Mutational Analysis of the Catalytic Subunit of Muscle Protein Phosphatase-1†

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Received December 14, 1995; Revised Manuscript Received March 14, 1996⊗

ABSTRACT: A mutational analysis of rabbit skeletal muscle protein phosphatase-1 was performed by site-directed mutagenesis of the recombinant protein expressed in *Escherichia coli*. The selection of the sites to be mutated was based on sequence alignments which showed the existence of a number of invariant residues when eukaroytic Ser/Thr protein phosphatases were compared with bacteriophage phosphatases and adenosinetetraphosphatase [Barton et al. (1995) *Eur. J. Biochem. 220*, 225−237]. In other studies, it had been shown that PP1 is a metalloprotein [Chu et al. (1996) *J. Biol. Chem. 271*, 2574−2577], and in this study, we have largely focused on invariant histidine and aspartate residues which may be involved in metal binding. The residues which were mutated were H66, H125, H173, H248, D64, D71, D92, D95, N124, and R96E. The results showed that mutation of H66, H248, D64, and D92 resulted in severe loss of catalytic function. Mutation of D95, N124, and R96 also led to loss of function, while attempts to mutate H125 and H173 led to production of insoluble, inactive proteins. The results of the mutational analysis are consistent with the involvement of conserved His and Asp residues in metal binding, and are discussed in the context of the recently described crystal structure of PP1 [Goldberg et al. (1995) *Nature, 376*, 745−753], which reveals that PP1 possesses a bimetallic center at the active site. The behavior of the D95, R96, and N124 mutants supports a catalytic mechanism involving nucleophilic attack by a hydroxide ion with H125 functioning as a proton donor to the leaving alcohol group.

The Ser/Thr protein phosphatases have been classified into four major classes—PP1, PP2A, PP2B, and PP2C,[1] according to their substrate specificity, metal-ion dependence, and sensitivity to phosphatase inhibitors [for reviews, see DePaoli-Roach et al. (1994), Bollen and Stalmans (1992), and Shenolikar and Nairn (1991)]. However, PP1, PP2A, and PP2B are members of the same protein family, whereas PP2C is structurally unrelated. The activities of PP1 and PP2A are independent of metal ions, whereas PP2B is Ca^{2+} and calmodulin dependent and PP2C is Mg^{2+}-stimulated. The catalytic subunit of PP1 is a 37 kDa protein (Silberman et al., 1984; Lee, 1995), and four mammalian isoforms have been identified (Sasaki et al., 1990). The rabbit muscle PP1α isoform is composed of 330 amino acid residues (Bai et al., 1988). Multiple sequence alignment and structure prediction analysis of 44 eukaryotic Ser/Thr phosphatases with 2 related bacteriophage phosphatases and *E. coli* adenosinetetraphosphatase have led to the identification of a conserved core which is located in the N-terminus of PP1 (residues 62−126) (Barton et al., 1994). Within this core, 14 invariant residues were found. Mutagenesis of residues in the bacteriophage λ phosphatase which are conserved in the mammalian phosphatases has demonstrated their importance in catalysis and metal binding (Zhuo et al., 1994). Deletion mutagenesis of PP1 has shown that the catalytic activity is little affected by deletion of the C-terminal 33 residues (Zhang et al., 1994a). Mutagenesis of the six cysteines conserved between PP1 and PP2A has shown that these are not essential for catalysis (Zhang et al., 1993a), clearly distinguishing the Ser/Thr protein phosphatases from the tyrosine protein phosphatases for which a reaction mechanism involving a cysteinyl phosphate intermediate was established (Guan & Dixon, 1991).

The issue of whether PP1 is a metalloprotein had been an unresolved issue for several decades as some forms of the isolated enzyme displayed a dependence on Mn^{2+} [reviewed in Bollen and Stalmans (1992)]. Previous experiments to determine whether the muscle enzymes contained bound metals showed the presence of substoichiometric levels of Fe and Zn in PP1 and PP2A (Yan & Graves, 1992). However, recombinant PP1, unlike the native protein, is dependent on Mn^{2+} for activity (Zhang et al., 1992, 1993a), and recently it has been shown that, unlike Mn^{2+} (Brautigan et al., 1982), $^{57}Co^{2+}$ can be stably incorporated into recombinant PP1 in a 1:1 stoichiometry (Chu et al., 1996). Another reason for considering that PP1 might be a metalloprotein arises by analogy with PP2B, which has stoichiometrically bound Fe^{3+} and Zn^{2+} (King & Huang, 1984). Vincent and Averill (1990a) had earlier suggested that the PP1/PP2A/PP2B family of Ser/Thr phosphatases are metalloproteins, based on a weak homology in their sequence alignment with the purple acid protein phosphatase (PAP). Mammalian PAP possesses an antiferromagnetically coupled Fe^{3+}/Fe^{2+} iron center, which in the plant PAP is an Fe^{3+}/Zn^{2+} pair (Vincent et al., 1990b). In this study, we have mutated some of the residues in PP1 found to be invariant by Barton et al. (1994) over a wide range of Ser/Thr phosphatases. In particular, we mutated histidine and aspartate residues because these are likely candidates as metal ion ligands. The results show that several of these invariant His and Asp residues are

