Mutational Analysis of Substrate Recognition by Protein Phosphatase 1[†]

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ABSTRACT: The role of residues that are involved in substrate recognition by rabbit muscle protein phosphatase 1α (PP1) was investigated by site-directed mutagenesis and kinetic analyses using phosphorylase *a*, RII peptide, Kemptide, and *p*-nitrophenyl phosphate as substrates. The atomic structure of PP1 has shown the active site to be at the confluence of three shallow grooves, a C-terminal groove, an acidic groove, and a hydrophobic groove. Mutations of residues D208, D210, D212, E218, D220, E252, D253, E256, E275, and D277 in the acidic groove, of R221, W206, and Y134, which have been suggested to be involved in substrate binding, and of residues C127, I130, and D197 in the hydrophobic groove were examined. Our results show that mutations in the acidic groove lead to modest changes in substrate binding, consistent with a role of the acidic residues in forming a negatively charged surface well for binding of peptides with basic N-termini. Severe effects on V_{max} were observed for mutants of R221, D208, and W206. These results are consistent with the proposal that the R221 plays an important role as a phosphate oxygen ligand that positions the substrate for catalysis. The kinetic behavior of mutants at W206 and D208 can be explained by the observation that, together with R221, these residues form the microenvironment which dictates the orientation of the imidazole ring of H248, one of the metal binding ligands, as well as contributing to the orientation of R221 itself.

Mammalian protein phosphatase 1 (phosphorylase phosphatase) has been extensively studied since its discovery over 50 years ago (Cori & Green, 1943) as the enzyme that converts phosphorylase *a* into phosphorylase *b*. Identification and isolation of the catalytic subunit of phosphorylase phosphatase as a protein of ca. 38 kDa (Brandt et al., 1974, 1975; Khandelwal et al., 1976; Gratecos et al., 1977; Silberman et al., 1984) paved the way for later studies that revealed an unusual enzymology in which the catalytic subunit forms complexes with a number of regulatory or targeting subunits [for reviews see Lee (1995), Brautigan (1994), Bollen and Stalmans (1992), Shenolikar and Nairn (1991), and Cohen (1989)]. These subunits include the glycogen binding subunit (Hubbard & Cohen, 1991), inhibitor 2 (Merlevede et al., 1984), a nuclear inhibitory protein, NIPP-1 (Jagiello et al., 1995), a ribosomal inhibitor, RIPP-1 (Beullens et al., 1996), and the myosin binding subunits (Chen et al., 1994).

The availability of the purified catalytic subunit led to the findings that PP1 was capable of dephosphorylating both glycogen synthase and phosphorylase kinase (Lee et al., 1980; Cohen, 1989). PP1 exhibits a broad specificity against a number of protein substrates, as opposed to findings for the various protein kinases (Pelech et al. 1984; Cohen, 1989; Kemp & Pearson, 1990). PP1 also hydrolyzes phosphopeptide substrates (McNall & Fisher, 1988; Pinna & Donella-Deana, 1994). Cardiac PP1 was found to be able to hydrolyze *p*-nitrophenyl phosphate (Li et al., 1979), as were rabbit skeletal muscle preparations of both PP1 and PP2A (Silberman et al., 1984). The molecular cloning of PP1 and the development of an expression system in *Escherichia coli* (Zhang et al. 1992) has allowed mutagenesis studies to be performed with PP1 and has permitted expression of the protein in sufficient amounts to allow its crystallization. The atomic structures of PP1α in a complex with microcystin and of the PP1γ1 isoform alone and in complex with tungstate (Egloff et al., 1995; Goldberg et al., 1995) have been determined. These studies provide insights into the catalytic mechanism of PP1, which involves a bimetal ion center (Egloff et al., 1995; Goldberg et al., 1995; Barford, 1996; Zhang et al., 1996a), and for its broad substrate specificity. The surface of PP1 has three shallow grooves radiating from the active site that are potential binding sites for substrates and inhibitors. These consist of a hydrophobic groove, a C-terminal groove, and an acidic groove (Goldberg et al., 1995). Microcystin, a cyclic heptapeptide inhibitor, binds in a manner such that it occupies the active site, while its ADDA side chain (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid) occupies the hydrophobic groove. It has been proposed that the hydrophobic groove and the acidic groove may represent the binding region for DARPP-32, a neuronal analog of inhibitor 1 (Goldberg et al., 1995), where the basic residues N-terminal to the phosphothreonine are accommodated in the acidic groove while the sequence C-terminal to the phosphothreonine would occupy the hydrophobic groove (Goldberg et al., 1995). This binding likely would represent a pseudosubstrate binding, since many of the protein substrates (as well as a number of synthetic peptide substrates) of PP1 (Pinna & Donella-Deana, 1994) carry a number of basic residues N-terminal to the phosphoserine or phosphothreonine.

