The Inositol Polyphosphate 5-Phosphatases and the Apurinic/Apyrimidinic Base Excision Repair Endonucleases Share a Common Mechanism for Catalysis*

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Inositol polyphosphate 5-phosphatases (5-phosphatase) hydrolyze the 5-position phosphate from the inositol ring of phosphatidylinositol-derived signaling molecules; however, the mechanism of catalysis is only partially characterized. These enzymes play critical roles in regulating cell growth, apoptosis, intracellular calcium oscillations, and post-synaptic vesicular trafficking. The UCLA fold recognition server (threader) predicted that the conserved 300-amino acid catalytic domain, common to all 5-phosphatases, adopts the fold of the apurinic/apyrimidinic (AP) base excision repair endonucleases. PSI-BLAST searches of GENPEPT, using the amino acid sequence of AP endonuclease exonuclease III, identified all members of the 5-phosphatase family with highly significant scores. A sequence alignment between exonuclease III and all known 5-phosphatases revealed six highly conserved motifs containing residues that corresponded to the catalytic residues in the AP endonucleases. Mutation of each of these residues to alanine in the mammalian 43-kDa, or yeast Inp52p 5-phosphatase, resulted in complete loss of enzyme activity. We predict the 5-phosphatase enzymes share a similar mechanism of catalysis to the AP endonucleases, consistent with other common functional similarities such as an absolute requirement for magnesium for activity. Based on this analysis, functional roles have been assigned to conserved residues in all 5-phosphatase enzymes.

The phosphoinositide signaling cascade regulates many essential cellular processes including secretion, cellular proliferation, actin polymerization, vesicular and protein trafficking, cell growth, and inhibition of apoptosis (1–5). The inositol polyphosphate 5-phosphatases (5-phosphatases)1 are a large family of enzymes that specifically hydrolyze the 5-position phosphate from the inositol ring from both inositol phosphates and phosphoinositides (5). Nine mammalian enzymes have been cloned and characterized, and four yeast homologues have been identified in Saccharomyces cerevisiae (6–8).

The 5-phosphatases play a significant role in the regulation of many phosphoinositide signaling events and in the pathogenesis of human diseases. Recent characterization of mice or humans lacking functional 5-phosphatase isoforms has identified the role specific 5-phosphatases play in regulating cell growth, post-synaptic vesicular trafficking, and apoptosis. The Src homology 2 domain containing 5-phosphatase SHIP is exclusively expressed in hematopoietic cells. Gene-targeted deletion of SHIP in mice leads to early death from a syndrome that resembles chronic myeloid leukemia (9). In primary cell lines derived from Philadelphia-positive chronic myeloid leukemia patients, the expression of SHIP is reduced or absent (10). The pre-synaptic 5-phosphatase synaptojanin associates with endocytic-coated intermediates and regulates synaptic vesicle recycling. Synaptojanin-deficient mice demonstrate neurological impairment and die shortly after birth (2). Lowe's occulocerebrorenal (OCRL) syndrome is a human disorder characterized by growth and mental retardation, cataracts, and renal failure resulting from loss of function mutations in an X-linked gene (OCRL1), which encodes a phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P2) 5-phosphatase (11). However, mice with gene targeted deletion of OCRL show no phenotype, while 5-phosphatase II-deficient mice show little phenotype with the only noted abnormality being testicular abnormalities. Mice deficient in both OCRL and 5-phosphatase II die in utero (12).

Underexpression of the 43-kDa 5-phosphatase using an antisense strategy results in enhanced intracellular calcium oscillations and cellular transformation (13, 14).

