Analysis of the Catalytic and Binding Residues of the Diadenosine Tetraphosphate Pyrophosphohydrolase from Caenorhabditis elegans by Site-directed Mutagenesis*

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The contributions to substrate binding and catalysis of 13 amino acid residues of the Caenorhabditis elegans diadenosine tetraphosphate pyrophosphohydrolase (Ap4A hydrolase) predicted from the crystal structure of an enzyme-inhibitor complex have been investigated by site-directed mutagenesis. Sixteen glutathione S-transferase-Ap4A hydrolase fusion proteins were expressed and their kinetic values determined after removal of the glutathione S-transferase domain. As expected for a Nudix hydrolase, the wild type k\textsubscript{cat} of 23 s\textsuperscript{-1} was reduced by 10\textsuperscript{5}-, 10\textsuperscript{3}-, and 30-fold, respectively, by replacement of the conserved P\textsuperscript{1}-phosphate-binding catalytic residues Glu\textsuperscript{56}, Glu\textsuperscript{52}, and Glu\textsuperscript{102} by Gln. K\textsubscript{m} values were not affected, indicating a lack of importance for substrate binding. In contrast, mutating His\textsuperscript{21} to Val or Ala and Lys\textsuperscript{39} to Met produced 10- and 16-fold increases in K\textsubscript{m} compared with the wild type value of 8.8 μM. These residues stabilize the P\textsuperscript{1}-phosphate. H31V and H31A had a normal k\textsubscript{cat} but K83M showed a 37-fold reduction in k\textsubscript{cat}. Lys\textsuperscript{56} also stabilizes the P\textsuperscript{1}-phosphate and a K36M mutant had a 10-fold reduced k\textsubscript{cat} but a relatively normal K\textsubscript{m}. Thus both Lys\textsuperscript{56} and Lys\textsuperscript{83} may play a role in catalysis. The previously suggested roles of Tyr\textsuperscript{27}, His\textsuperscript{35}, Lys\textsuperscript{39}, and Lys\textsuperscript{81} in stabilizing the P\textsuperscript{2} and P\textsuperscript{3}-phosphates were not confirmed by mutagenesis, indicating the absence of phosphate-specific binding contacts in this region. Also, mutating both Tyr\textsuperscript{76} and Tyr\textsuperscript{121}, which clamp one substrate adenosine moiety between them in the crystal structure, to Ala only increased K\textsubscript{m} 4-fold. It is concluded that interactions with the P\textsuperscript{4}, and P\textsuperscript{3}-phosphates are minimum and sufficient requirements for substrate binding by this class of enzyme, indicating that it may have a much wider substrate range than previously believed.

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Ap\textsubscript{4}A\textsuperscript{1} hydrolases are enzymes that hydrolyze dinucleoside polyphosphates. Structurally and mechanistically, they fall into two groups. The symmetrically cleaving enzymes (EC 3.6.1.41), such as Escherichia coli ApaH, generate 2-ADP from Ap\textsubscript{4}A, whereas the asymmetrically cleaving enzymes (EC 3.6.1.17) produce AMP and ATP (1, 2). The latter are members of the Nudix hydrolases, a family of structurally and catalytically similar enzymes that act upon a wide range of different nucleotide substrates. Some are highly specific whereas others appear to have a broad substrate range in vitro (3–5). The Nudix Ap\textsubscript{4}A hydrolases can be further subdivided into “plant” and “animal”-types, according to their primary structure (6).

The plant-type includes enzymes from the Proteobacteria that have in some cases been shown to be associated with the invasion of mammalian cells, whereas the animal-type includes putative Ap\textsubscript{4}A hydrolases from Archaea (6–10). Early studies of both animal and plant Ap\textsubscript{4}A Nudix hydrolases employing a combination of substrate analogues and labeling with heavy isotopes of oxygen revealed the mechanism of hydrolysis to involve in-line nucleophilic attack of a water molecule at the P\textsuperscript{4} (P\textsuperscript{1}O\textsuperscript{4}) phosphate with subsequent breakage of the P\textsuperscript{4}-O\textsuperscript{P} bond (8, 11–14). Recently, the catalytic residues of the lupin Ap\textsubscript{4}A hydrolase involved in this process were identified by a combination of structural analysis and site-directed mutagenesis (15–17). This study supported the catalytic mechanism previously described in detail for the prototypical Nudix hydrolase, the E. coli MutT 8-oxo-dGTPase.