With regard to the binding of the phosphoserine moiety, the atomic structure of the PP1−tungstate complex has shown that R96, N124, and R221 are involved in binding to phosphate oxygens (Egloff et al., 1995). R221 and R96 form

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salt bridges with two of the phosphate oxygens while the amino group of N124 is hydrogen-bonded to the third oxygen. D208 may be important in maintaining the orientation of R221 as it is involved in a salt bridge interaction with R221. Thus, these residues are proposed to be involved in the recognition of phosphate and also the proper orientation of the phosphate during the formation of the transition complex during catalysis (Egloff et al., 1995). Egloff et al. (1995) have also pointed out that W206 and Y134 are well positioned to interact with the Ser or Thr carrying the phosphate residue. In order to experimentally test the importance of these residues in structure−function relationships, we have systematically mutated them and examined the kinetic behavior of the mutant proteins toward several substrates.

MATERIALS AND METHODS

Materials. RII peptides and Kemptide were purchased from BIOMOL Co. Plasmids for performing mutagenesis, pALTER-1, were obtained from Promega Co.

Site-Directed Mutagenesis. The PP1 mutants were all based on the rabbit muscle PP1α isoform cDNA (Bai et al., 1988). All mutants were constructed by the Altered Sites II in vitro mutagenesis system (Promega). The pALTER-1 vector has an ampicillin-resistance gene that has been inactivated. The ampicillin repair oligonucleotide and mutagenic oligonucleotide were annealed to the single- or double-stranded DNA template at the same time. Subsequent synthesis and ligation of the mutant strand was followed by selection of mutants by selection in ampicillin-containing growth medium. The correctness of the mutated cDNAs was confirmed by DNA sequencing. The mutant cDNAs were excised and subcloned into the pTACTAC vector for expression as previously reported (Zhang et al. 1996b).

Purification of Mutant Enzymes. E. coli DH5α host cells were transformed with the pTACTAC vector containing the mutated PP1α sequences as described previously (Zhang et al. 1992). The PP1 wild-type and mutant constructs were grown in *E. coli* host cells in 1 L of Terrific medium (24 g of yeast extract, 12 g of tryptone, 10 g of NaCl, 4 mL of glycerol, pH 7.4) with 0.2 mM $MnCl_2$. Protein expression was induced with 0.5 mM isopropyl β-thiogalactopyranoside (Zhang et al., 1992, 1996b). The cells were harvested by centrifugation and disrupted using a French press. The PP1 proteins were purified to near-homogeneity by the column chromatography procedures described by Zhang et al. (1993). The procedures involved successive chromatographies on DEAE-Sepharose, heparin−Sepharose, Q-Sepharose, and polylysine−agarose. Final yields of protein were about 5 mg. Preparations were assayed for phosphatase activity as described below and for protein content by the procedure of Bradford (1976).