Although the 5-phosphatase enzymes are defined by their ability to remove the 5-position phosphate from the inositol ring, the catalytic mechanism and substrate-binding sites of the 5-phosphatases have yet to be defined. The various isoforms show specificity for discrete substrates, which include the water-soluble inositol phosphates inositol 1,4,5-trisphosphate (Ins (1,4,5)P3) and inositol 1,3,4,5-tetrakisphosphate (Ins (1,3,4,5)P4), and phosphatidylinositol-derived messenger molecules PtdIns (4,5)P2, phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5)P3) and phosphatidylinositol 3,5-bisphosphate (PtdIns (3,5)P2). For example the 43-kDa 5-phosphatase only hydrolyzes Ins (1,4,5)P3 and Ins (1,3,4,5)P4. The molecular mechanisms governing each enzyme’s distinct substrate specificity have not been determined. All 5-phosphatases contain a conserved 300-amino acid central region designated the “5-phosphatase domain,” which demonstrates approximately 30% amino acid identity between family members apart from the 43-kDa 5-phosphatase (5-phosphatase I), which is more exten-

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1The abbreviations used are: 5-phosphatase, inositol polyphosphate 5-phosphatase; OCRL, ocuclocrebrorenal; AP, apurinic/apyrimidinic; PtdIns (4,5)P2, phosphatidylinositol 4,5-bisphosphate; Ins (1,4,5)P3, inositol 1,4,5-trisphosphate; Ins (1,3,4,5)P4, inositol 1,3,4,5-tetrakisphosphate; PtdIns (3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns (3,5)P2, phosphatidylinositol 3,5-bisphosphate; SHIP, SH2-containing inositol 5-phosphatase.

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sively diverged. It has been proposed that two short amino acid motifs, WXC0DRRX and PXWC0DRXL, located 60 amino acids apart in the 5-phosphatase domain, define the enzyme family and contain critical residues for both catalysis and substrate binding (15). Mutation of conserved residues within these two motifs results in loss of enzyme activity (16–18).

In this study, significant amino acid sequence similarities between the 5-phosphatases and apurinic/apyrimidinic (AP) endonucleases have been identified. Furthermore, we have shown the active site residues in the AP endonucleases correspond to absolutely conserved residues in all 5-phosphatases. Mutation of these residues in mammalian and yeast 5-phosphatases results in complete loss of enzyme activity. Based upon these data, we have been able to assign functional roles to conserved residues in the 5-phosphatase family and propose a catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA modifying and restriction enzymes were obtained from New England Biolabs. [32P]Orthophosphate was from PerkinElmer Life Sciences. Oligonucleotides were from Bresatec (Adelaide, Australia) and the Department of Microbiology, Monash University (Victoria, Australia). All reagents used were from Sigma, unless otherwise stated.

**Database Searching**—PSI-BLAST searches (19) of GenBank™ using members of the 5-phosphatase superfamily as probes failed to identify significant sequence similarity to any protein family of known structure. The amino acid sequence of the 5-phosphatase domain from synaptojanin, SHIP1, SHIP2, and Inp52p was submitted to the UCLA fold prediction server (20). In each case the Gnonpreds^+MULT method identified the Escherichia coli AP endonuclease, exonuclease III as the top scoring fold with the endonuclease DNAse I also returning a significant score. Exonuclease III and DNAse I are homologous proteins that share very low (1%) sequence identity (21). We performed further PSI-BLAST searches of GenBank™ (current to April 10, 2000) using the sequence of exonuclease III as a probe and an expect value for inclusion in the next iteration (h) of 0.001. The search was performed to convergence and identified all members of the 5-phosphatase family with highly significant scores (Table I). A search using the sequence of the human homolog of exonuclease III, AP endonuclease I (APE1), as a probe also identified the 5-phosphatase family with highly significant scores.

**Alignments**—Nine members of the 5-phosphatase family (OCRL, the 5-phosphatase II, Inp52p, 43-kDa 5-phosphatase (also called 5-phosphatase I), SHIP1, SHIP2, synaptojanin, pharbin, and the 107-kDa 5-phosphatase (also called PIPP)) were aligned using CLUSTALW (22). The 43-kDa 5-phosphatase is extremely diverged compared with other family members; therefore, pairwise alignments produced by PSI-BLAST align the type I 43-kDa 5-phosphatase to the domains of other sequences. The pairwise alignment between exonuclease III and OCRL produced by PSI-BLAST was used to guide the alignment of exonuclease III to the 5-phosphatases.

