Zoological Studies **34**(3): 149-163 (1995)

Zoological Studies

Review Article

The Enzymology of Phosphorylase Phosphatase (Protein Phosphatase-1) — A Personal Perspective

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CONTENTS

ABSTRACT

Ernest Y.C. Lee (1995) The enzymology of phosphorylase phosphatase (protein phosphatase-1) — a personal perspective. *Zoological Studies* **34**(3): 149-163. In this paper we review studies of phosphorylase phosphatase (protein phosphatase-1) that have been the focus of our work for the past two decades. The enzymology of the enzyme is complex, and the description of its properties has taken a great deal of effort. Key discoveries in this process were the isolation of the catalytic subunit of the enzyme, and the discovery of inhibitory proteins, one of which (inhibitor-2) forms a complex with the catalytic subunit. Later it was discovered that a number of holoenzyme forms existed, and that these consisted of complexes of the catalytic subunit with different regulatory subunits. In recent years, the cloning of the cDNAs for the catalytic subunit has demonstrated that it is a very highly conserved protein and that it plays a critical role in mitosis. Expression of the catalytic subunit in *E. coli* has opened the way for structure-function studies. The region involved in the binding of toxins which inhibit the enzyme have been identified by mutagenesis.

Key words: Protein phosphatase, Metabolic control, Mutagenesis.

INTRODUCTION

The protein phosphatases are integral components of regulatory mechanisms involving protein phosphorylation. Protein phosphorylation cycles are regulatory elements in many areas of metabolism and cell function. The phosphatases impart the crucial property of reversibility to these systems, and the modulation of their activities can also govern the overall phosphorylation state (and functional level) of a given phosphorprotein (for reviews, see Cohen 1989, Shenolikar and Nairn 1991, Bollen and Stalmans 1992, Cohen 1994). The protein phosphatases which dephosphorylate phosphoserine/phosphothreonine residues in proteins are now classified as protein phosphatases

1, 2A, 2B and 2C, respectively, based on substrate specificity and inhibitor sensitivity (Cohen 1989). The prototype for the ser/thr phosphatases is mammalian phosphorylase phosphatase (protein phosphatase-1 or PP1). In this review article I will present a personal perspective of research on phosphorylase phosphatase (for earlier reviews see Lee et al. 1976 1980). Phosphorylase phosphatase is indeed an "old" enzyme, and was first described in 1943 (Cori and Green 1943). It was originally discovered by its ability to convert phosphorylase *a* to phosphorylase *b*, and it was only in 1956 that its mode of action was determined to be that of a protein phosphatase by Wosilait and Sutherland (1955). The enzymology of phosphorylase phosphatase is complex, and took a great deal of effort over several decades by many laboratories to unravel (Lee et al. 1980, Ballou et al. 1985, Bollen and Stalmans 1992, Cohen 1994).

ISOLATION OF THE CATALYTIC SUBUNIT OF PHOSPHORYLASE PHOSPHATASE

In 1972, I embarked on the isolation of liver phosphorylase phosphatase. The liver enzyme had previously been studied by Earl W. Sutherland Jr., who reported a partial purification of the enzyme based on classical precipitation methods (Wosilait and Sutherland 1956). The focus of interest in the area in those days was largely to solve the isolation problem, so that the basic physicochemical properties of the protein could be determined, as well as its substrate specificity. The latter issue was important because it was then not clear whether different phosphatases were involved in the dephosphorylation of glycogen synthase, phosphorylase kinase and phosphorylase, and in fact research groups in the area could be divided into two groups, one studying glycogen synthase phosphatase and another studying phosphorylase phosphatase. There were numerous reports of partially purified phosphatase preparations of widely differing apparent molecular weights, and also conflicting reports regarding substrate specificity (see Lee et al. 1976 1980). Initial efforts in my laboratory using ion-exchange chromatography and gel filtration procedures presented us with problems that were encountered by other investigators in the area. Thus, the enzyme activity was extremely unstable, and heavy losses of activity were sustained after even simple isolation steps. In addition, gel filtration revealed a very polydisperse profile, suggesting that there were multiple protein phosphatases which could dephosphorylate phosphorylase *a*, or that these were generated by proteolysis.

The turning point in understanding the complex enzymology of the phosphatase came when we discovered a means for the activation of liver phosphorylase phosphatase by exposure of the crude enzyme to denaturing levels of ethanol (Brandt et al. 1974). In this procedure, liver extracts were precipitated with 70% ammonium sulfate, and the precipitate redissolved and denatured with five volumes of room temperature 95% ethanol. The phosphorylase phosphatase activity could then be extracted from the alcohol precipitate. This procedure resulted in a marked activation of phoshorylase phosphatase activity, and gel filtration experiments showed that there was a concomitant reduction of the apparent molecular weight of the enzyme to a form of ca. 35 kDa. This key experiment is shown in Fig. 1. We proposed the hypothesis that this could be explained by the existence of an inactive holoenzyme composed

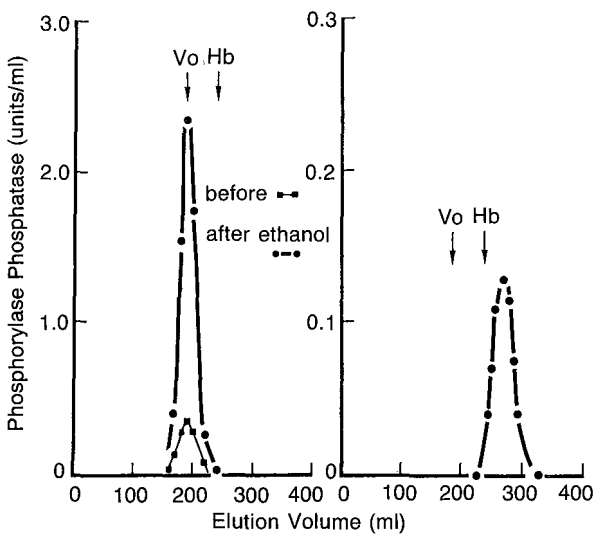

Fig. 1. Effect of alcohol treatment on the size and activity of rat liver phosphorylase phosphatase.
The data show the effects of treatment by precipitation with 5 volumes of 95% ethanol on phosphorylase phosphatase activity in a rat liver extract. The liver extract (5 ml) was chromatographed on a Sephadex G-75 column (2.5 × 90 cm). Fractions were collected and assayed for phosphorylase phosphatase activity (left panel, solid squares). Samples (2 ml) of each fraction were precipitated with ammonium sulfate, the precipitate redissolved and precipitated with ethanol. The precipitate was extracted with buffer and then assayed for phosphorylase phosphatase activity (solid circles). The treated enzyme fractions from the peak of activity were pooled, and rechromatographed on the same column (right panel). Vo and Hb refer to the void volume and elution volume for hemoglobin. Data are taken from Brandt et al. (1974) with permission of Academic Press Inc.

of a catalytic subunit in a complex with a protein inhibitor (Brandt et al. 1974). This was supported by our discovery that there were heat-stable protein inhibitors of phosphorylase phosphatase activity that were trypsin sensitive (Brandt et al. 1975b). At about the same time, the effects of trypsin on causing an activation and reduction in molecular weight of phosphorylase phosphatase were also described by Kato et al. (1974).