Preparation of Phosphorylated Substrates and Enzyme Assays. The procedure for the preparation of phosphorylated RII and Kemptide peptides used was based on that of Perrino et al. (1992; B. Perrino, personal communication). The RII peptides and Kemptide (1 mM in a final volume of 200 μL of 20 mM HEPES [*N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 2 mM magnesium acetate, 1 mM dithiothreitol, and 2 mM ATP, pH 7.0) were phosphorylated using 1 μg of catalytic subunit of cAMP-dependent kinase (PKA) at 30 °C with 200 μCi of [γ-^{32}P]ATP. After 1 h the reaction was stopped by adding glacial acetic acid to a final concentration of 30% (v/v). The ^{32}P-labeled peptide was separated from free [γ-^{32}P]ATP by passage through a Dowex 1-X8 anion-exchange resin column (2 × 0.5 cm) equilibrated with 30% acetic acid. The phosphopeptide solution was lyophilized and dissolved in 20 mM Tris/HCl, pH 7.4, and 1 mM DTT. The degree of phosphorylation of the peptides was greater than 90%. The specific activity of the ^{32}P incorporated in the peptides was assumed to be equal to that of the [γ-^{32}P]ATP and the concentration of the ^{32}P-labeled peptide was based on this assumption. Assays for phosphorylase phosphatase and *p*-nitrophenyl phosphatase activities were performed essentially as described by Zhang et al. (1992). The dephosphorylation of RII and Kemptide substrates were performed in a reaction volume of 20 μL in the same assay buffer as for phosphorylase phosphatase assays (Zhang et al., 1992). Reactions were terminated by addition of 180 μL of 75 mM phosphoric acid. The mixtures were then passed through 0.5 mL columns of Dowex AG50W-X8 ion-exchange resin to remove unreacted phosphopeptide and counted (Blumenthal et al., 1986).

All the kinetic data were analyzed by the Sigma Plot program using nonlinear regression fit to the Michaelis−Menten equation to obtain K_m, V_{max}, and standard error values.

RESULTS

Preparation of Mutant Enzymes and Kinetic Analyses. All mutants of PP1α were expressed in *E. coli* and purified to near-homogeneity (Materials and Methods). The expression levels and yields of the mutants were all comparable to those of the wild-type enzyme. The kinetic properties of the mutants were determined (K_m and V_{max}) using four substrates. These were phosphorylase *a*, RII peptide, Kemptide, and *p*-nitrophenyl phosphate (PNPP). Kemptide (L**RR**A**S**LG) is a commonly used PKA peptide substrate (Kemp & Clark, 1978; Roskoski, 1983). Peptide RII (DLDVPIPGRFD**RR**V**S**-VAAE) comprises residues 81−99 of the regulatory subunit of cAMP-dependent protein kinase (Blumenthal et al., 1986; Perrino et al., 1992). These two substrates contain the RRXS substrate recognition motif for PKA (basic residues and phosphorylated serine in bold). Rabbit muscle phosphorylase *a* is phosphorylated at Ser-14 (Nolan et al., 1963), which lies in the sequence SDQE**KRK**QI**S**VRGLAGVENV (residues 5−23). Data for PNPP were obtained as the behavior of PP1 mutants with this substrate could provide a useful comparison with the peptide and protein substrates which have more extended binding interactions.

Mutations in the Acidic Groove of PP1. The location of the residues selected for mutation is shown in Figure 1. There are ten acidic residues (D208, D210, D212, E218, D220, E252, D253, E256, E275, and D277), which line both sides of the acidic groove. The following mutants were studied: D208A, D210A, D212A, E218A, D220A, E252A, D253N, E256A, E275R, and D277G. The latter two mutants were constructed in a previous study (Zhang et al., 1996b). The results of the kinetic analyses of these mutants using phosphorylase *a* and PNPP are listed in Table I. In general, mutations of the acidic residues had little effect on the kinetic parameters for phosphorylase *a* and for PNPP, with the exception of the mutation of D208A, for which a 7-fold increase in K_m was observed. This effect was confirmed by