† This work was supported by NIH Grant DK18512.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: PP1, PP2A, PP2B, and PP2C, protein phosphatase-1, -2A, -2B, and -2C, respectively; PAP, purple acid phosphatase; PCR, polymerase chain reaction; pNPP, *p*-nitrophenyl phosphate.

S0006-2960(95)02954-0 CCC: $12.00

important for catalytic activity, and the findings are discussed in the context of the recently reported crystal structures of PP1 (Goldberg et al., 1995) and PP2B (Griffith et al., 1995) which show that both proteins contain a bimetal center at the active site.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from New England BioLabs. Inhibitor-2 was the recombinant protein isolated as described by Zhang et al. (1994b). Inhibitor-2−Sepharose was prepared as described by Zhang et al. (1994b). A rabbit polyclonal antibody to the C-terminal 14 residues of PP1α was a generous gift of Dr. K. K. Schlender, Medical College of Ohio.

Site-Directed Mutagenesis. The rabbit muscle PP1α cDNA (1.3 kb; Bai et al., 1988) was cloned into M13mp19. Site-directed mutagenesis was performed with the Sculpter in vitro mutagenesis system (Amersham Life Science). Primers used for each specific mutation were as follows: H66N, 5′-TGCGGTGACATC**AAC**GGCCAGTACTAC; H66K, TGCGGTGACATC**AAA**GGCCAGTACTAC; H125N, CTGCGCGGGAAC**AAC**GAGTGCGCCAGC; H125K, CTGCGCGGGAAC**AAA**GAGTGCGCCAGC; H173N, ATATTCTGCTGC**AAC**GGCGGCCTCTCC; H173K, ATATTCTGCTGC**AAA**GGCGGCCTCTCC; H248N, ATCTGCCGGGCG**AAC**CAGGTGGTGGAG; H248K, ATCTGCCGGGCG**AAA**CAGGTGGTGGAG.

Mutants of H125 and H173 were generated by use of the following degenerate primers. Mutation to Phe, Cys, Tyr, and Ser, respectively was performed using CTGCGCGGGAAC**TNC**GAGTGCGCCAGC for H173 and ATATTCTGCTGC**TNC**GGCGGCCTCTCC for H173. Mutation to Gly, Asp, Val, and Ala, respectively, was performed using CTGCGCGGGAAC**GNT**GAGTGCGCCAGC for H125 and ATATTCTGCTGC**GNT**GGCGGCCTCTCC for H173.

Primers for the generation of other mutants were as follows: D64N, AAGATCTGCGGT**AAC**ATCCACGGCCAG; D92N, CTGTTCCTGGGT**AAC**TACGTGGACCCGC; D95N, GGTGACTACGTG**AAC**CGCGGCAAGCAG; N71N, GGCCAGTACTAC**AAC**CTGCTGCGGCTG; R96E, GACTACGTGGAC**GAG**GGCAAGCAGTCC; N124D/A, CTGCTGCGCGGG**GA/CC**CACGAGTGCGCC.

The underlined bases indicate the changes in sequence from the wild-type PP1. The sequences of the mutated cDNAs were confirmed by dideoxynucleotide termination DNA sequencing and cloned into the expression vector pTACTAC and expressed as described by Zhang et al. (1992).

Expression and Isolation of the Mutants. The mutant cDNAs in the pTACTAC vector were used to transform *E. coli* DH5α cells. One liter cultures were grown as described by Zhang et al. (1992). The cell lysates were centrifuged at 10000*g*, and the extracts were purified by affinity chromatography on an inhibitor-2−Sepharose column (15 cm × 1 cm, 12 mL volume) as described by Zhang et al. (1993a, 1994b). This column had a capacity for PP1 of about 2 mg of protein. The affinity chromatography procedure was used for isolation of most of the mutants. The H66K, H248N, N124D, and N124A mutants were purified by conventional chromatography on DEAE-Sepharose, Q-Sepharose, heparin−Sepharose, and polylysine−agarose (Zhang et al., 1993b).