**Model Building**—The x-ray crystal structure of exonuclease III (Protein Data Bank identifier 1AKO) was obtained from the protein data bank (21, 23). The program MODELLER (24) was used to build a homology model of the human 5-phosphatase OCRL based upon the structure of exonuclease III. We were able to predict the core structure and active site of human OCRL with confidence; however, no attempt was made to predict the structure of the loops surrounding the active site, since these were extensively diverged from the template structure.

**Site-directed Mutagenesis and Purification of Recombinant Proteins**—Site-directed mutagenesis of the murine 43-kDa and yeast Inp52p 5-phosphatases was performed by using sequential polymerase chain reactions. The sequence of all mutants was verified by dideoxy sequencing of both strands. The wild type and mutant 5-phosphatase recombinant proteins were expressed as His tag fusion proteins in the pTrcHis B (murine 43-kDa 5-phosphatase) and pMalC2 (yeast Inp52p) expression systems in E. coli and purified by Talon affinity chromatography previously (17). Inp52p recombinant proteins contained only the central 5-phosphatase domain and the C-terminal proline rich region (nucleotides 1609–3552), and lacked the N-terminal SacI domain. Recombinant proteins were assessed by SDS-polyacrylamide gel electrophoresis and Coomasie staining, and immunoblot analysis using anti-His tag antibodies. The structural integrity of all wild type and mutant recombinant proteins were assessed by far UV CD spectroscopy using a Jasco 810 spectropolarimeter. Spectra were collected at 20 °C in a 0.01-cm cell using a bandwidth of 1 nm, a scanning speed of 20 nm/min, and a 1-s time response. For each enzyme preparation, the final spectrum is the result of five accumulated scans.

**RESULTS AND DISCUSSION**

PSI-BLAST searches of GenPEPT using members of the 5-phosphatase superfamily as probes failed to identify any significant sequence similarity to any protein family of known structure. In order to identify a putative homologue of known structure, the amino acid sequence of the conserved 5-phosphatase domain from synaptojanin, SHIP1, SHIP2, and yeast 5-phosphatase Inp52p was submitted to the UCLA hybrid fold recognition server. In each case the domain was predicted to adopt an AP base excision repair endonuclease-like fold. In addition, exonuclease III was identified as the top scoring fold when these 5-phosphatase sequences were submitted to the hybrid fold recognition server (data not shown) (20). The AP enzyme family includes the mammalian APE1 endonucleases and E. coli exonuclease III, both of which play a crucial role in the base excision repair pathway, cleaving the DNA sugar phosphate backbone 5' to AP sites to prime DNA repair synthesis (28, 29). The AP endonucleases share a common fold and similar catalytic mechanism to DNAse I, despite extremely low amino acid sequence identity (14%) for exonuclease III versus DNAse I (21, 30, 31).

We performed PSI-BLAST searches of the GenPEPT data bank using the amino acid sequence of exonuclease III as a probe. The search converged in the 9th iteration, and all members of the 5-phosphatase family were identified with expect scores between 3 × 10⁻¹⁴ (5-phosphatase II (INP53B)) and 6 × 10⁻¹⁷ (5-phosphatase I, or 43-kDa 5-phosphatase) (Table I). These are highly significant scores, well below the threshold of 1 × 10⁻⁶ as previously defined by Park et al. (32).

**Pairwise sequence alignments (PSI-BLAST)** were used to construct a multiple sequence alignment between exonuclease III and members of the 5-phosphatase family (Fig. 1). We identified six highly conserved motifs (termed motifs 1–6, respectively), comprising four novel sequences (XW, QXQE, XNH, and SDXHYPV) and the two previously identified “phosphatase motifs” (GDXXRR and PXWXR), which have been used to define the 5-phosphatase enzyme family (15) (Table II and III). Each motif contains amino acids absolutely conserved in all 5-phosphatases that correspond to essential catalytic mechanism.