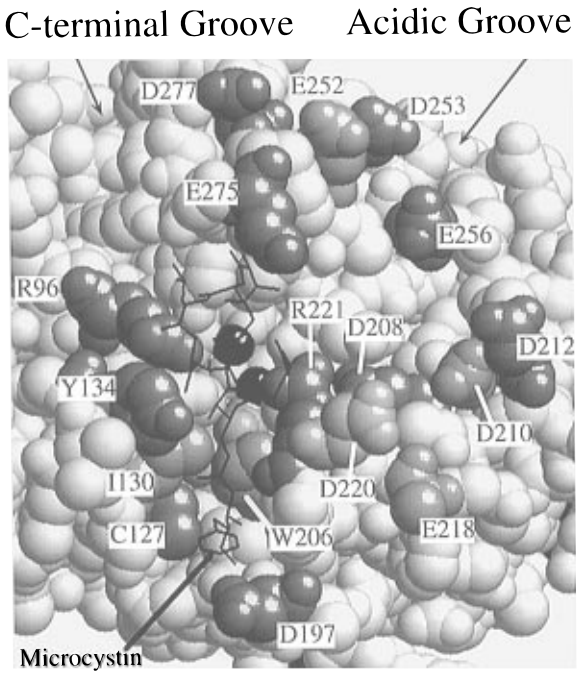

FIGURE 1: Location of the residues in PP1 selected for mutation. The figure shows the orientation of the three shallow grooves of PP1α (Goldberg et al., 1995), which radiate from the active site, and the location of the residues that were mutated. Residues D208, D210, D212, and E218 are located on β strands 7 (207−209) and 8 (216−218) which, together with β strand 9, comprise β sheet 3. Acidic residues E252 and D253 are located on the loop (247−254) connecting β strands 10 and 11. Residue E256 is on β strand 11 (Goldberg et al., 1995). Residues E275 and D277 are part of the loop connecting β strands 12 and 13, which we have previously shown to be important in the binding of toxins to PP1 (Zhang et al. 1996b). The two metals at the active site are shown as black spheres, and the active site and hydrophobic groove are delineated by the binding of microcystin, shown in wireframe form in black. The diagram was produced using the RasMol molecular graphics program (Sayle & Milner-White, 1995) using the coordinates for rabbit muscle PP1α (Goldberg et al., 1995).

examination of a second mutation, D208N (Table 1), for which a 3-fold increase in K_m was obtained. Otherwise, the K_m values for phosphorylase *a* were not significantly different, except for D277G, which exhibited a 2-fold increase. The K_m values for PNPP were not altered except for the E275R and the D208N mutants, which were reduced from 10 to 6 mM. In the case of E275R, this may be due to an improved binding of the *p*-nitrophenyl moiety of PNPP. E275 is located on the loop connecting β strands 12 and 13, which directly overhangs the active site, and is centrally located in the overhang. Only relatively small changes in V_{max} values for phosphorylase *a* and PNPP were observed with all of the mutants. These were reduced by about 50% for E252A and D208N for both substrates. (However, changes of 20−30% in V_{max} may not be important as this falls within the range of variability from preparation to preparation of the wild-type enzyme.) Besides the changes noted, the overall effects of mutation of individual acidic residues show that they promoted very little change in the kinetic parameters for phosphorylase *a* and for PNPP. The lack of effect on PNPP hydrolysis was expected, but the lack of effect on phosphorylase *a* was unexpected. Two double mutants were studied, these being D210N/D212N and E252Q/D253N, in order to determine if a greater effect could be observed on removal of two acidic residues. The results show that these were not significantly different in their behavior from the parent single mutants.

The acidic groove mutants were then studied using RII peptide and Kemptide as substrates, as it was thought that these substrates might be more revealing, as they represent increasingly truncated substrates that might be more sensitive to changes in the acidic groove. This expectation was realized as modest but significant increases (2−3-fold) in K_m were observed with the majority of the mutants with RII, except for mutations at D210 and D212. With Kemptide, similar effects of increases of 2−5-fold in K_m were also observed, but only for the mutants D210A, D212A, E252A, and D208A and the double mutants D210N/D212N and E252Q/D253N. Only modest changes in V_{max} were seen with the majority of the mutants. V_{max} values were reduced to about a third of the wild-type value for mutants D210 and D212 with RII peptide, and to a sixth for D277G with Kemptide. The striking exceptions were the two mutations of D208, where dramatic losses of V_{max} were observed with both Kemptide and RII peptide. Thus, mutation of the acid residues results in a clear sensitivity to loss of individual acidic residues when tested against the peptide substrates, as opposed to the results obtained with phosphorylase *a*. This suggests that the difference may be due to the ability of the protein substrate to make more extended contacts with PP1. This is consistent with the greater effects observed with the shorter peptide, Kemptide, when compared to RII. It was also evident that while there was a common response, this was different for the two peptides. The major unexpected findings were the apparently anomalous behavior of the D208 mutations in relation to the changes in V_{max}, which were only observed for the peptide substrates. This behavior is further discussed below in relation to the findings for mutation of R221.