Detailed structural studies of E. coli MutT first showed the importance of the highly conserved residues in the loop-helix-loop Nudix motif (Fig. 1). Glu\textsuperscript{53}, Glu\textsuperscript{56}, Glu\textsuperscript{52}, and Glu\textsuperscript{58} (outside the linear motif but structurally close), and the carbonyl of Gly\textsuperscript{58} coordinate an enzyme-bound Mg\textsuperscript{2+} ion. A water ligand of this ion is oriented or deprotonated for nucleophilic attack by Glu\textsuperscript{53}, which is itself oriented by Arg\textsuperscript{52}. A second metal ion is complexed to the substrate and neutralizes the charge on the attacked phosphate while Lys\textsuperscript{89} activates the NMP leaving group (A–B1). The importance of Glu\textsuperscript{57} was indicated by a 10\textsuperscript{5}-fold reduction in k\textsubscript{cat} in an E57Q mutant (19). The contributions of the other residues to catalysis were also confirmed by site-directed mutagenesis: E59Q, E56Q, and E44Q led to 10\textsuperscript{4.7-}, 10\textsuperscript{3}-, and 30-fold decreases in k\textsubscript{cat}, respectively (20), whereas K39Q and R52Q produced 8-fold and >10\textsuperscript{3}-fold reductions, respectively (22). The principle of this catalytic mechanism

1 The abbreviations used are: Ap\textsubscript{4}A, diadenosine 5’-P\textsuperscript{1}-P\textsuperscript{4}-tetraphosphate; AppCH\textsubscript{2}ppA, diadenosine 5’-P\textsuperscript{2}-P\textsuperscript{4}-methylene-P\textsuperscript{3}-P\textsuperscript{3}-tetraphosphate; Nudix, nucleoside diphosphate linked to X; GST, glutathione S-transferase.
appears to be well conserved among the Nudix hydrolases, including the lupin and *Bartonella bacilliformis* Ap4A hydrolases (15–17, 25), human MTH1 (24), yeast Dcp2p (25), and human NUDT3 (DIPP1) (26).

Among the asymmetrically cleaving Ap4A hydrolases, identification of residues responsible for substrate binding as well as catalysis is of interest for two reasons. First, it will help our understanding of the evolution of substrate specificity among the Nudix hydrolases. Second, if the plant-type Ap4A hydrolase of invasive pathogenic bacteria is to be considered as a target for new antibacterial agents, the design of such agents will require knowledge of the subtle differences between the plant and animal types if selectivity is to be achieved. Recently we reported the crystal structure of an animal Ap4A hydrolase from the nematode *Caenorhabditis elegans* in both free form and after crystallization in the presence of the substrate analogue, AppCH2ppA (27, 28). The structure of the resulting binary complex allowed some predictions to be made about the importance of certain residues for substrate binding and catalysis and comparisons to be drawn with the lupin enzyme. Here, we extend these studies to include the effects of 19 site-specific mutations on Ap4A binding and hydrolysis by the *C. elegans* enzyme.

**EXPERIMENTAL PROCEDURES**

**Synthesis of* C. elegans* First Strand cDNA Library**—Total RNA was isolated and purified from washed adult nematodes (*C. elegans* strain N2) using Trizol solution (Invitrogen) according to the manufacturer’s instructions. Full-length *C. elegans* first strand cDNA was synthesized from this RNA using a first strand cDNA synthesis kit (MI Fermen-
tas). RNA (2 µl of 2.5 µg/µl) was added to 10 µl of RNase-free dH2O. The solution was mixed gently, incubated at 70 °C for 5 min, and chilled on ice for 3 min before adding to a mixture containing 4 µl of 5× reaction buffer (250 mM Tris-HCl, pH 8.3, at 25 °C, 375 mM KCl, 15 mM MgCl₂, 5 mM dithiothreitol), 1 µl of ribonuclease inhibitor (20 units/µl), 2 µl of oligo(dT)₁₈ (0.5 µg/µl), 2 µl of dNTPs (10 mM each), and 2 µl of Moloney murine leukemia virus reverse transcriptase (20 units/µl, Promega). The reaction was incubated at 42 °C for 1 h and then heated to 90 °C for 5 min. The library was stored at –20 °C.