It was subsequently shown by Walter Glinsmann and his colleagues that there were two protein inhibitors: inhibitor-1 (I-1) and inhibitor-2 (I-2) (Huang and Glinsmann 1976). These two inhibitors were extensively studied by Phil Cohen, who purified and sequenced both of these inhibitors. Inhibitor-1 is inhibitory only when phosphorylated by cAMP-dependent protein kinase, and may play an important physiological role in regulating the activity of PP1 (Cohen 1989, Shenolikar and Nairn 1991). Inhibitor-1 and I-2 are small proteins (165 and 204 residues, respectively) which have been sequenced; both appear to have little ordered structure and their behavior on SDS-PAGE as well as gel filtration is anomalous. The cDNAs for inhibitor-1 (Elbrecht et al. 1990), and inhibitor-2 (Zhang et al. 1992a, Park et al. 1994) have been cloned. The discovery of the existence of heat-stable, trypsin-sensitive protein inhibitors of PP1 in our laboratory played an important role in the unraveling of the enzymology of PP1. Our original ideas (Brandt et al. 1974) that the 35 kDa enzyme was the catalytic subunit of a larger holoenzyme, and that the inhibitor proteins might be components of a multisubunit phosphatase, were later shown to substantially correct.

The 35 kDa enzyme obtained after the ethanol treatment was readily purified to homogeneity, and the same procedure was also found to be suitable for the isolation of the muscle enzyme (Brandt et al. 1975a). The muscle enzyme was isolated as a ca. 35 kDa form by Ed Fischer's laboratory (Gratecos et al. 1977). We purified phosphorylase phosphatase activity to homogeneity, in the form of a ca. 35 kDa protein from both rabbit liver and skeletal muscle (Brandt et al. 1975, Silberman et al. 1984). This same enzyme was also isolated from liver by Khandelwahl et al. (1976). The isolation of this enzyme allowed us to make the rigorous demonstration that phosphorylase phosphatase dephosphorylates both glycogen synthase (Killilea et al. 1976) and the β-subunit of phosphorylase kinase (Ganapathi et al. 1981). The original 35 kDa enzyme was later shown by several laboratories to be a proteolytic product of a slightly larger 37 kDa protein (DeGuzman and Lee 1988).

Extensive studies by Phil Cohen's laboratory were focused in detail on the specificities of the muscle protein phosphatases. Their approach was to classify the protein phosphatases on the basis of substrate specificities and sensitivity to the protein inhibitors. Since their earlier studies were performed with partially purified enzyme preparations (Cohen et al. 1978) this led to some initial confusion but has given rise to the nomenclature that has now reached common currency. In addition to phosphorylase phosphatase, which became known as PP1, the other phosphatases in this classification are PP2A, PP2B (calcineurin) and PP2C (Cohen 1989). It may be noted that the nomenclature of protein phosphatases, originally defined by substrate specificity and inhibitor sensitivity, has no basis in terms of structural relationships as was later confirmed when their cDNAs were cloned.

During the study of heart protein phosphatases, Heng-Chun Li isolated a 35 kDa form of phosphorylase phosphatase, but established also its ability to dephosphorylate *p*-nitrophenyl phosphate at alkaline pH (Li 1982). In our studies of muscle phosphorylase phosphatase, we found that we could in fact isolate two low molecular protein phosphatases, the first being the original phosphorylase phosphatase, and the second of which had a much higher activity toward *p*-nitrophenyl phosphate. This enzyme we called phosphatase C-II (Silberman et al. 1984). We demonstrated that phosphatase C-II had a specificity broadly similar to that of PP1, but it exhibited a higher level of activity toward *p*-nitrophenyl phosphatase and dephosphorylated the α-subunit of phosphorylase kinase more rapidly than the β-subunit (Ganapathi et al. 1984). In the classification of Cohen, this was a "type-2A" phosphatase. Our studies indicated that phosphatase C-I (PP1) and C-II (PP2A) were structurally related, based on the cross reactivity of monoclonal antibodies (Speth et al. 1984). Studies of smooth muscle contractile protein phosphorylation have shown this catalytic subunit to be associated with myosin light chain phosphatases (Pato and Kerc 1987). PP2A has also been shown to have functions that are important in cell growth processes (for review see Mumby and Walter 1993).

CHARACTERIZATION OF THE HOLOENZYME FORMS OF PHOSPHORYLASE PHOSPHATASE

We proposed that the ca. 35 kDa form of

phosphorylase phosphatase is the catalytic subunit of a larger holoenzyme, based on the observations that high molecular weight forms of the enzyme were converted to the 35 kDa species by treatments with either trypsin or denaturing levels of alcohol (Brandt et al. 1974). Initially, this hypothesis met with some resistance, as when we presented this at a meeting of the Biochemical Society in London (Lee et al. 1978). Attempts to isolate and define the holoenzyme forms of phosphorylase phosphatase frustrated a number of laboratories and only partially purified preparations were initially obtained (Cohen 1978, Mellgren et al. 1979, Brautigan et al. 1982). Part of the difficulty was the sensitivity of the holoenzyme to proteolysis; the other difficulty, not immediately realized at the time, was that there was more than one form of holoenzyme.

Our attempts to isolate an active holoenzyme form of phosphorylase phosphatase activity from rabbit skeletal muscle cytosol were unsuccessful. What we were able to isolate was an enzyme which had phosphorylase phosphatase activity but was in fact more like PP2A in its behavior. This enzyme had subunits of 70 and 35 kDa, and behaved rather like PP2A than PP1 (Paris et al. 1984). During the same period Mary Pato and Bob Adelstein at the NIH were working on the isolation of smooth muscle myosin light chain phosphatase. They made major contributions to the isolation of the type 2A holoenzymes, and they isolated the holoenzyme form of PP-2A as a myosin light chain phosphatase (Pato and Adelstein 1983, Pato and Kerc 1987).