Mutation of Residues Involved in Phosphate and Phosphoserine Recognition at the Active Site. The following mutants were studied: R221Q, W206F, N124D, and Y134F. We have previously mutated R96 and N124 and examined their activities on PNPP and phosphorylase *a* (Zhang et al., 1996a). Data for the N124D mutant were also obtained in this study for Kemptide and RII and are shown in Table I. Mutation of R221 to R221Q led to a ca. 3-fold increase in the K_m for phosphorylase *a* and a 20-fold reduction in V_{max} (Table 1). With RII as the substrate, the K_m was increased 2-fold, while the V_{max} was reduced by 3500-fold. Similar effects of a modest increase in K_m and a large reduction in V_{max} were observed with the Kemptide substrate. These results provide experimental support for the proposed role of R221 as having dual functions in substrate binding and in catalysis (Egloff et al., 1995). The dramatic effects on V_{max} on mutation of R221 that are seen with the peptide substrates are consistent with the proposal that R221 is critical for appropriate positioning of the phosphate intermediate during catalysis. In the case of PNPP, the K_m was unchanged with a modest decrease in V_{max}. As noted above, the D208A mutant exhibited significant changes in V_{max} with the peptide substrates, as did the D208N mutant (Table 1). This behavior is more likely explained on the basis of the interaction of D208 with R221 rather than its function as a charged residue. (In addition, D208 is partially buried and is less surface- accessible than the other acidic residues.)

W206 and Y134 have also been proposed to be likely candidates for interaction with the serine or threonine carrying the phosphate residue (Egloff et al., 1995). W206F

Table 1: Kinetic Analysis of PP1 Mutants[a]

	phosphorylase *a*			PNPP			RII			Kemptide		
enzyme	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m
WT	2.8	14000	3200	10	18000	1.1	51	14000	170	140	4600	20
D210A	3.2	16000	3200	11	14000	0.8	52	5000	60	500	3600	4.5
D212A	3.4	15000	2700	11	12000	0.7	73	6000	50	400	2600	4
E218A	3.7	18000	3000	10	14000	0.9	99	11000	67	210	3000	9
D220A	3.3	10000	1900	11	14000	0.8	130	16000	75	200	3600	11
E252A	3.6	5600	1000	10	12000	0.8	120	10000	50	340	2500	4.6
D253N	3.5	11000	2000	10	13000	0.8	95	12000	76	200	4300	13
E256A	2.3	10000	2800	13	19000	0.9	136	16000	76	150	3000	13
E275R	3.6	15000	2600	6	13000	1.3	150	13000	53	110	2100	13
D277G	5.2	17000	2000	14	12000	0.6	120	14000	70	150	760	3
D210N/D212N	3.7	6600	1100	12	10000	0.5	63	8000	80	260	2300	5.6
E252Q/D253N	3.3	11000	2100	11	10000	0.6	140	10000	47	240	4000	10
D208A	19	15000	490	11	15000	0.9	106	180	1	280	9	0.02
D208N	9	6200	440	6	9000	0.9	157	240	1	100	4.3	0.03
R221Q	11	700	40	11	6400	0.4	107	4	0.02	430	25	0.04
W206F	56	500	6	2.4	6300	1.6	470	83	0.1	170	8.3	0.03
N124D	10	4600	280	65	200	0.002	350	200	0.4	610	9	0.01
Y134F	2.6	3100	740	7	4600	0.4	100	1700	10	370	67	0.1
I130A	1.5	1200	480	8	900	0.07	110	2500	15	150	340	1.4
C127S	2.4	2400	620	9	4300	0.3	100	3600	23	170	360	1.3
D197N	3.9	10000	1640	8	10000	0.8	63	15000	150	280	1700	3.8

[a] Kinetic analyses of the mutants were performed as described in Materials and Methods K_m values for phosphorylase *a*, RII and Kemptide are in micromolar, while those for PNPP are in millimolar. V_{max} values for all substrates are in nanomoles of P_i released per minute per milligram of protein. Values for k_{cat}/K_m values are in $(moles)^{-1}$ $(seconds)^{-1} \times 10^{-3}$.

exhibited the largest increase in K_m for phosphorylase *a* observed for any mutant in this study (20-fold) and a 30-fold decrease in V_{max} (Table 1). Changes in K_m for RII were smaller (10-fold increase) and no change in K_m was observed with Kemptide. With PNPP, an interesting result was obtained in that the K_m was decreased about 4-fold. The effects on K_m with phosphorylase *a* and RII are consistent with the proposal that W206 is involved in interaction with the phosphate-bearing moiety (Ser or Thr) and is also consistent with the effect on PNPP binding. In the latter case, substitution of the less bulky aromatic Phe residue may permit a better interaction with the *p*-nitrophenyl moiety. The result was that this mutant exhibited a higher catalytic efficiency than the wild-type enzyme (1.4-fold increase in k_{cat}/K_m). V_{max} values for the polypeptide/peptide substrates were all consistently reduced, the effect being more pronounced for the peptides, similar to results obtained for R221Q and D208A/N mutants.