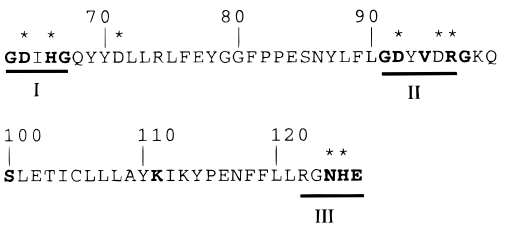

FIGURE 1: Conserved core region of PP1α. The sequence of PP1α is shown from residues 63 to 126. This region contains all 14 of the invariant residues identified in a multiple sequence alignment of 44 eukaryotic Ser/Thr protein phosphatases, bacteriophage λ, and φ80 protein phosphatases, and *E. coli* adenosinetetraphosphatase (Barton et al., 1994). Residues in boldface are the 14 invariant residues found in the catalytic core (E126 is an Asp in *E. coli* tetraphosphatase). The underlined regions (I, II, and III) represent the motif identified by Zhou et al. (1994) as being common to both the phosphatases and several exonuclease enzymes. Asterisks denote residues mutated in this study.

Assay of PP1. PP1 activity was assayed using ^{32}P-labeled phosphorylase *a* as described by Zhang et al. (1992). Kinetic analyses of the PP1 mutants were performed as previously described (Zhang et al., 1992, 1993a). *p*-Nitrophenyl phosphatase activity of PP1 was assayed as described previously (Zhang et al., 1992).

Electrophoresis and Western Blotting. SDS−PAGE was performed using 12% acrylamide gels. Western blotting was performed using a rabbit polyclonal antibody developed against the C-terminal (14-mer) of PP1. Bound antibody was detected using an alkaline phosphatase-conjugated second antibody.

RESULTS

Expression and Isolation and Characterization of Mutants. The N-terminal region (63−126) of PP1 containing the invariant residues found among a number of Ser/Thr phosphatases (Barton et al., 1994) is shown in Figure 1. We chose to mutate H66, H125, D64, D92, N124, and R96 from within this region. In addition, D71, D95, H173, and H248 were also chosen for mutation because they are conserved between eukaryotic PP1, PP2A, and PP2B. Mutations were constructed and expressed in the pTACTAC vector, which we have previously shown to give good expression (ca. 4% soluble protein) of the wild-type PP1 (Zhang et al., 1992). The first set of mutants made were H66N, H66K, H125N, H125K, H173N, H173K, H248N, H248K, D64N, D71N, D92N, D95N, N124D, N124A, and R96E. In all cases, expression of the mutants was monitored by Western blot analysis of both the soluble and particulate (20000*g* pellet) fractions of the *E. coli* lysates. This is illustrated for some of the mutants in Figure 2. Soluble proteins were produced in all cases except for the Asn and Lys mutants of H125 and H173. Attempts to perform the renaturation of these insoluble mutant proteins as described by Zhang et al. (1992) were unsuccessful.

The enzyme activity of the majority of the soluble mutants described was barely detectable or undetectable in the lysates, except for D71N and N124D. Purification of the mutant enzymes was performed by affinity chromatography on inhibitor-2−Sepharose (Zhang et al., 1993). This provided near-homogeneous proteins after a single step. The use of inhibitor-2−Sepharose affinity chromatography was advantageous from a practical point of view and also because the ability of the mutants to bind to inhibitor-2−Sepharose provided some assurance that they were properly folded

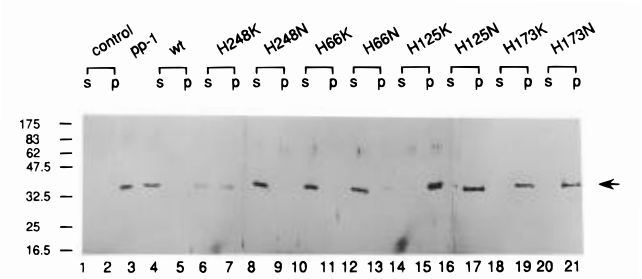

FIGURE 2: Western blot analysis of PP1 mutants expressed in *E. coli*. The supernatants (s) or the pellets (p) of the *E. coli* lysates expressing the mutant PP1 enzymes were separated on a 12% SDS−polyacrylamide gel. Approximately 1 μg of protein was loaded onto each lane. Proteins were transferred to nitrocellulose membranes, and Western blot analysis was performed as described under Materials and Methods. Positions of the prestained protein standards are shown at the left. The arrow at the right shows the position of the 37 kDa band of PP1. Lane 3 is recombinant PP1 which was expressed in *E. coli* and purified to homogeneity (Zhang et al., 1992). "wt" refers to an *E. coli* lysate expressing the wild-type PP1. Mutants are as indicated.