**Cloning of* C. elegans* Ap4A Hydrolase as a Glutathione S-Transferase (GST) Fusion Protein**—A cDNA corresponding to the *C. elegans* Y37H9A.6 Ap4A hydrolase gene (6) was amplified from the cDNA library by PCR using the forward primer d(CAGCCGCGATGTTGCTGGTATGAAGCCCGCGG) and d(GAAATTCCTCGAGAAGATTTGCTATTCCGCGC), respectively. These primers provided an EcoRI restriction site at the start of amplified cDNA and a Xhol site at the end. After amplification with Taq DNA polymerase, the DNA was recovered, digested with EcoRI and Xhol, and the required restriction fragment ligated between the EcoRI and Xhol sites of the pGEX-6P-3 vector (Amersham Biosciences). The resulting construct, pGEX-Y37H9A.6, encoded the 137-amino acid Ap4A hydrolase fused to the 25 terminus of GST through a 6-aminocaproic acid linker.

**Generation of Site-specific Mutants**—Site-directed mutagenesis was performed by PCR using the QuickChange™ site-directed mutagenesis kit (Stratagene). PCR reactions contained pGEX-Y37H9A as template, Pfu Turbo DNA polymerase, and pairs of complementary oligonucleo-
tide primers 37 to 43 nucleotides long containing the required mutations (Table 1). Each reaction volume was 50 µl and contained the following: 50–100 ng of plasmid DNA, 125 ng of each mutagenic primer, 200 µM dNTPs, 10 mM KCl, 6 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 0.1% Triton X-100, 10 µg/ml bovine serum albumin, and 2.5 units of Pfu Turbo DNA polymerase. The PCR reaction protocol consisted of 2 min at 95 °C followed by 16 cycles of 95 °C for 1 min, 55 °C for 1 min, 68 °C for 14 min, followed by a final incubation at 72 °C for 15 min. Parental DNA was digested with 10 units of DpnI to degrade the methylated parental strands and the remaining plasmid DNA was used to transform *E. coli* XLI-Blue cells. For production of the Y76A/ Y121A double mutant, the Y76A DNA construct was used as template in a PCR containing the Y121A mutagenic primers (Table 1). The identities of all mutants were verified by complete sequencing of both DNA strands.

**Expression and Purification of* GST*-Ap4A Hydrolase Fusion Proteins**—*E. coli* strain BL21(DE3) was transformed with pGEX-Y37H9A or its mutant derivatives. Cultures (250 ml) in LB medium containing 50 µg/ml ampicillin were grown to an *A*₅₅₀ of 0.7 at 37 °C. Isopropyl-1-thio-β-δ-galactopyranoside was added to 1 mM and incubation continued for 2 h. Induced cells (approximately 1.6 g) were harvested by centrifugation at 10,000 × *g*, washed, and resuspended in 10 ml of ice-cold breakage buffer: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 µM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64, Sigma). Cell suspensions were sonicated and the resulting lysates cleared by centrifugation at 15,000 × *g* and 4 °C for 10 min. Supernatants were recovered and applied to columns containing 2.5 ml of glutathione-SEpharose 4B (Amersham Biosciences). Columns were washed with 25 ml of phosphate-buffered saline, followed by 25 ml of PreScission cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol). Following complete elution of the buffer, the outlets were closed and 100 units of PreScission protease in 2.5 ml of cleavage buffer added to the resin and incubated for 18–20 h with gentle rocking at 4 °C. Cleavage of the GST domain from the Ap4A hydrolases was complete after 20 h. Columns were remounted, the resin left to settle, and the free Ap4A hydrolases containing the N-terminal extension GPLGLSPNS eluted.