**Table I**

| Name of 5-phosphatase | Expect score in 9th iteration (convergence) |
|-----------------------|------------------------------------------|
| 5-phosphatase II (75 kDa 5-phosphatase) | 3 × 10⁻⁴⁴ |
| OCRL (human) Lowe’s protein | 4 × 10⁻⁴¹ |
| 107-kDa (PIPP) | 2 × 10⁻⁵⁸ |
| Synaptojanin (human) | 7 × 10⁻⁵⁸ |
| Inp52p (S. cerevisiae) | 1 × 10⁻⁷⁷ |
| SHIP (human) | 4 × 10⁻³¹ |
| Pharbin (rat) | 1 × 10⁻³⁰ |
| SHIP-2 (human) | 7 × 10⁻³⁰ |
| 43-kDa 5-phosphatase (5-phosphatase I) | 6 × 10⁻¹⁷ |
FIG. 1. Sequence alignment between exonuclease III and inositol polyphosphate 5-phosphatases. Multiple sequence alignment between exonuclease III (commencing amino acid 1; GenBank® accession no. P09030), human OCRL (commencing amino acid 317; GenBank® accession no. Q01968), yeast Inp52p (commencing amino acid 593; GenBank® accession no. NP-014293), human 43-kDa 5-phosphatase (commencing amino acid 12, GenBank® accession no. S45721), human 5-phosphatase II (commencing amino acid 296; GenBank® accession no. P32019), human SHIP1 (commencing amino acid 405; GenBank® accession no. NP-005532), human SHIP2 (commencing amino acid 426; GenBank® accession no. JC5765), synaptojanin (commencing amino acid 535; GenBank® accession no. O43426), rat 107-kDa (commencing amino acid 420; GenBank® accession no. BAA90553), and rat pharbin (commencing amino acid 303; GenBank® accession no. BAA82150). The conserved motifs (see also Tables II and III) are numbered above the alignment. Conserved catalytic residues are boxed and are in purple. Hydrophobic residues are shaded yellow, polar non-charged residues are green, acidic residues are red, and basic residues and histidine are blue.
conserved catalytic residues in the AP endonucleases (Table II and Fig. 1).

It had previously been noted that the AP-endonucleases share sequence similarity with the endonuclease domain of long interspersed nuclear element-1 (LINE-1 or L1) retrotransposons and siringomyelinases (36, 37). A more recent sequence analysis identified the conservation of fold and catalytic residues between the Mg$^{2+}$-dependent endonucleases and a variety of signaling proteins including the inositol polyphosphate 5-phosphatases and the yeast carbon catabolite repressor protein Ccr4p (38). Dlakic (38) suggests that these enzymes may share a similar mechanism of catalysis and arose from a common ancestor via divergent evolution. Our sequence alignment is in broad agreement with that of Dlakic; however, in this study we focus upon conservation within all the 5-phosphatase family members with comparison specifically with the AP-endonucleases and investigate via site-directed mutagenesis the role of conserved residues in 5-phosphatase enzyme function (38).

Three high resolution x-ray crystal structures of human APE1 in complex with abasic DNA have been resolved allowing a detailed reaction mechanism for the AP endonucleases to be proposed (33) (Table II). The target phosphate is orientated by a His/Asp pair and a conserved Asn. Nucleophilic attack is initiated via a conserved Asp, which activates a water molecule, forming the attacking nucleophile. Cleavage is achieved via inversion of the phosphate configuration. A conserved Glu coordinates a Mg$^{2+}$ ion that stabilizes the transition state and leaving group. An alignment of all known 5-phosphatases reveals these catalytic residues critical for endonuclease activity correspond to absolutely conserved residues in all 5-phosphatases (data not shown).

It is noteworthy that both the AP endonucleases and 5-phosphatases share an absolute dependence on magnesium ions for activity and both attack a sugar phosphate substrate. We therefore hypothesized that these conserved 5-phosphatase motifs may perform similar catalytic roles to those described for AP endonucleases. To test this hypothesis, we mutated residues in the 43-kDa 5-phosphatase, an Ins (1,4,5)P$_3$ 5-phosphatase, and the yeast Inp52p 5-phosphatase (N478A) to Asp-349 in APE1 (motif 5) and forms a hydrogen bond to scissile phosphate.