The concept of a holoenzyme form of PP1 involving the catalytic subunit we had originally isolated was important as it provided the impetus for the isolation of the native enzyme forms. This concept was firmly established by the isolation of two holoenzyme forms containing the same catalytic subunit but different regulatory subunits. The first was the ATP/Mg dependent protein phosphatase, which had been studied since the 1960's by Wilfried Merlevede and his colleagues (Merlevede and Riley 1966). This was an inactive form of phosphorylase phosphatase which could be stimulated by the addition of ATP/Mg^{++}. Surprisingly, this enzyme turned out to be a 1:1 complex of PP1 with inhibitor-2. The ATP/Mg dependent PP1 is complex of PP1 and I-2. It is activated via phosphorylation of I-2 by a protein kinase, F_A, also known as glycogen synthase kinase-3 (GSK-3) (Yang et al. 1980, Ballou et al. 1983, Cohen 1989). The mechanism for its regulation is complex, involving transient phosphorylation of I-2, which results in the activation of PP1 (see Fig. 2). Thus, the term "inhibitor-2" is a misnomer as its primary function is that of a regulatory subunit, and it has more properly been referred to as the modulator protein. When inhibitor-2 is added to PP1, it has an ability to immediately inhibit its activity. However, its primary role within the complex is different, in that it serves to drive PP1 between active and inactive conformations in a process that requires ATP consumption. (Bollen and Stalmans 1992, Merlevede et al. 1984, Ballou et al. 1985).

The second holoenzyme form is one which is bound to glycogen. The presence of this enzyme was foreshadowed by earlier studies on the behavior of glycogenolytic enzymes in "glycogen particle," which established that phosphorylase, phosphorylase phosphatase and phosphorylase kinase were associated with glycogen (Meyer et

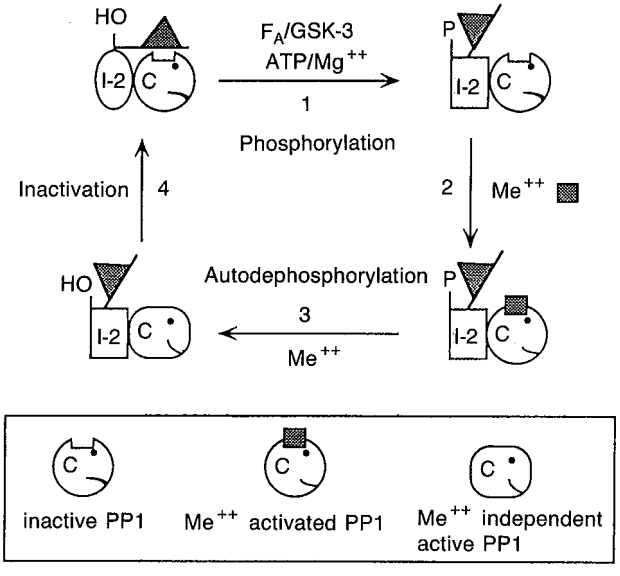

Fig. 2. Model for the activation-inactivation cycle of the ATP/Mg dependent protein phosphatase.
This diagram is based on current views of the regulation of ATP/Mg dependent PP1 (Bollen and Stalmans 1992). The ATP/Mg complex is normally inactive; PP1 in the complex is in an inactive conformation (Merlevede et al. 1984, Villa-Moruzzi et al. 1984). The activation cycle ("1") is initiated by phosphorylation of I-2 at Thr-72 by glycogen synthase kinase 3 (GSK-3), also known as F_A. This by itself does not lead to activation, which requires the presence of a divalent cation and autodephosphorylation ("3") (Li et al. 1985). Bollen and Stalmans (1992) have suggested that phosphorylation makes available a metal binding site, which allows that activation of the inactive PP1. In binding to metal, ("2"), PP1 is then capable of autodephosphorylation ("3") with consequent activation of PP1 to a non-metal dependent form. The activated state does not immediatley return to the inactive state on dephosphorylation of I-2, i.e., the system exhibits hysteresis (Bollen and Stalmans 1992).

al. 1970, Haschke et al. 1970). Studies from Willy Stalmans' laboratory (Laloux et al. 1978) also focused attention on the observations that glycogen synthase phosphatase and phosphorylase phosphatase activities were differentially distributed between the cytosol and the glycogen particles. The glycogen-bound phosphatase was isolated from muscle by Phil Cohen (Stralfors et al. 1985). This holoenzyme consists of a 1:1 complex of PP1 and a glycogen binding subunit, R_G (Stralfors et al. 1985). The complex is active, and PP1 is dissociated by phosphorylation of R_G by cAMP dependent protein kinase (Fig. 3). Binding to R_G and glycogen also results in effects on substrate specificity (Hubbard and Cohen 1991), providing an explanation for studies which showed an apparent dissociation between synthase and phosphorylase phosphatase activities. Phil Cohen proposed the important hypothesis that the catalytic subunit is associated with different targeting subunits, which serve to direct the enzyme to specific subcellular localities, in addition to serving regulatory functions (Cohen 1989). Two other forms of PP1 involving targeting subunits have been described, one being a myosin bound form, "ppase-1M" that is localized to muscle thick filaments (Chisholm and Cohen 1988) and one associated with the sarcoplasmic reticulum (Hubbard et al. 1990). R_G has been cloned and expressed, and contains a hydrophobic region that may function as a transmembrane domain (Tang et al. 1991). The concept that binding to glycogen is a required facet for in vivo function

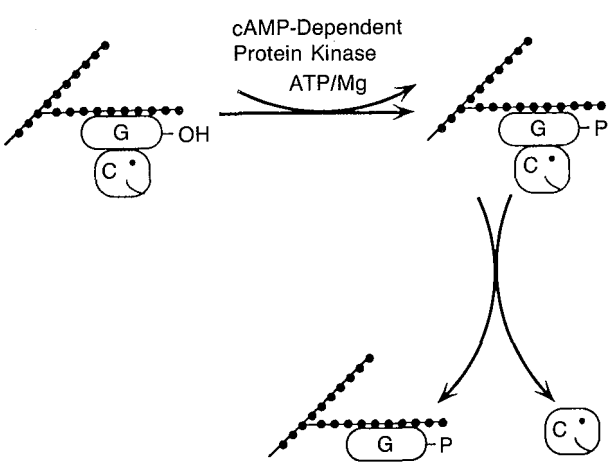

Fig. 3. Model of the targeting and regulation of PP1 by R_G. In this model, the glycogen binding subunit (R_G) serves to bind PP1 to glycogen. Control of the enzyme is mediated by cAMP-dependent protein kinase which results in dissociation of the PP1 from the glycogen.

of PP1 has been established by findings that a PP1 mutant which lost its ability to bind to R_G in yeast also fails to accumulate glycogen, presumably due to an ability to activate its glycogen synthase by dephosphorylation (Stuart et al. 1994).