**Ap4A Hydrolase Assay**—Ap4A hydrolase activity was measured using a luciferase-based bioluminescence assay as previously described (6). One ng enzyme protein was used in each case, except for K83M (10 ng), K79M (20 ng), E92Q and E109Q (60 ng), and E56Q (600 ng). This sensitive, continuous assay permits direct evaluation of initial rates. The increase in luminescence was linear for several minutes for each enzyme.

**Other Methods**—Protein concentrations were estimated by the Coomassie Blue binding method (29) and protein molecular masses were determined by electrospray mass spectrometry as previously described (30).

**RESULTS**

**Expression and Purification of Wild Type and Mutant GST- Ap4A Hydrolase Fusion Proteins**—A cDNA corresponding to the *C. elegans* Y37H9A.6 Ap4A hydrolase gene was amplified from a cDNA library by PCR and inserted into the pGEX-6P-3 GST fusion vector to generate the recombinant plasmid pGEX-Y37H9A. When *E. coli* BL21(DE3) cells were transformed with this plasmid and induced with isopropyl-1-thio-β-δ-galactopy-
ranoside, a major soluble 43-kDa band corresponding to the expected GST-Ap4A hydrolase fusion protein was detected (data not shown). The GST domain of this protein was readily removed by on-column cleavage with PreScission protease, resulting in the free Ap4A hydrolase containing the N-terminal extension GPLGLSPNS and mass 16.6 kDa.

Specific mutations were introduced into the Ap4A hydrolase coding region of pGEX-Y37H9A by PCR (Table 1). The mutations were confirmed by DNA sequencing. A total of 16 single mutants and one double mutant involving 13 different residues was generated in this way. Their positions in the primary structure of the Ap4A hydrolase are shown in Fig. 2a and the locations of their α-carbon atoms in the three-dimensional structure of the binary complex are shown in Fig. 2b. Each was expressed as a GST fusion protein and purified after on-column cleavage and elution as described for the wild type. The predicted masses of the cleaved proteins were confirmed by mass spectrometry. With the exception of five (K36M, Y76A, Y76A/ Y121A, K79M, and W32G) mutant proteins were substantially expressed in the soluble fraction (at least 40% of the total

**Figure 1.** The consensus Nudix motif and the actual motifs of *E. coli* MutT protein, *C. elegans* (Ce) Ap4A hydrolase, and *Lupinus angustifolius* (La) Ap4A hydrolase. The numbers indicate the positions in each primary structure.
C. elegans Diadenosine Tetraphosphatase