**Table II**

| Motif | Sequence | Catalytic role in APE1 |
|-------|----------|-----------------------|
| 1     | TWN      | Asn hydrogen bonds to catalytic Asp in motif 4 |
| 2     | GXXQ    | Glu coordinates Mg$^{2+}$ |
| 3     | NXXH    | His substituted by Tyr in APE1 family but is a conserved catalytic His in related DNase I family |
| 4     | GDXXRX  | Catalytic Asp; Asn hydrogen bonds to scissile phosphate |
| 5     | PXWXXD  | Asp paired with His in motif 6 |
| 6     | SDHXPV  | His paired with Asp in motif 5 and forms a hydrogen bond to scissile phosphate. |

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Secondary structure analysis of the mutant forms compared with wild type 43-kDa enzyme using far UV CD spectroscopy showed no change in the overall structure of any of the mutants (data not shown). We assayed the wild type 43-kDa and Inp52p and all mutant 5-phosphatases for their ability to hydrolyze Ins (1,4,5)P$_3$, or PtdIns (4,5)P$_2$, respectively, and expressed enzyme activity as a percentage of that observed in the wild type enzyme. Mutation of residues that correspond to primary catalytic residues in the AP-endonucleases in either the 43-kDa (D232A in motif 4, and H385A in motif 6), or Inp52p (D476A in motif 4) demonstrated complete loss of Ins (1,4,5)P$_3$ or PtdIns (4,5)P$_2$ 5-phosphatase activity respectively (Figs. 2 and 3). Other conserved residues that we predict play a primary role in enzyme catalysis have been investigated and reported in previous studies (16–18). 5-phosphatase II mutants (N478A) in motif 4 and D553A (corresponds to Asp-349 in the 43-kDa 5-phosphatase) in motif 5 are absolutely essential for 5-phosphatase enzyme activity (18).

A molecular model of the human 5-phosphatase OCRL was built using the crystal structure of exonuclease III as a template (Fig. 4). The majority of residues conserved between the two families map to the core of the structure, enabling the active site to be modeled with confidence. However, no attempt was made to model the loops surrounding the active site since sequence similarity is minimal in these regions. We have predicted functional roles for the conserved motifs in the 5-phosphatases as assigned by analogy with the structure function studies recently reported for human APE1 (Fig. 4) (33). We propose that in the 43-kDa 5-phosphatase His-385 (motif 6) hydrogen bonds to Asp-349 (motif 5) to form a His/Asp pair that together with Asn-234 (motif 4, corresponds to Asn-478 in 5-phosphatase II) orientates the 5-phosphate group of the inositol ring. Asp-232 (motif 4) corresponds to the catalytic Asp in the AP endonucleases. We predict that this residue in the 5-phosphatase enzymes is responsible for generating an attacking nucleophile. Cleavage may be achieved via inversion of configuration, with the Mg$^{2+}$ ion stabilizing the transition state and the Inp51 (1,4)P$_2$ leaving group.

In addition to the primary catalytic residues, we have identified residues within the active site that are absolutely conserved in both 5-phosphatases and AP endonucleases. These include residues in motif 6, Pro-387 and Val-388, which in the 43-kDa 5-phosphatase are absolutely essential for enzyme activity (Fig. 2). The x-ray crystal structure of APE1 reveals the catalytic Asp is orientated by forming a hydrogen bond to the side chain of a conserved Asn (33). The corresponding residue (Asn-18) in the 43-kDa 5-phosphatases is located in motif 1 (Tables I and II). Mutation of this residue in the 43-kDa 5-phosphatase (N18A) results in complete loss of enzyme activity.