The existence of a nuclear form of PP1 is likely, based on the identification of a new heat-stable inhibitory protein(s) in calf thymus nuclei (Buellens et al. 1992). Two proteins, NIPP1a and NIPP1b, of molecular weights of 18 and 16 kDa, were isolated. These are extremely potent inhibitors of PP1 (K_i estimated as 1 pM). Their potential to act in a regulatory fashion is suggested by evidence that phosphorylation by cAMP dependent protein kinase reduces the inhibition (Buellens et al. 1993). The PP1/NIPP complex may thus represent another holoenzyme form with specific functions in nuclear events, including transcriptional regulation.

Thus, PP1 is an example of an enzyme whose role in various cellular functions may be dictated by its noncatalytic subunits, which can serve both the functions of targeting of the enzyme to its functional location, as well as providing the basis for selective individual responses to different signal transduction pathways. The requirement for targeting subunits for PP1 makes it an additional example of the paradigm of molecular localization by protein-protein interactions as a mechanism for enzyme regulation. The precedent for this perspective is to be found in the growing number of proteins, including members of the tyrosine phosphatase family, which possess *src* homology (SH2) domains (Mayer and Baltimore 1992). The SH2 domains confer the ability for binding to phosphorylated tyrosine kinase sites, and the system thus allows regulated protein-protein interactions. This context is relevant to the protein phosphatases since there is now evidence that both PP1 and 2A are to be found in complexes with proteins other than their known regulatory subunits. An important recent finding is that PP1 interacts with Rb, the retinoblastoma tumor suppressor protein (Durfee et al. 1993). This finding provides further evidence that implicates PP1 in cell cycle and growth regulation, since regulation of Rb may involve phosphorylation by cyclin-dependent kinase-cyclin complexes (Cobrinik et al. 1992). The association of PP1 with Rb is cell cycle dependent, and both PP1 and PP2A have been implicated in Rb dephosphorylation (Alberts et al. 1993). Interestingly, two occurrences of the sequence motif LXSXE were identified in PP1; this resembles the motif LXCXE which is known to be

important in binding to Rb, and is present in viral Rb associated proteins and in two other Rb binding proteins (Durfee et al. 1993). Rb (Cobrinik et al. 1992) and PP2A (Pallas et al. 1990) both bind to T antigen; the PP2A catalytic subunit was in fact identified as a factor involved in SV40 replication and acts through T-antigen (Virshup et al. 1992).

The effect of molecular targeting is to provide selective signal transduction responses, avoiding the contradiction that a single phosphatase catalytic subunit is involved. For example, activation of GSK-3 should result in selective activation of the cytosolic PP1-I-2 complex, while at the same time phosphorylation of glycogen synthase by GSK-3 would be expected to reduce synthase activity. Since glycogen synthase is activated by PP1, this might be considered a contradiction, but is explained by virtue of the fact that the PP1 involved in synthase regulation is the R_G-PP1 glycogen bound enzyme. Seen in the context of the relatively large number of protein kinases involved in signal transduction, this system of a single catalytic subunit which combines with a number of different regulatory/targeting subunits provides an elegant mechanism for extending the cellular repertoire of PP1.

MOLECULAR CLONING OF PP1

We reported the cloning of a cDNA for rabbit muscle PP1 in 1988 (Bai et al. 1988). This cDNA encoded a protein of 330 amino acids and differed at the 5′ end from that of a cDNA ["PP1β"] described a year earlier by Berndt et al. (1987), such there were differences in the N-terminal sequences of the two encoded proteins. Patricia Cohen (1988) isolated the same cDNA as we had, and proposed that there was a single gene for PP1 and that the two cDNAs were related by alternative splicing at the 5′ end of the cDNA. We explored this question, by use of oligonucleotide probes to the variant 5′ end of the sequence reported for "PP1β". We could find no evidence for its mRNA by Northern blotting; Southern genomic blots were also ambiguous regarding the question of the existence of a single gene. We concluded that the single isolate sequenced by the Cohens' laboratories (Berndt et al. 1987) might be a cloning artifact (G. Bai, Ph.D. Thesis, University of Miami, 1990). A second "PP1β" rabbit cDNA was subsequently isolated (Dombradi et al. 1990). The new PP1β sequence in fact corresponds to the PP1δ isoform as defined by Sasaki et al. (1990). Subsequent work (see below) shows that the cDNA we isolated is identical in protein sequence to the rat PP1α isoform isolated by Sasaki et al. (1990).

The use of molecular genetics has had a major impact on our knowledge of PP1 during the past few years. Since the initial cloning of the cDNAs for rabbit muscle PP1 (Bai et al. 1988, Cohen 1988) and PP2A (Green et al. 1987), a large number of ser/thr protein phosphatase genes and cDNAs have been cloned from a wide range of phyla (for review, see Cohen et al. 1990). The existence of at least four isoforms of mammalian PP1 was established by Sasaki et al. (1990) who isolated four cDNAs, PP1α, 1γ1, 1γ2, and 1δ. Multiple PP1 genes have also been isolated from Drosophila and plants (Dombradi et al 1993, Smith and Walker 1993). In addition to these closely related PP1 isoforms, which exhibit $>$ 80% identity, Patricia Cohen's laboratory has described a number of other phosphatase cDNAs which show $<$ 70% identity to either PP1 or PP2A. PCR amplification experiments have identified additional ser/thr PP1 genes, bringing the total to 11, 15, and 12 in *S. cerevisiae*, *D. melanogaster* and *H. sapiens*, respectively (Chen et al. 1992). It is now evident that PP1 and PP2A are members of a large protein family. Both PP1 and PP2A are remarkably conserved throughout evolution, and are among the most conserved proteins known (MacKintosh et al. 1990, Chen et al. 1992).

It is now recognized that PP1 may play important functions in mediating the dephosphorylation of phosphoproteins that are crucial to a number of cellular functions, including the cell cycle (Walker et al. 1992). Genes from yeast and fungi mutants blocked in mitosis were found to encode highly conserved PP1 sequences. A gene from an *A. nidulans* mutant which was blocked in mitosis was isolated (BimG11). We were contacted by John Doonan prior to the appearance of our published work on the PP1 cDNA and we found that its encoded protein sequence had 86% similarity to muscle PP1 (Doonan et al. 1989). It was subsequently shown that expression of the rabbit muscle PP1 in the aspergillus mutant allowed its rescue (Doonan et al. 1991). In the yeast *Saccharomyces pombe*, studies of two mutant cell cycle genes, sds21 and dis2, which are blocked in mitosis at restrictive temperatures, showed that they both encoded putative PP1 sequences with 82 and 74% identity with rabbit PP1 (Ohkura et al. 1989). It was suggested that they play a part in controlling the process of chromosomal condensation or in the control of the mitotic spindle

during cell division. In addition to the significant genetic evidence for a role of PP1 in the cell cycle discussed above, there have been an increasing number of studies that have implicated PP1 and/or PP2A in other major cellular processes (Bollen and Stalmans 1992, Mumby and Walker 1993). Considering the number of complex regulatory pathways ranging from metabolism to the cell cycle that involve protein phosphorylation-dephosphorylation cycles, this is perhaps not surprising.