proteins which retained their functional ability to interact with inhibitor-2. Some of the mutants (Materials and Methods) were purified by conventional methods; in these instances, separate experiments confirmed that these mutants were able to bind to inhibitor-2−Sepharose. An additional advantage of the affinity chromatography procedure was that several of the mutants were observed to be significantly less stable on storage than the wild-type enzyme, so that analysis of the mutants could be performed before loss of activity occurred. (Care was taken to avoid cross-contamination with wild-type enzyme since the latter was, in some instances, more than 10000-fold more active than the mutants. All column media used for the isolation of the mutants were those that had not been exposed to the wild-type enzyme or to mutants with high PP1 activity.) Recoveries of protein from the cells derived from 1 L cultures were as follows for the mutants: H66N (0.6 mg), H66K (3.2 mg), H248N (5.4 mg), H248K (0.3 mg), D64N (0.6 mg), D92N (1 mg), D95N (0.7 mg), D71N (0.7 mg), N124D (0.4 mg), N124A (4.5 mg), and R96E (0.16 mg). A typical recovery for the wild-type enzyme is 5 mg per liter of culture (Zhang et al., 1992).

The systematic mutation of H125 and H173 to F, C, Y, S, G, D, V, or A was performed in the attempt to obtain mutants which could be expressed in a soluble form. No enzyme activity was detectable in the lysates from any of these mutants. Expression of immunoreactive protein was monitored by Western blotting of the soluble and particulate fractions of the *E. coli* lysates with an antibody against PP1 (Figure 3). All the mutants of H173 were produced as insoluble proteins. This behavior suggested that His173 is crucial for either folding or stability of PP1. All of the H125 mutants were produced as insoluble proteins, except for H125S and H125A, where a small fraction of the expressed protein was present in the soluble fraction (Figure 3). The soluble enzymes were purified by affinity chromatography on inhibitor-2−Sepharose with yields of 0.14 mg and 0.13 mg of protein for the H125S and H125A mutants, respectively. Neither mutant exhibited any detectable activity. It was concluded that the H125S and H125A mutants were null mutants, but also suffered from a lack of stability and/or ability to fold properly.

Kinetic Analyses of the Mutants. The purified mutants were analyzed kinetically using phosphorylase *a* as the

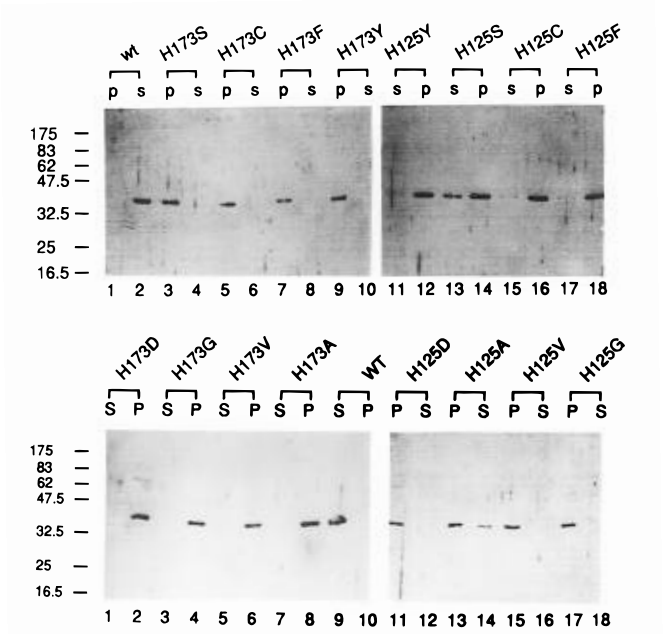

FIGURE 3: Western blot analysis of PP1 mutants at His125 and H173 expressed in *E. coli.* The supernatants (s) or the pellets (p) of the *E. coli* lysates expressing the mutant PP1 enzymes were separated on a 12% SDS−polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, and Western blot analysis was performed as described under Materials and Methods.