The Y134F mutant exhibited slightly decreased K_ms for phosphorylase *a* and PNPP, while those for RII and Kemptide were increased 2−3-fold (Table 1). The changes in K_m for the peptide substrates are consistent with the proposal that it is involved in substrate recognition. However, as for mutation of W206, there are significant effects on V_{max}, whose values were modestly decreased for phosphorylase *a* and PNPP (3−5-fold) and reduced 8-fold for RII and 70-fold for Kemptide. Data for the N124D mutation showed that K_ms for phosphorylase *a* and PNPP were increased for all substrates. The V_{max} for PNPP was drastically reduced (ca. 100-fold), while those for the peptide substrates followed a similar pattern as for the R221Q, Y134F, and D208N/A mutants.

Mutation of Hydrophobic Groove Residues. Three residues in the hydrophobic groove were chosen for mutation. The residues that form one side of the hydrophobic groove are Y134, I130, and C127. The latter two were mutated as well as D197, which forms the end of the groove. The I130A mutant exhibited a *decrease* in K_m for phosphorylase *a*, while the K_m with RII was increased and the K_m with Kemptide was unchanged. With the C127S mutant, only the K_m for RII was increased (Table 1). The V_{max} values for I130 and C127S were significantly affected in all cases. Mutation of D197 had no effects on any of the substrates except with Kemptide, where the K_m was increased 2-fold. The lack of general sensitivity of mutation of D197 is consistent with its position at the end of the hydrophobic groove and suggests that it defines the end of this groove (Goldberg et al., 1995).

The properties of the C127S mutant were of additional interest as crystallographic analysis has shown that C127 is oxidized to cysteic acid in the recombinant protein (Goldberg et al., 1995). It has been speculated that this may account for the differences in behavior of the recombinant protein compared to the wild-type enzyme (Goldberg et al., 1995), in particular its Mn^{2+} dependence (Zhang et al., 1992). No differences in Mn^{2+} sensitivity of the C127S mutant were found (not shown).

DISCUSSION

We have systematically mutated residues in the acid groove, the active-site region, and the hydrophobic groove to assess the importance of these structural features in substrate recognition. The selection of mutants was based on the available crystal structures of PP1, which reveal the active site as the nexus of three shallow grooves in PP1 (Goldberg et al., 1995; Egloff et al. 1995). The best substrate for PP1 is phosphorylase *a* , and its phosphorylation site, although very specifically phosphorylated by phosphorylase kinase, carries some similarity to the PKA motif in having three basic residues N-terminal to the phosphoserine. PP1, like PP2A, exhibits a broad specificity that encompasses a number of proteins that are phosphorylated by PKA (Cohen, 1989), and studies of the specificity requirements of PP1, PP2A, and PP2B using model peptides have shown that the presence of basic residues amino-terminal to the phosphoserine/phosphothreonine is a general determinant (Pinna &

Donella-Deana, 1994; Donella-Deana et al., 1994). The two PP1 inhibitors inhibitor 1 (Endo et al., 1996) and DARPP-32 both carry four basic residues N-terminal to the phosphothreonine residue. Binding of peptide/polypeptide substrates to PP1 can be considered to be composed of three elements: interaction of the basic residues N-terminal to the phosphoserine with the acidic residues in the acid groove, binding of the phosphoserine to the active-site region, and interaction of the region C-terminal of the phosphoserine to the hydrophobic groove.