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT MUSCLE PP1α

We made the development of a useful expression system for rabbit muscle PP1 a major priority of our research efforts. We were successful in expressing PP1 in the pET3a vector. PP1 was overexpressed in this vector as an inactive, insoluble protein in inclusion bodies (Zhang et al. 1992b). We optimized conditions for its renaturation and found that these included the requirements for Mn^{++}, dithiothreitol, high salt, and high dilution. However, only 5% of the expected specific activity was observed following renaturation, even after purification of the protein in the presence of urea. During the progress of our work, Berndt and Cohen (1990) had reported the expression rabbit muscle PP1 in the baculovirus expression system in which it was expressed as inclusion bodies. They noted that they had failed to obtain any expresion in *E. coli* systems — suggesting that either the enzyme was toxic or that it required the presence of its subunits for the stable formation of the folded protein. The baculovirus enzyme could be renatured to the full expected specific activity and in yields of 10 mg protein/liter of cell culture, but also required very high dilutions during the renaturation process. We estimated on the basis of their data and purification protocols that the material from 1 liter of cells required dilution to 1,000 liters.

The expression of PP1 in the pET3a vector as insoluble material, with the attendant problems of renaturation and isolation made it an unwieldy system, so that we explored the use of other vector systems. We turned to the pTACTAC vector which had been shown to allow the expression of rabbit muscle phosphorylase (Browner et al. 1991) in a soluble form, as opposed to its expression in the pET vector as inclusion bodies. The key points of this work were the use of induction at a lowered temperature of 26-28°C, rather than 37°C, and the use of a weaker promoter, stratagems based on findings that expression of foreign proteins at lowered temperature and induction rates sometimes increased the fraction expressed in the soluble state. The use of this vector led to the successful expression of PP1α in a soluble state. The use of this vector led to the successful expression of PP1α in a soluble form in *E. coli* (Zhang et al. 1992b). The expression of PP1 in *E. coli* in the pTACTAC vector is shown in Fig. 4. Yields of 20 mg per 2 liters of culture were routinely obtained after a simple purification procedure. We have recently developed faster methods of isolation of recombinant PP1 by a single chromatography step on inhibitor-2-Sepharose (Zhang et al. 1994c).

Four cDNAs for rat PP1 isoforms have been isolated, as noted above. The primary structures of the four PP1 isoforms are highly conserved, and they exhibit 87-100% identity with rabbit PP1α. They differ mainly in the N- and C-terminal regions (Fig. 5). In a collaborative effort with Dr. Minako Nagao we expressed these isoforms in *E. coli* (Zhang et al. 1993a). We found that the isoforms were expressed at similar levels to those found for the rabbit muscle PP1α cDNA, with the ex-

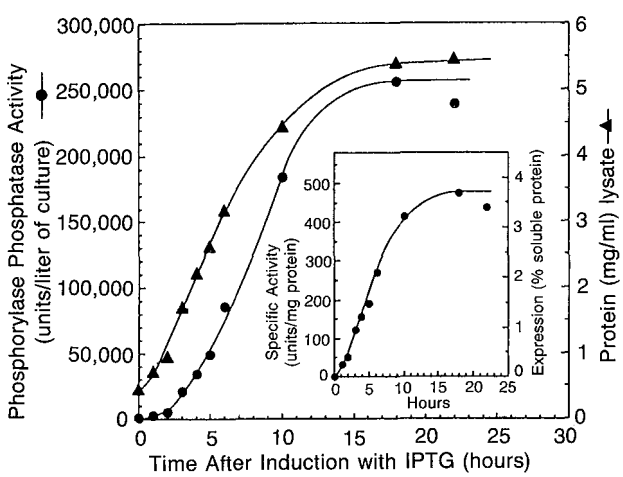

Fig. 4. Expression of PP1α in *E. coli*.
The diagram shows the time course of induction of active phosphorylase phosphatase activity in a culture of *E. coli* harboring the pTACTAC plasmid into which the PP1 coding sequence had been inserted. Samples (50 ml) from a 2 liter culture were taken at various time intervals after addition of IPTG. The cells were harvested by centrifugation, and lysates prepared and assayed for phosphorylase phosphatase activity (●) and protein content (▲). The insert shows the specific activity of the phosphatase activity, and the estimated level of expression based on a specific activity for the pure protein of 13,000 units/mg. Assays for phosphorylase phosphatase were performed in the presence of $MnCl_2$. Data are taken from Zhang et al. (1992b).

ception of PP1δ which was more poorly expressed. These studies revealed that the basic enzymatic properties of all four isoforms are highly similar, i.e., the structural differences do not reflect gross functional differences in these proteins in terms of specificity or sensitivity to okadaic acid or inhibitor-2. Thus, it appears that the *raison 'être* of the isoforms is not related to obvious differences in substrate specificities or their interactions with I-2.

THE Mn^{++} DEPENDENCE OF RECOMBINANT PP1

The functional behavior of recombinant PP1 was similar to that of the PP1 isolated from rabbit muscle, with one notable exception. We discovered early on in our work with the pTACTAC vector that recombinant PP1 is a Mn^{++}-dependent enzyme (Fig. 6). Moreover, even prolonged storage in the presence of Mn^{++} did not lead to permanent activation of the enzyme (as determined by assay in the presence of EDTA). Thus, more precisely, the effect is one of stimulation, not activation. Our findings were unexpected and significant since almost all previously isolated PP1 catalytic subunits were independent of Mn^{++} when assayed on phosphorylase *a* (Bollen and Stalmans 1992, Ballou et al. 1985). Furthermore, all of the recombinant forms of PP1 that we have produced are Mn^{++} dependent. This includes the isoforms of PP1, as well as all of the mutants of PP1 that we subsequently generated.