substrate. The K_m and k_{cat} values are shown in Table 1. As can be seen, little significant change in K_m was observed for the mutants listed in Table 1, except for the D71N mutant. Dramatic losses of catalytic efficiency by factors of 10^3−10^4 were observed for the H66N, H248N, D64N, and D92N mutants. Mutation of H66 and 248 to Lys had more severe effects than the more conservative mutation to Asn. The effect of mutation of these His and Asp residues is consistent with their identification as metal ligands at the active site (see Discussion). The R96 mutant, R96E, exhibited a 700-fold lower catalytic efficiency. Thus, these residues are critical for the catalytic function of PP1. Mutation of D95 resulted in a 71-fold lower catalytic efficiency, due to an effect on k_{cat}, as the K_m was unchanged. Thus, D95 also appears to be important for catalysis. In the case of the D71N mutation, a 11-fold difference in catalytic efficiency was observed. This was not due to a change in k_{cat}, but due to a 10-fold increase in K_m, suggesting that it may be important in substrate binding.

The N124D and N124A mutants gave contrasting results, as the N124D mutant showed a 10-fold decrease in k_{cat}, while the N124A mutant exhibited a dramatic loss in k_{cat}. The latter result is consistent with a role of the N124 residue in catalysis, but the lesser effect of the Asn to Asp mutation indicates that it is the carbonyl function that is important. The further observation was made that the N124D mutant was devoid of activity toward *p*-nitrophenyl phosphate (pNPP). This small molecule is hydrolyzed by PP1 (Li et al., 1979; Silberman et al., 1984). In the case of recombinant PP1, the k_{cat} for pNPP is roughly comparable to that for phosphorylase *a*, since the specific activities toward the two substrates are comparable (Zhang et al., 1992). In order to confirm that the loss of activity was not a pH-dependent phenomenon, the pH optima of the N124D and N124A mutants were determined using both phosphorylase *a* and *p*-nitrophenyl phosphate as the substrates. No activity toward pNPP could be elicited at any pH. However, it was observed

Table 1: Kinetic Analysis of PP1 Mutants[a]

enzyme	K_m	V_{max}	k_{cat}	$k_{cat}/K_m \times 10^{-3}$	relative k_{cat}/K_m (WT/mutant)
WT	3.5	22800	14255	4072	1
H66N	2.4	3.94	2.46	1.03	4175
H66K[b]					
H125S[b]					
H125A[b]					
H248N	5.8	29.5	18	3.18	1312
H248K	4.8	0.93	0.58	0.12	33933
D64N	2.4	0.8	0.50	0.21	19390
D92N	10	4.5	2.8	0.28	14542
D95N	3	274	171	57	71
D71N	42.8	25200	15755	368	11
N124D	6.6	4270	2670	404	10
N124A	7.4	4.2	2.6	0.35	1163
R96E	8.9	86	54	6.1	667
Mutants expressed as insoluble proteins:					
H125 to F, Y, C, G, D, or V					
H173 to F, Y, C, S, G, D, V, or A					

[a] Kinetic analyses were performed as described under Materials and Methods using phosphorylase *a* as the substrate. Values of K_m are in μM; V_{max}, nmol of P_i released (mg of protein)$^{-1}$ min^{-1}; k_{cat}, $s^{-1} \times 10^3$; k_{cat}/K_m, s^{-1} M^{-1}. WT refers to the wild-type recombinant PP1. [b] No detectable activity.

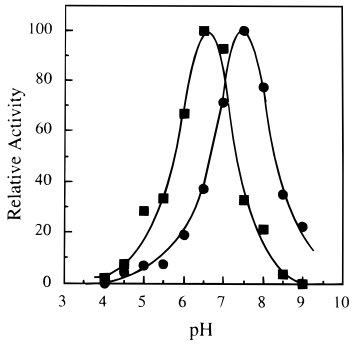

FIGURE 4: pH optima of the N124D mutant. The activity of the N124D mutant was assayed using phosphorylase *a* as the substrate as described under Materials and Methods. Activity of the wild-type enzyme is shown as circles, and of the N124D mutant as squares. Buffers used were (50 mM) were sodium acetate, pH 4−5.5; Bistris, pH 5.5−7.5; and Tris-HCl, pH 7.5−9.

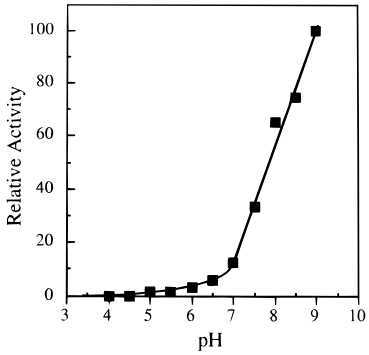

FIGURE 5: pH optima of the N124A mutant. The activity of the N124A mutant was assayed using phosphorylase *a* as the substrate as described under Materials and Methods. Buffers used were as for Figure 4. Data are shown as relative activities.

that the N124D mutant had a pH optimum of 6.5 with phosphorylase *a* as the substrate, compared to an optimum of pH 7.5 for the wild-type enzyme (Figure 4). For the N124A mutant, an unusual pH behavior was observed, in that this was a monophasic curve showing increasing activity with pH (Figure 5).