**Table II**

| Motif | Sequence | Catalytic role in APE1 |
|-------|----------|-----------------------|
| 1     | TWN      | Asn hydrogen bonds to catalytic Asp in motif 4 |
| 2     | GXXQ    | Glu coordinates Mg$^{2+}$ |
| 3     | NXXH    | His substituted by Tyr in APE1 family but is a conserved catalytic His in related DNase I family |
| 4     | GDXXRX  | Catalytic Asp; Asn hydrogen bonds to scissile phosphate |
| 5     | PXWXXD  | Asp paired with His in motif 6 |
| 6     | SDHXPV  | His paired with Asp in motif 5 and forms a hydrogen bond to scissile phosphate. |
indicating that this residue may play a similar role to that shown for APE1. We noted that, within motif 1, two residues N-terminal to the catalytic Asn is a threonine residue that is conserved in most 5-phosphatases, but not endonucleases. Mutation of this residue in the 43-kDa 5-phosphatase (T16A) had minimal effect on enzyme activity, consistent with our contention this does not represent a catalytic residue. Other mutations in residues outside the putative catalytic motifs such as F392A in the 43-kDa 5-phosphatase not present in endonucleases do not result in significant (more than 50%) loss of enzyme activity (Fig. 2).

The x-ray crystal structures of the AP endonucleases and DNase I have shown that the essential Mg²⁺ ion is coordinated by a conserved Glu (30, 31, 33). We identified the corresponding residue in the 5-phosphatases. Mutation of the proposed magnesium binding residue (E55A or E55Q in motif 1) in the 43-kDa 5-phosphatase, or E631A in the yeast Inp52p resulted in complete loss of enzyme activity. Previous studies have shown enzyme activity of DNase I recombinant protein with this magnesium-binding residue mutated can be recovered using high concentrations of magnesium (35). However, we noted that both the 43-kDa (E55A) and Inp52p (E631A) mutant

![Fig. 2](image2.png)

**FIG. 2.** Hydrolysis of Ins(1,4,5)P³ by wild type and mutant 43-kDa 5-phosphatase. Wild type and mutant 43-kDa 5-phosphatase were expressed and purified. Hydrolysis of Ins[¹³⁵P]₄[¹³⁵P]₅P₃ was measured by extraction of released [³²P]ο₄ using a substrate concentration of 30 μM and three linear protein concentrations in triplicate (26). Enzyme activity is expressed as a percentage of that observed in wild type enzyme (μmol/min/mg of recombinant protein) and is representative of at least four similar experiments. Mutants are as indicated on the bottom. WT, wild type. All enzyme assays were performed using 2.5 mM Mg²⁺ except where indicated.

![Fig. 3](image3.png)

**FIG. 3.** Hydrolysis of PtdIns(4,5)P₂ by wild type and mutant Inp52p. PtdIns(4,5)P₂ 5-phosphatase activity was performed on wild type and mutant Inp52p. Enzyme activity is expressed as nanomoles of PtdIns(4,5)P₂ hydrolyzed/min/mg of recombinant wild type or mutant protein (27). WT, wild type. All enzyme assays were performed using 2.5 mM Mg²⁺ except where indicated.