The fact that recombinant PP1 is Mn^{++} dependent suggested that it represents a known form of PP1, this being the inactive conformer of PP1 present in the ATP/Mg dependent holoenzyme consisting of 1:1 complex of PP1 and I-2 (see Fig. 2). It is now well recognized that interaction of active PP1 with I-2 results in a complex in which the catalytic subunit is converted into an inactive or latent form that is dependent on Mn^{++} for activity. It is implicit in this mechanism that there must be two conformers of PP1, one which is inactive, and one which is active. The existence of these two conformers was demonstrated by their isolation from the complex by FPLC (Villa Moruzzi et al. 1984). Thus, recombinant PP1 resembles the inactive conformer of PP1 present in the ATP/Mg

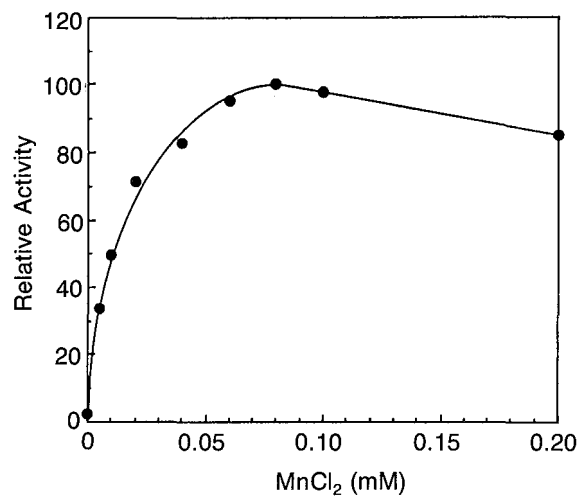

Fig. 6. Dependence of recombinant protein phosphatase-1 activity of the presence of Mn^{++}.
Purified recombinant PP1α was diluted in buffers in the absence of Mn^{++}, and assayed for phosphorylase phosphatase activity in the presence of increasing amounts of added $MnCl_2$. Data are taken from Zhang et al. (1992b).

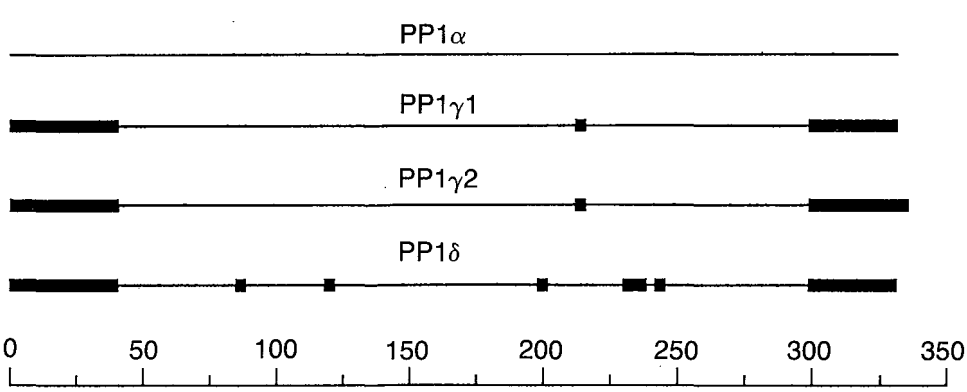

Fig. 5. Diagrammatic representation of the differences of the PP1 isoforms.
The diagram shows the protein sequence of PP1α as a solid line. Single differences in the primary sequence of the other three isoforms with PP1α are shown as solid squares. Solid bars at the N and C-termini are regions with multiple differences. The diagram is based on the data of Sasaki et al. (1992).

dependent complex in its metal ion dependency. We have proposed that recombinant PP1 represents the conformation of the enzyme that is present in the PP1·I-2 complex (Zhang et al. 1993b). This view that PP1 represents a natural conformation of PP1 is supported by the discovery of a latent Mn^{++}-dependent PP1 catalytic subunit which has recently been purified to homogeneity from cardiac myofibrils (Chu et al. 1994).

We have pointed out that the apparently invariant expression of PP1 and its mutant forms as the Mn^{++} dependent enzyme suggests that this is the preferred folding state of the protein, at least within the milieu of the *E. coli* cytoplasm (Zhang et al. 1993b). This poses a conundrum since the isolated mammalian PP1 catalytic subunit and holoenzyme forms other than in the PP1/I-2 complex are fully active enzymes. The only known method for the interconversion of these two forms is that which takes place in the ATP-Mg dependent holoenzyme. However, if the consistency with which all forms of PP1 which we have expressed in *E. coli* are folded in a Mn^{++} dependent form, is also true in the milieu of the mammalian cytosol, then indeed some mechanisms must be present which result in the conversion of this stable conformer to that the active conformer present in PP1 holoenzymes. Should this require a mandatory transit (and activation by glycogen synthase kinase-3) through the PP1/inhibitor-2 complex, it would represent a novel example of protein trafficking (Fig. 7). Because inhibitor-2 would then function to modulate the conformation of PP1, its function would then more closely fit that of the "molecular matchmaker" class of proteins that have been defined by Sancar and Hearst (1993).

Phil Cohen has proposed that I-2 is a chaperone protein (Alessi et al. 1993) in studies in which they have reproduced the expression system that we developed and also observed recombinant PP1 is Mn^{++} dependent. They also considered that recombinant PP1 was had differences in properties from the muscle enzyme, specifically in that the recombinant enzymes were insensitive to I-1 and exhibited a markedly different substrate specificity in possessing a much higher activity toward histone. However, we have reexamined the activities of native and recombinant PP1 using histone as a substrate (Zhao et al. 1994), and have found that its relative activities to histone and phosphorylase *a* are very similar to that for muscle PP1 (Silberman et al. 1984).

The hypothesis (Alessi et al. 1993) that I-2 functions as a chaperone appears unlikely to be correct. While the functions of chaperone proteins have been extensively studied in relation to prokaryotes and mitochondrial proteins (Kelly and Georgopoulos 1992), their role in regard to the cytosol of mammalian cells is less defined (Nilsson 1991). Moreover, they are considered to bind proteins in a molten globule state or intermediate folding state (Kelly and Georgopoulos 1992). No folding studies have been done which demonstrate such an intermediate for PP1. Recombinant PP1 does not qualify as a folding intermediate, based on its full functional ability and its resistance to trypsin.