DISCUSSION

In this study of rabbit muscle PP1α, we selected residues that were invariant among a number of widely distributed phosphatases related to PP1, focusing on histidine and aspartate residues as these were the most likely to be involved in metal binding. Our findings, which are the first report of the mutagenesis of these invariant residues in a mammalian Ser/Thr phosphatase, show that several of the His and Asp residues (H66, D64, D92, and H248) are important for catalysis. These findings, taken on their own, are generally consistent with the view that these are likely to be metal ligands, and are similar to results obtained by mutagenesis of the bacteriophage λ phosphatase (Zhou et al., 1994). The issue of whether PP1 is a metalloenzyme was resolved during the course of this work by the reports of the crystal structures of recombinant rabbit muscle PP1α (Goldberg et al., 1995) and PP2B (Griffith et al., 1995) which both possess two metal ions at the active site. These developments, including the solution of the crystal structure of a member of the purple acid phosphatase (PAP) family (Sträter et al., 1995), provide a structural context in which to evaluate the behavior of the PP1 mutants that we have studied.

The PP2B and PP1 structures are conserved in the region of the catalytic core, and both enzymes possess a bimetal center. In the case of PP1, this is presumed to be two Mn^{2+} ions, since the recombinant enzyme is expressed and isolated in the presence of this metal ion. The nature of the metal ions in wild-type muscle PP1 is unknown, although it can be speculated that this may be an Fe^{3+}/Zn^{2+} pair as in PP2B (King & Huang, 1984; Griffith et al., 1995; Chu et al., 1996). The Ser/Thr phosphatases thus resemble the purple acid protein phosphatases in having a bimetal center at the active site (Sträter et al., 1995). The coordination spheres of the metals in PP2B are highly similar to that of PAP (Griffith et al., 1995; Sträter et al., 1995).

The role of the oxide-bridged bimetal center in the PAP's has been extensively studied by enzyme kinetics and

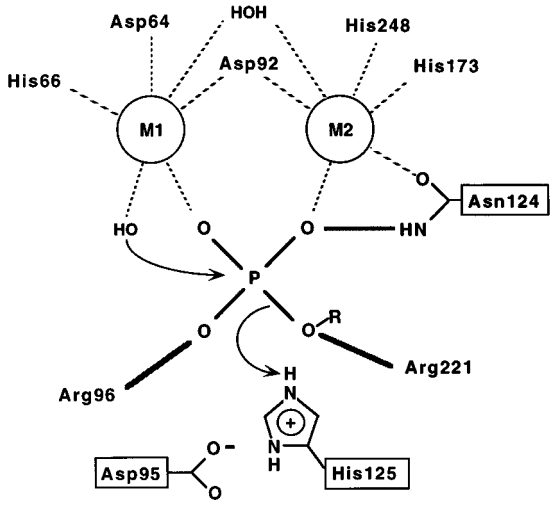

FIGURE 6: Schematic diagram of the active site of PP1. The active site of PP1 is shown in purely diagrammatic form. Ligands for the metal ions (M1 and M2) are shown as dashed lines and include the indicated amino acid residues as well as two water molecules (Goldberg et al., 1995). The hydrogen bonds of the phosphorus oxygens to N124, R96, and R221 are shown as thin lines. The suggested general catalytic mechanism, based on studies of PAP and PP2B (Dietrich et al., 1991; Griffith et al., 1995; Strater et al., 1995), is indicated in the diagram. Binding of the substrate would involve interaction with the metals, displacing a third water molecule (not shown in the diagram). The interaction of the phosphorus oxygens could involve both metal ions, or only one of them (Dietrich et al., 1991). The proposed reaction mechanism involves nucleophilic attack by a hydroxide ion, with H125 providing the proton for the leaving alcohol group as proposed for PP2B (Griffith et al., 1995). The ion pairing of H125 with D95 which stabilizes H125 in the protonated form is also shown.

