity is 2-3 fold lower than that of the full length CN, implying the tighter affinity of the mutant for RII phosphopeptide. These results are complicated by the fact that Etzkorn et al., in their coupled assay, found bovine brain CN to have a \(K_{\text{m}}\) of 110 \(\mu\text{M}\) for RII phosphopeptide (almost 5-fold higher than the \(K_{\text{m}}\) determined for the same substrate and enzyme by the standard \(^{32}\text{P}\)-release assay; Chan et al., 1986) which is similar to the \(K_{\text{m}}\) found for the \(\Delta 378/\text{CNB}\) enzyme in this study. Possible differences may have resulted from differences in the reconstitution buffer for bovine brain CN. Etzkorn et al. report a 6-hour incubation to achieve full activity, likely in the presence of \(\text{Mn}^{2+}\), whereas in this
(1) **first step**: dephosphorylation of Rγ phosphopeptide substrate by calcineurin.

\[
\text{DLDBPIPGFDRRV} \rightleftharpoons \text{OPO}_3^2^- \rightleftharpoons \text{CALCINEURIN} \rightarrow \text{HOPO}_3^2^- + \text{OH}\]

\[
\text{DLDBPIPGFDRRV} \rightleftharpoons \text{VAAE} \]

(2) **Second step**: purine ribonucleoside phosphorylase scavenges inorganic phosphate and catalyses the breakage of the glycosidic bond, which causes a shift in \(\lambda_{\text{max}}\) characteristic of the free base.

\[
\text{HOH}_2C \rightleftharpoons \text{C}_\text{S} \rightleftharpoons \text{PRPase} \rightarrow \text{HOPO}_3^2^- + \text{OH}\]

\[
\text{HOH}_2C \rightleftharpoons \text{C}_\text{S} \rightleftharpoons \text{PRPase} \rightarrow \text{HOPO}_3^2^- + \text{OH}\]

Figure 5. Coupled Assay.
Marked Wavelengths

\begin{align*}
\text{Seg A: L} & \sim 342 = 0.26173 \\
\text{Seg A: L} & \sim 344 = 0.2615
\end{align*}
Figure 6. Crude Methylthioguanosine, prepared according to the method of Webb (1992) was purified by preparatory HPLC, with the active substrate for purine ribonucleotide phosphorylase eluting slightly later than a large contaminating peak in an isocratic elution of 20 mM KOAc pH 5.5 which contained 10% methanol. The MESG displays a UV absorbance profile as shown in the inset.
Marked Wavelengths
Reg A: L 258 = 0.74586
Figure 7. RII peptide purification by preparatory HPLC according to the method of Etzkorn et al. (1994). The phosphorylated peptide substrate is eluted at 17% B, while the unphosphorylated peptide is eluted at 22% B (the peptides were resolved with a gradient from 100% A to 75% A/25% B where buffer A contained a 9:1 proportion of 20 mM NH₄OAc: Acetonitrile and buffer B contained a 1:9 proportion of 20 mM NH₄OAc: Acetonitrile.
Figure 8. Kinetic parameters were determined by analysis of Lineweaver-Burke reciprocal plots of a set of individual kinetic assays. Shown here is a representative plot of the reciprocal initial velocity of the Δ378 enzyme (not reconstituted with CNB) in the presence of 2mM MnCl₂ vs. the reciprocal Rₚ phosphopeptide substrate concentrations, in units of M⁻¹. The standard deviation associated with each point did not exceed 3%.
study, calcineurin purchased from Sigma was reconstituted in the absence of any added metal cations for 6 hours. It is possible that the longer treatment of Mn\(^{2+}\) results in a more activated form of the enzyme with altered $K_{cat}$ and $K_m$ values. This interpretation is consistent with the notion of a slow conformational change of calcineurin to a more active form in the presence of Mn\(^{2+}\) (Ballou and Fischer, 1986). If so, extended Mn\(^{2+}\) incubation times become critical in order to compare results obtained from different studies (a suggestion also made by Chan et al., 1986). In our study, A378 reconstituted with CNB in Ca\(^{2+}\) has a slightly higher $K_m$ than the same enzyme reconstituted in Mn\(^{2+}\) (117 $\mu$M vs. 88 $\mu$M), as well as exhibiting only 65% of the specific activity found with the Mn\(^{2+}\)-reconstituted enzyme (which was incubated in Mn\(^{2+}\) for extended periods of time beyond the 4-minute preincubation in Mn\(^{2+}\) included in the assay).

The positive effect of CNB on both the $K_m$ and $K_{cat}$ with RII phosphopeptide as substrate demonstrated in this study corroborate results obtained by Perrino et al. (1995) on a Δ420 enzyme containing both the CNB-binding- and CAM-binding-domains in a 32-P-release RII phosphopeptide assay. They find that their deletion mutant exhibits Ca\(^{2+}\)-independent activity in Mg\(^{2+}\) basal stimulation assays, with only a slightly higher $V_{max}$ attained in the presence of Ca\(^{2+}\)/CAM combined than in the presence of one or the other (perhaps alluding to the possibility of additional inhibitory residues in the CAM binding domain, alluded to in the proteolysis experiments of Hubbard and Klee (1989). Direct comparison of their results with ours in order to further clarify this issue and assess the effects of the further C-terminal truncation on both specific activity and $K_m$ values are difficult because the coupled assay is unable to detect Ca\(^{2+}\)/Mg\(^{2+}\)-stimulated activity.

**References**