Another question raised by the behavior of the recombinant forms of PP1 is an old question, that of whether it is a metalloenzyme. There have been a number of observations over the years on the effects of Mn^{++} and also Co^{++} on PP1 activity (reviewed by Bollen and Stalmans 1992). These effects are not fully understood, since most of the muscle PP1 catalytic subunit preparations studied were generally independent of metal ions for their activity on phosphorylase *a*. A further complication is that muscle PP1 activity toward substrates other than phosphorylase *a* can be stimulated by Mn^{++}, as is the case with histone (Zhao et al. 1994) and phosphorylated inhibitor-1 (Foulkes et al. 1983). Studies of the

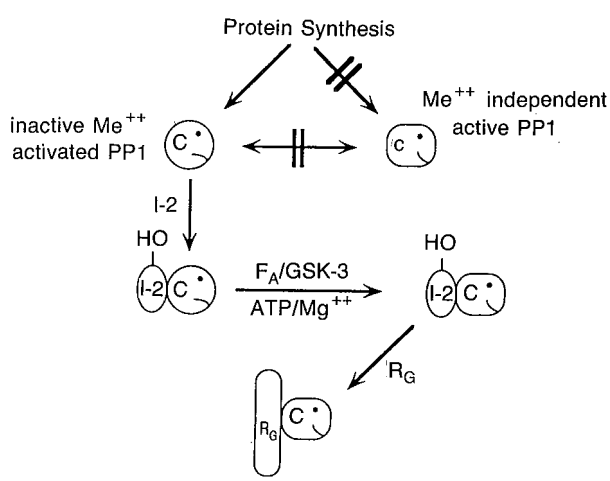

Fig. 7. Hypothetical consequences of the expression of PP1 as the inactive conformer.
The potential consequences of the expression of PP1 as the inactive conformer if this were to take place in mammalian cells are illustrated in this diagram. First, the active form is not formed; second, the spontaneous interconversion of the two isoforms has not been observed. In this case, it can be postulated that the I-2 functions to activate PP1 and that this may be a mode of subunit interchange that is necessary for supplying active PP1 subunits to the other holoenzyme forms.

inactivation of PP1 by ATP, PPi and fluoride have revealed the phenomenon where it is stably reactivated (rather than stimulated) by Mn^{++} or Co^{++} (Hsiao et al. 1978, Yan and Graves 1982). This suggested the stable incorporation of Mn^{++}. However, attempts to demonstrate binding of radioactive Mn^{++} have failed (Brautigan et al. 1982) and stoichiometric levels of metal ions could not be found in PP1 preparations (Yan and Graves 1982). The latter authors proposed the interesting possibility that Mn^{++} effects a conformational change which might facilitate a covalent modification (which must be reversible), the most likely being the formation or reduction of an internal disulfide bond, in order to account for a stable activation to a Mn^{++} independent form. This possibility is intriguing because of the marked stabilities exhibited by both forms of PP1.

It is still possible, and likely that PP1 activity may require an interaction with metal ion, since many phosphoryl transfer reactions involve coordination of the phosphoryl moiety with divalent metal ions (Vincent et al. 1992). A novel view of the potential role of histidine residues in catalysis by protein phosphatases has been proposed by Vincent and Averill (1990a) from the viewpoint of their role in metal binding. They had previously studied the purple acid protein phosphatases which are metalloproteins possessing a spin-coupled binuclear iron unit at the active site (Vincent and Averill 1990b). They aligned the sequences of purple acid phosphatases with the protein phosphatases (1, 2A, 2B, 2C) and have found a weak region of homology, but nevertheless one in several residues which could be involved in metal ion binding are conserved. Critical evaluation of the hypothesis must take into account the fact that the purple acid protein phosphatases contain stoichiometric amounts of iron, and are unquestionably metalloproteins; on the other hand, there has been little evidence that muscle PP1 is a metalloprotein.

STRUCTURE-FUNCTION STUDIES OF PP1 BY MUTAGENESIS

Properties of Cys to Ser mutants

A central goal of our work has been to utilize molecular cloning methods for studies of the properties of the protein phosphatases. With the acquisition of a good expression system we initiated studies toward probing structure function relationships of PP1α by site-directed mutagenesis. Little work has been done on the chemical modification of PP1, with the exception of its modification by sulfhydryl reagents. It has been known for many years that PP1 is sensitive to sulfhydryl reagents and to inactivation by disulfides, e.g., oxidized glutathione (Bollen and Stalmans 1992, Usami et al. 1980). We had previously examined the reactivity of the cysteine residues of PP1 and PP2A catalytic subunits, and had shown that the kinetics of inactivation were consistent with the presence of a single reactive sulfhydryl residue (Nemani and Lee 1993). This suggested, but did not prove, the possibility that cysteine might be involved in the active site via a mechanism involving the formation of a cysteinyl phosphate intermediate, such as has been shown to be the case for the 35 kDa soluble tyrosine phosphatase (Guan and Dixon 1991). Since both PP1 and PP2A were sensitive to sulfhydryl reagents, and had 46% identity in amino acid sequence, it was reasoned that if they shared a common catalytic mechanism involving a cysteine residue it would be one of the six cysteines that are conserved. These conserved cysteines were mutagenized to serine residues. The six mutants were purified to homogeneity by affinity chromatography on I-2-Sepharose (Zhang et al. 1993c). Analyses of the expression levels of the mutants showed that these were all similar. However, all six mutants displayed enzymatic activity, and maintained an ability to interact with I-2, as gauged from their successful isolation by

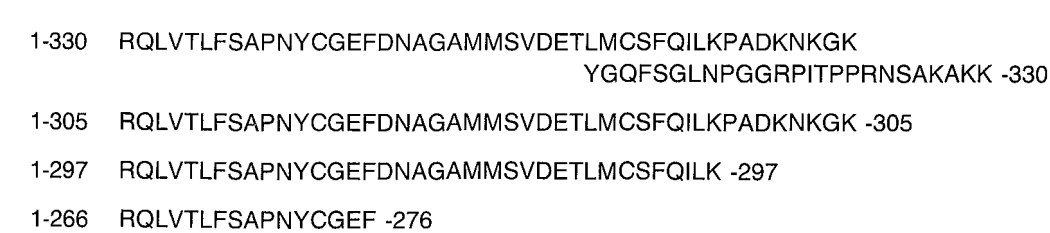

Fig. 8. Deletion mutants of PP1.
This figure shows the C-terminal sequence of PP1α and for the deletion mutants containing only the first 305, 297, and 276 residues.

I-2-Sepharose chromatography as well as by inhibition assays (Zhang et al. 1993c). These findings showed that the presence of a cysteine residue essential for catalytic function in PP1 is unlikely, since none of the mutants was totally inactive.

Delection mutagenesis of PP1α

We have completed a survey of the core region of PP1α that is required for catalytic activity and I-2 binding by the construction of deletion mutants (Zhang et al. 1994a). Deletion of the first 20 N-terminal amino acids led to loss of expression of PP1. Because of the strong resistance of the N-terminus to proteolysis, we suspect that the N-terminus is tightly integrated into the core structure of PP1. When the C-terminus was truncated (Fig. 8), two deletion mutants PP1[1-305] and PP1[1-297] were found to be fully active. These two mutants were expressed at levels similar to that of the full length protein. The inhibition of these two deletion mutants by okadaic acid, microcystin and I-2 were found to be similar to that of the wild type enzyme, although there was some slight reduction in affinity. Further truncation of the C-terminus beyond residue 297 led to loss of expression and activity. Thus it appears that the limit for truncation is in the 20 amino acid residues from 277-297. These studies indicate that the C-terminus is not significantly or overtly involved in either catalytic function, I-2 interaction, or in toxin binding, and are consistent with our studies on the effects of trypsin on PP1 (Zhang et al. 1993b). The deletion of 33 of the C-terminal residues without loss of function, and the strong resistance of the core to further proteolysis suggests these residues are not integrated into the core. This view is also consistent with the fact that this C-terminal region (Fig. 8) contains 10 charged residues (9 basic) out of 34 and is consonant with the findings that this region is the one with the greatest variations found in PP1 sequences from different phyla.