biophysical methods, and has led to the development of models for their function in catalysis (Dietrich et al., 1991; Vincent et al., 1992a,b; Mueller et al., 1993; Suerbaum et al., 1993). The metals serve as ligands for the phosphate residue which allow its stabilization and orientation in the catalytic site, and for the generation of a hydroxide nucleophile involved in catalysis. Kinetic solvent isotope and isotope exchange studies of PP2B also support a role for the metal ion as the source of hydroxide ions (Martin & Graves, 1994). The role of the metal ion pair minimally involves binding of the phosphate by one or both of the metals, as well as the generation of the hydroxide nucleophile which is involved in catalysis [see Griffith et al. (1995) and Sträter et al. (1995) and references cited therein]. The role of the metal ion in assisting the dissociation of water at lower pH values is consistent with that which is known for alkaline phosphatase (Coleman, 1992), and for the classic example of carbonic anhydrase (Lindskog & Coleman, 1973). While several variants of a reaction mechanism involving a nucleophilic attack by a hydroxide ion on the phosphorus are possible, a reasonable mechanism has been proposed for PAP and PP2B (Griffith et al., 1995; Sträter et al., 1995). The two metals are bridged by a water molecule and serve as the source of a hydroxide ion which initiates a nucleophilic attack on the phosphorus via an S_N2 mechanism that involves the formation of a pentacoordinate transition state. A protonated histidine (H151 in PP2B) functions to donate a proton to the leaving serine side chain and is stabilized by a salt bridge to an Asp residue (D121 in PP2B). This general mechanism is illustrated in Figure 6. Alternate mechanisms involving a nucleophilic attack by H125 (Goldberg et al., 1995) appear unlikely, as there is experimental evidence against the formation of a transient phosphoenzyme intermediate for PP2B (Martin & Graves, 1994), and the PAP reaction has been shown to take place with inversion of configuration (Müeller et al., 1993). Tyr-272 has been suggested as a candidate nucleophile, but in other studies we have eliminated it as a participant in catalysis by PP1 (Zhang et al., 1996). There have been no mechanistic studies of PP1, but, based on similarities in the metal ion center at the active sites and the conservation of metal and phosphate ligands, it is likely that the general mechanism will be similar to that described above for PP2B and the PAP. These conclusion have been reinforced by the very recent solution of the crystal structure of the PP1γ1 isoform as a complex with tartrate, a phosphate analog; in addition, the use of proton-induced X-ray emission spectroscopy revealed the presence of Mn^{2+} and iron in the ratio 1 to 0.5 in the PP1γ1 crystals (Egloff et al., 1995).

The six residues which serve as ligands for the metal ions, which are identical for PP1 and PP2B (Griffith et al., 1995; Goldberg et al., 1995), are located in highly conserved regions of the two proteins. In PP1, the two metals are designated as M1 and M2 (Goldberg et al., 1995), and would correspond to Fe^{3+} (or Fe^{2+}) and Zn^{2+} in PP2B (Griffith et al., 1995). In PP1, the ligands are His66, H173, H248, D64, D92, and N124. These account for all of the His and Asp residues in the group of invariant residues identified by Barton et al. (1994) except for H125, and include the two His residues (H173 and H248) that are conserved in PP1, PP2A, and PP2B. Thus, we have mutated all of the metal binding ligands, of which one, H173, was not expressed. H125, which corresponds to H151 in PP2B (Griffith et al., 1995) and is proposed to be the proton donor for the leaving alcohol group, and D95, to which H125 is salt-bridged, are also invariant and were mutated in this study.

Our mutational data support the role of the residues proposed to be involved in metal ion binding, since mutation of H66, D64, D92, and H248 led to severe loss of catalytic function. This provides another example of the validity of sequence analysis for the identification of functionally important residues within a protein family. The loss of catalytic function observed on mutation of the His and Asp metal ligands is expected, given that both metal ions may play a key role in catalysis. It is likely that mutations involving the metal ligands will result in loss of metal binding, which would be consistent with the effects on catalysis that were observed.

Mutation of H125 in our studies, as with H173, did not result in readily expressed proteins, although small amounts of the H125S and H125A mutants could be isolated. These mutants were devoid of measurable activity. Mutation of D95, which stabilizes the protonated H125 by a salt bridge in PP1, did not affect the K_m but resulted in significant (71-fold) reduction in catalytic activity. These findings support the idea that H125, stabilized by a salt bridge to D95, is essential for catalysis because it functions as the donor of a proton for the leaving alcohol group, as is proposed for PP2B (Griffith et al., 1995).