Our findings show that mutation of any of the acidic residues in the groove did not markedly perturb binding of phosphorylase *a*, although effects were observed with the peptide substrates. Strong effects on the kinetic parameters for PP1 were only observed for mutation of residues involved in phosphate binding and orientation at the active site. Thus, it appears that for PP1, the interaction of the N-terminal region of the phosphopeptide substrate is of lesser importance than the binding at the active-site region. The interaction of the basic residues of the substrates may be relatively flexible and may depend on the presence of the negatively charged surface well in the acidic groove (Egloff et al., 1995; Goldberg et al., 1995). That the loss of any one of the acid residues did not produce large effects is in keeping with the proposal that this element of the substrate recognition of PP1 is dictated by a general electrostatic interaction presented by the acidic groove and is in keeping with the broad specificity exhibited by PP1 (Egloff et al., 1995; Goldberg et al., 1995). The basis for our findings that effects are observed on mutation of the acidic residues with the phosphopeptide substrates and not with the protein substrate requires some comment. This can be attributed to the likely possibility that the phosphopeptide region of phosphorylase *a* may have a more favorably constrained orientation as well as the likelihood that the protein substrate has the potential for a more extended interaction with PP1 than the peptide substrates. It has been previously observed that phosphopeptides are much poorer substrates than their parent proteins (McNall & Fischer, 1988; Pinna & Donella-Deana, 1994); similarly, phosphopeptides derived from inhibitor 1 are much less efficient inhibitors (Endo et al., 1996). In addition, our studies show the importance of using peptide substrates as well as the protein substrate in the analysis of substrate recognition by PP1 mutants.

Our findings provide direct experimental support for the proposed roles of R221, D208, W206, and Y134F in substrate binding and catalysis as proposed on the basis of the crystal structure of PP1 (Egloff et al., 1995; Goldberg et al., 1995). The effects of mutation of D208, particularly in relation to its effects on the peptide substrates, are consistent with the proposal that it is important in the positioning of R221. A surprising result of our mutational studies was the large effects on V_{max} observed for mutation of D208 and W206 that are not explicable by its proposed role in substrate binding. The dramatic effects on V_{max} on mutation of W206 and D208 can be understood in the context of their microenvironment. W206 is packed close to R221, D208, S207, and G174. These residues form a cusp into which the imidazole ring of H248 is tucked, such that the plane of the ring is held in its correct orientation (Figure 2). H173 forms another contact with H248. Both H173 and H248 are ligands for one of the metal ions, M2. Thus it appears that W206, R221, D208, S207, and G174 are likely to be critical

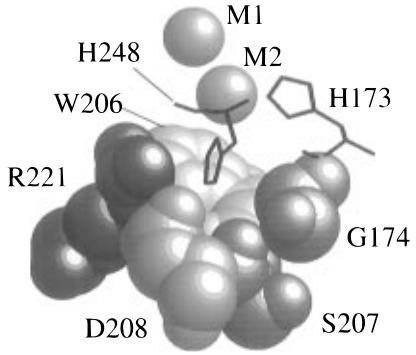

FIGURE 2: Arrangement of R221, D208, and W206 in relation to the imidazole ring of H248. R221, D208, W206, S207, and G174 are shown as CPK images, while H173 and H248 as shown as wireframes. M1 and M2 refer to the two metal ions at the catalytic site (Egloff et al., 1995; Goldberg et al., 1995).

in dictating the angular disposition of the imidazole ring of H248, and also of H173 by virtue of its abutment to H248. W206 is also positioned directly adjacent to N124, which is a third ligand for M2. Of these residues, N124, W206, R221, D208, and H173 are invariant in eukaryotic Ser/Thr protein phosphatases, while S207 is highly conserved, being replaced by Ala in yeast PP2B (Barton et al., 1994).

The mutants in the hydrophobic groove that we studied were C127S, I130A, and D197N. Modest changes in K_m for C127S and I130A were observed, but only for the peptide substrates. Our findings are generally consistent with the view that substrate binding involves the hydrophobic groove (Goldberg et al., 1995) but show that modification of individual residues in the groove did not markedly perturb the binding of the protein substrate.

In summary, our mutational studies provide direct experimental evidence supporting the proposed role of the acid groove in substrate binding and for R221, D208, and W206 in substrate binding and orientation. In particular, the data support the importance of the salt bridge interaction between D208 and R221 and the role of W206 in substrate recognition as proposed by Barford and colleagues (Egloff et al., 1995). In addition, our data also support the view that R221, D208 and W206 are important in its function because they form a architectural element that is important for the appropriate orientation of the histidine metal ligands of M2.

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