Toxin inhibitors

Besides the protein inhibitors of PP1, several structurally unrelated toxins produced by marine and freshwater organisms have been identified as inhibitors of PP1 and PP2A. Okadaic acid, a fatty acid polyketide, which is produced by a marine dinoflagellate, accumulates in certain sponges and shellfish and causes seafood poisoning. The discovery that it is a potent inhibitor of PP1 and PP2A (Bialojan and Takai 1988) has made it a widely used tool for the perturbation of phosphorylation systems in intact cells. It inhibits PP2A (IC_{50} = 1 nM) much more strongly than PP1 (IC_{50} = 200 nM). The microcystins (Carmichael 1992) are a family of cyclic heptapeptide toxins produced by fresh water cyanobacteria (blue green algae) which are potent inhibitors of both PP1 and PP2A at nanomolar concentrations (MacKintosh et al. 1990). These are of considerable importance for freshwater ecology and can be a significant problem for livestock. Both okadaic acid and microcystins are tumor promoters, underscoring the potential role of PP1 and/or PP2A in the regulation of cell growth; if these phosphatases play a role in maintaining controlled cell growth, then the tumor promoting actions of these toxins can be rationalized through the inhibition of phosphatase activity. A striking feature of all the inhibitors of PP1/PP2A

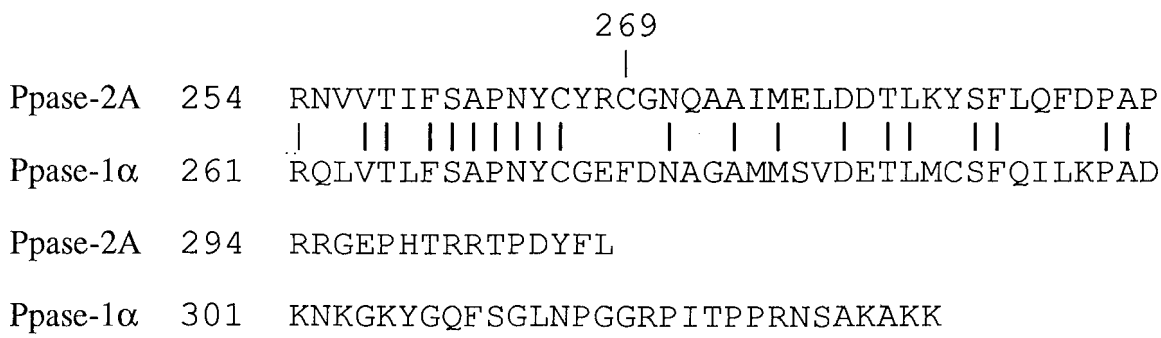

Fig. 9. Alignment of the C-terminal regions of PP1 and PP2A.
The diagram shows the location of residue Cys269 of PP2A which is mutated to gly in a muant CHO cell line (Sasaki et al. 1990). In the alignment with PP1 it is seen that this is in a region of four residues where four residues (GEFD) of PP1 differ from those of PP2A (YRCG). A chimeric mutant of PP1 was constructed in the residues GEFD of PP1 were replaced by YRCG (Zhang et al. 1994c).

is their extreme potencies, which approach those involved in receptor binding to the extent that PP1 and PP2A can be regarded as the cellular receptors for these toxins.

In collaboration with Dr. M. Nagao, National Cancer Institute, Tokyo, we have performed mutagenesis of a site which may be involved in toxin binding of PP1 and PP2A (Zhang et al. 1994b). This arose out of observations (Shima et al. 1994) that CHO cell lines with an increased resistance to okadaic acid contianed a mutation in the PP2A gene, such that cys-269 was mutated to gly (Fig. 9). We have constructed the chimeric PP1 mutant in which the sequence YRCG was substituted for the sequence GEFD in PP1. This mutant was expressed and purified to near homogeneity. The resultant mutant exhibited a 10 fold increase in okadaic sensitivity, consistent with the fact that PP2A is more sensitivity to okadaic acid (Zhang et al. 1994b). The success of this experiment provides a foundation for the further exploration of the determinants necessary for okadaic acid binding by site-directed mutagenesis.

Acknowledgements: I thank Dr. Jen-Leih Wu, Dr. Ren-Jye Ho, and the Institute of Zoology, Academia Sinica, Taipei, for the opportunity to participate in the "Symposium on Cellular Signal Transduction Systems: Recent Advances and Examples," May 1994, and to which this paper owes its inspiration. I thank the students and post-doctoral fellows who have worked in my laboratory over the years, and whose talent and dedication have been responsible for the work discussed in this review. In particular, I am grateful to Drs. G. Bai and Z. Zhang, who undertook as students to bring the tools of molecular biology into a largely enzymological laboratory, and who succeeded beyond all expectations. I also acknowledge the contributions of my current associates, Dr. Sumin Zhao, Ms. Lifang Zhang, Ms. Jun Zhang, Mr. Fengxiang Long and Mr. Stephen Deans-Zirattu. The work described here has been continuously supported by NIH grant DK 18512 since 1975.

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磷酸解酶磷酸酶(蛋白質磷酸酶-1)之酶學-個人之回顧

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本文回顧過去二十年來我們實驗室在肝醣磷酸解酶磷酸酶(glycogen phosphorylase phosphatase)(蛋白質磷酸酶-1)之研究結果。此酶之酶學非常複雜，有關其特性之研究，著實花了一番功夫。在此實驗過程中，最主要的發現爲純化其催化亞單元(catalytic subunit)，及發現其抑制蛋白質(inhibitory proteins)，其中抑制蛋白質-2與催化亞單元可形成一複合體。後來又陸續發現許多不同型式之全酶存在，包括不同之催化單元與不同之調控單元(regulatory subunits)之結合。近年來，利用基因選殖技術證明催化單元是一保留性很高之蛋白質，並且在細胞有絲分裂時扮演著重要角色。利用大腸桿菌基因表現系統，已開啟研究此酶之構造-功能關係之門，此酶與其抑制性毒素之結合部位亦已經基因突變法確定出來。

關鍵詞：蛋白質磷酸酶，代謝作用之調控，基因突變法。

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