Mutation of R96 resulted in a 700-fold loss in catalytic efficiency with a small effect on K_m. R96 is one of the three residues (the other two being N124 and R221) which on the basis of the crystal structure are proposed to be involved in hydrogen bonding with the phosphorus oxygens, and would

function in stabilizing a pentacoordinate intermediate (Figure 6). The results are consistent with such a role, and the lesser effect on K_m indicates that this hydrogen bonding is more critical for catalysis than substrate recognition. Mutation to N124A led to a 1200-fold loss in catalytic efficiency. The changes in kinetic properties of the N124A mutant are similar to those for the R96E mutant. However, interpretation of the effects of mutation of N124 is complicated by the fact that it is also one of the metal ligands, providing an alternate explanation for the loss of catalytic efficiency as being related to the loss of metal binding. This appears more likely based on the behavior of the N124D mutation (see below). The pH optimum of the N124A mutant was examined, and found to be unusual as it simply showed increasing activity with pH. This pH effect could reflect an increase in hydroxide ion concentration so that in this mutant, the solvent hydroxide concentration is sufficient to promote the low level of catalysis observed. The hydroxide ion in the reaction comes directly from the solvent rather than being generated by the action of the metal ion. This explanation is consistent with a reaction mechanism involving nucleophilic attack by a hydroxide ion.

The properties of the N124D mutant were surprising, in that only a small effect on k_{cat} was observed, such that this remained at ca. 20% of the wild-type enzyme, in contrast to the N124A mutant. This can be explained on the basis that there is a retention of the carbonyl function of N124 which is involved in metal binding (Figure 6), and which is lost in the N124A mutation; i.e., the effects of the N124A mutation are due to the loss of its function in metal binding rather than its possible role in binding to the phophorus oxygen. The presence of a carboxylate residue rather than the neutral amide function in the N124D mutant would have been expected to cause a significant loss of catalytic activity, considering the results obtained with the R96E mutant. Further examination of the N124D mutant showed that the pH optimum is acid-shifted by 1 pH unit. It can be speculated that the pH shift reflects protonation of the phosphorus oxygen combined with an effect on the pK_a for dissociation of the metal-bound water. The apparent pK_a of the group contributing to the alkaline portion of the pH curve is 7.3, which is consistent with the expected second pK_a (7.2) of the phosphate and the relatively small effect on k_{cat}. The involvement of the carbonyl function of N124 in metal binding could also explain the shift in the acid portion of the pH optimum, as a result of perturbation of the dissociation of the metal-bound water. This provides an explanation for the maintenance of the catalytic activity of the N124D mutant, but also questions the degree of importance of the N124 residue as a ligand for stabilization of the pentacoordinate phosphate intermediate during catalysis (Figure 6).

The observation that the N124D mutant exhibited a complete loss of activity toward pNPP in contrast to its activity toward phosphorylase *a* requires further explanation. The crystal structure shows the active site to be at the intersection of a Y-shaped groove, one arm of which is a hydrophobic groove that is likely to be the peptide binding groove. Binding of the protein substrate involves the adjoining peptide region in addition to the phosphoserine, in contrast to the smaller pNPP substrate. This is reflected in the poor binding of pNPP. The K_m for PNPP is in the millimolar range (Zhang et al., 1992; Silberman et al., 1984), while that for the polypeptide phosphate substrate is in the micromolar range, and those for phosphopeptide substrates range from 55 to 139 μM (McNally & Fischer, 1988). Modification of the active site region would therefore be expected to have a greater effect on pNPP binding than on phosphopeptide binding. The presence of the negative carboxylate group would be expected to result in charge repulsion of the substrate, which would be more severe for pNPP than for the peptide substrates.

The D71N mutant was the only one studied which showed a large effect on the K_m, without any effect on k_{cat}, suggesting that it is involved in substrate binding. This residue is conserved in PP1, PP2A, and PP2B, but not in the bacteriophage λ phosphatase. Cursory examination of its location in the crystal structure shows that it is located in the C-terminal groove of PP1. This is surprising, since it is more likely that substrate binding will involve the hydrophobic groove as proposed by Goldberg et al. (1995), and raises the possibility that the binding of a polypeptide substrate may extend beyond the catalytic center into the C-terminal groove.

In summary, studies of mutations of the invariant residues conserved in the Ser/Thr protein phosphatase family in PP1 support the roles proposed for the metal ligands in PP1, and serve to clarify the potential role of residues that may be involved in catalysis. Our studies support a general catalytic mechanism for PP1 that is in concert with those proposed for PP2B and for PAP.

ACKNOWLEDGMENT

We thank Dr. K. K. Schlender for the antibody to PP1, and Drs. J. Goldberg and J. Kuriyan for providing the coordinates for the structure of the PP1/microcystin complex.

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BI952954L