### Table III

| Motif | 5-Phosphatase | Mutant | Study |
|-------|---------------|--------|-------|
| (1) TWN | 43-kDa | T16A | This study |
| | 43-kDa | N18A | This study |
| (2) GXQE | 43-kDa | E55A | This study |
| | Inp52p | E631A | This study |
| | 43-kDa | E55Q | This study |
| (3) NXH | 43-kDa | H184A | This study |
| | Inp52p | H730A | This study |
| (4) GDXXNR | 43-kDa | D232A | This study |
| | 43-kDa | D232N | This study |
| | Inp52p | D476A | This study |
| 5-Phosphatase II | D476A | Jeffress and Majorus (18); no enzyme activity |
| 5-Phosphatase II | N478A | Jeffress and Majorus (18); no enzyme activity |
| (5) PXWXDR | 5-Phosphatase II | W551A | Jeffress and Majorus (18); decreased enzyme activity |
| | 5-Phosphatase II | D553A | Jeffress and Majorus (18); no enzyme activity |
| | Inp52p | R554A | Jeffress and Majorus (18); no enzyme activity |
| SHIP | D460A | Jeffress and Majorus (18); decreased enzyme activity |
| 43-kDa | R350A | Communi et al. (16); no enzyme activity; increased Kₘ Ins(1,4,5)P₃ |
| (6) SDHXPV | 43-kDa | D384A | This study |
| | 43-kDa | H385A | This study |
| | 43-kDa | P387A | This study |
| | 43-kDa | V388A | This study |
| | 43-kDa | F392A | This study |
| 5-Phosphatase II | W551A | Jeffress and Majorus (18); no enzyme activity |
| 5-Phosphatase II | D553A | Jeffress and Majorus (18); no enzyme activity |
| Inp52p | R554A | Jeffress and Majorus (18); no enzyme activity |
| SHIP | D460A | Jeffress and Majorus (18); decreased enzyme activity |
| 43-kDa | R350A | Communi et al. (16); no enzyme activity; increased Kₘ Ins(1,4,5)P₃ |
| 5-Phosphatase II | W551A | Jeffress and Majorus (18); no enzyme activity |
| 5-Phosphatase II | D553A | Jeffress and Majorus (18); no enzyme activity |
| Inp52p | R554A | Jeffress and Majorus (18); no enzyme activity |
| SHIP | D460A | Jeffress and Majorus (18); decreased enzyme activity |
| 43-kDa | R350A | Communi et al. (16); no enzyme activity; increased Kₘ Ins(1,4,5)P₃ |
5-phosphatases, even in the presence of increasing magnesium concentrations up to 100 mM, demonstrated no recovery of 5-phosphatases, even in the presence of increasing magnesium concentrations up to 100 mM, demonstrated no recovery of enzyme activity (Figs. 2 and 3).

Both the AP endonucleases and DNase I family contain a conserved Asp, one residue N-terminal to the active site His (motif 6). The role of this residue in endonuclease function has been firmly assigned; however, it may play a role in coordinating the Mg$^{2+}$ ion (35). This position corresponds to Asp-384 in the 43-kDa 5-phosphatase (motif 6), which we show in this study is absolutely essential for enzyme activity (Fig. 2).

A significant difference between the AP endonucleases and DNase I is the presence of a second His/Glu pair located within the active site of DNase I, which is proposed to act as a general acid stabilizing the leaving group (30). Mutagenesis of this second His in DNase I has confirmed that this residue is required for catalytic activity (34). Mutagenesis of the glutamine reduced, but did not abolish, catalytic function. Comparison of AP endonucleases and the 5-phosphatase family with the sequence of DNase I reveals that all 5-phosphatases also contain this second absolutely conserved histidine (His-184 in the 43-kDa 5-phosphatase in motif 3). However, analysis of the 5-phosphatase sequence alignment and our molecular model shows that no conserved Glu or Asp is available to pair with His-184. Mutation of H184A in the 43-kDa 5-phosphatase and the corresponding residue in Inp52p (H730A) results in complete loss of catalytic activity (Figs. 2 and 3). The x-ray crystal structure of DNase I reveals that this histidine residue is in close proximity to the O$_p$ atom of the target phosphate. We predict that, in the 43-kDa 5-phosphatase, His-184 in motif 3 hydrogen bonds to and further polarizes the scissile phosphate (see Fig. 4).

In conclusion, we have identified six conserved motifs in the 5-phosphatase family and have shown via site-directed mutagenesis that conserved residues within these motifs are crucial for catalytic activity. Based upon similarity to the AP endonucleases, we have been able to assign functional roles to these conserved motifs and propose a catalytic mechanism for the 5-phosphatase family consistent with our mutagenesis data.

In the absence of high resolution crystallographic data, the use of threading in conjunction with motif-based data base searching has allowed a working model of this important family of enzymes to be proposed, upon which further experiments investigating the substrate specificity can be based.

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