Table I

| Mutation | Primer name | Primer sequences | Length |
|----------|-------------|-----------------|--------|
| Y27A     | Y27A upstream | CTCCCTGCTTCAAGCCACACACATCGACGCACCC | 41     |
| TAT → GCG | Y27A downstream | GGTTGACCTGATGATGATGTCAGGTCCAGGGAAG | 41     |
| Y27D     | Y27D upstream | CTCCTGCTTCAAGCCACACATCGACGCACCC | 41     |
| TAT → GAC | Y27D downstream | GGTTGACCTGATGATGATGTCAGGTCCAGGGAAG | 41     |
| H31A     | H31A upstream | GCTTCAATCCACACATGCTGAGCACCACCAAAAGGTC | 40     |
| CAC → GCC | H31A downstream | GACCTTTCAGGTTGTGGATGGTTAGAGG | 40     |
| H31V     | H31V upstream | GCTTCAATCCACACATGCTGAGCACCACCAAAAGGTC | 40     |
| CAC → GTC | H31V downstream | GACCTTTCAGGTTGTGGATGGTTAGAGG | 40     |
| W32G     | W32G upstream | CTTATCCACACATCGGACCACCCCAAAAGGTC | 40     |
| TGG → GGG | W32G downstream | CTTATCCACACATCGGACCACCCCAAAAGGTC | 40     |
| K36M     | K36M upstream | CACCCACATCAGGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| AAA → ATG | K36M downstream | CACCCACATCAGGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| H38G     | H38G upstream | CACTGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| CAC → GGG | H38G downstream | CACTGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| H38K     | H38K upstream | CACTGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| CAC → AAG | H38K downstream | CACTGACCACCAAAAGGTCACGTTGACGCCATG | 41     |
| E52Q     | E52Q upstream | GCCAATTCGTGAGACTAAGGAACAAGCAATATTAACAAAG | 38     |
| GAG → CAG | E52Q downstream | GCCAATTCGTGAGACTAAGGAACAAGCAATATTAACAAAG | 38     |
| E56Q     | E56Q upstream | GCCAATTCGTGAGACTAAGGAACAAGCAATATTAACAAAG | 38     |
| GAA → CAA | E56Q downstream | GCCAATTCGTGAGACTAAGGAACAAGCAATATTAACAAAG | 38     |
| Y76A     | Y76A upstream | GAATGGCAGGCGGCAATTCGTCAGACTAAGGAAGAAGC | 40     |
| TAT → GCT | Y76A downstream | GAATGGCAGGCGGCAATTCGTCAGACTAAGGAAGAAGC | 40     |
| K79M     | K79M upstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| AAA → ATG | K79M downstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| K81M     | K81M upstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| AAG → ATG | K81M downstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| K83M     | K83M upstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| AAA → ATG | K83M downstream | GAGGCAAAAGGGGAAGCCAATGTCAGTGAAATATTGGC | 37     |
| E103Q    | E103Q upstream | CAGAGCTTCAATCTCTCTTCATCAACATGGAAATATTGGC | 40     |
| GAA → CAA | E103Q downstream | CAGAGCTTCAATCTCTCTTCATCAACATGGAAATATTGGC | 40     |
| Y121A    | Y121A upstream | GATGCTATCAAAATTGCCGATGCCGCTGAAATGGGCAGCC | 57     |
| TAC → GCC | Y121A downstream | GATGCTATCAAAATTGCCGATGCCGCTGAAATGGGCAGCC | 57     |

![Diagram](image)

**Nudix motif sequence**

Amino acid residues of the C. elegans Ap4A hydrolase changed by site-directed mutagenesis. A, the positions of residues in the amino acid sequence; conserved residues of the Nudix motif, residues which were mutated, and the resulting mutations are shown in **bold**. Numbers indicate the positions in the primary sequence. B, the locations of the α-carbon atoms of the mutated residues in the three-dimensional structure of the binary complex determined to a resolution of 1.8 Å (27) are indicated. The positions of the bound adenosine moiety (Ado), the P^γ- and P^δ-phosphates and the four bound Mg^{2+} ions are also shown.

FIG. 2. Amino acid residues of the C. elegans Ap4A hydrolase changed by site-directed mutagenesis. A, the positions of residues in the amino acid sequence; conserved residues of the Nudix motif, residues which were mutated, and the resulting mutations are shown in **bold**. Numbers indicate the positions in the primary sequence. B, the locations of the α-carbon atoms of the mutated residues in the three-dimensional structure of the binary complex determined to a resolution of 1.8 Å (27) are indicated. The positions of the bound adenosine moiety (Ado), the P^γ- and P^δ-phosphates and the four bound Mg^{2+} ions are also shown.

Expressed recombinant protein) and the yields of purified proteins were nearly the same as for the wild type. The first four exceptions yielded about 5% of the recombinant protein in a soluble form, whereas W32G was completely insoluble when expressed. All kinetic data were determined using enzymes nearly the same as for the wild type, indicating the stability of the mutants under assay conditions. *K*ₘ and *k*ₐₙ values were then determined for each mutant enzyme to determine the effects of each mutation on substrate binding and catalysis (Table II). For this enzyme, *K*ₘ can be taken to approximate the dissociation constant of the ES complex(es), and hence as an inverse measure of affinity, based on the lack of effect of active site (*k*ₐₙ) mutants on the value of *K*ₘ. Previous mutational studies with Nudix hydro- lases have highlighted the importance of the Glu residues within the Nudix motif for catalysis (Fig. 1) (16, 20, 25, 26, 31, 32). Not surprisingly, therefore, the E56Q mutation was found to result in a 10^5-fold reduction in *k*ₐₙ and virtual abolition of detectable enzyme activity, exactly as was found for the equivalent residue (Glu^205) in the lupin Ap4A hydrolase; in contrast, the *K*ₘ was unaffected, indicating that the mutation has no...
effect on substrate binding. Similarly, neutralization of the charge on Glu52, the second of the three highly conserved Glu residues within the Nudix motif, by conversion to Gln (E52Q) reduced k$_{cat}$ by a factor of 10$^3$ but again had little effect on K$_m$ (Table II). On the basis of the 10$^3$-fold reduction in k$_{cat}$, we previously proposed that Glu52 was most likely to be the catalytic base that deprotonates the attacking water molecule. However, the structural equivalents of Glu52 in the lupin Ap4A hydrolase (Glu55) and in the E. coli MutT protein (Glu53) have been proposed as the deprotonating base (11, 16, 20). Glu103, although not in the Nudix motif, is positioned close to it in the three-dimensional structure and coordinates two of the four Mg$^{2+}$ ions located in the catalytic site (27). E103Q has a 30-fold lower k$_{cat}$ than the wild type and a similar K$_m$. The equivalent mutations in E. coli MutT (E98Q) and the lupin Ap4A hydrolase (E125Q) produced 6.3- and 140-fold reductions in k$_{cat}$, respectively (16, 20). Whereas these values suggest that Glu103 and its equivalents are unlikely to be the catalytic base in these enzymes, a detailed structural analysis of E. coli MutT protein and/or Lys53 and/or Lys55 could neutralize the developing negative charge on the ATP leaving group, in much the same way as has been proposed for MutT Lys39 (18, 20). Therefore, appropriate mutants were generated to test these suggestions. Surprisingly, of the mutants analyzed (Y27A, Y27D, H38G, H38K, K79M, and K81M), only K79M showed a significant change in any kinetic constant, a 31-fold increase in K$_m$ and the 2.5-fold higher K$_{cat}$. However, Lys79, like Tyr76, is well conserved as a basic residue in animal and plant Ap4A hydrolases. However, its mutation to Met resulted in a 30 s$^{-1}$ increase in k$_{cat}$ (10-fold) but a relatively normal K$_m$. These results confirm the predictions of the crystal structure and indicate the importance of Lys36 and Lys83, which is positioned such that it could also stabilize the P$^3$-phosphate, to catalysis (27).

As there was no interpretable electron density for either the P$^2$ or P$^3$-phosphates in the binary complex, it was suggested that the side chains of His53 (within the Nudix motif) plus Lys79, Lys81, and Tyr27 (outside the Nudix motif) might be in appropriate positions to participate in P$^2$- and P$^3$-phosphate stabilization either by direct interaction or by metal coordination. The main chain amide of His53 is also the only direct protein contact with P$^4$ via a hydrogen bond to one of the oxygen atoms (27). Potentially, His53 (structurally equivalent to Lys53 in the E. coli MutT protein) and/or Lys53 and/or Lys81 could neutralize the developing negative charge on the ATP leaving group, in much the same way as has been proposed for MutT Lys39 (18, 20). Therefore, appropriate mutants were generated to test these suggestions. Surprisingly, of the mutants analyzed (Y27A, Y27D, H38G, H38K, K79M, and K81M), only K79M showed a significant change in any kinetic constant, a 140-fold reduction in k$_{cat}$. However, Lys79, like Tyr76, is not well conserved among the Ap4A hydrolases, so this reduced activity may reflect a slight structural alteration in the protein rather than an important catalytic role. In contrast, Lys81 is well conserved as a basic residue in animal and plant Ap4A hydrolases. However, its mutation to Met resulted in a slight increase in k$_{cat}$ to 30 s$^{-1}$, so it seems unlikely to be involved in stabilizing the leaving group. H38G and H38K also showed slight increases in k$_{cat}$ such that the k$_{cat}$/K$_m$ ratio was 2.5-fold higher than the wild type. The equivalent residue in plant Ap4A hydrolases is a Gly, so, unlike Lys81 in MutT, His53 does not appear to be important for catalysis either. As all other residues in the region are too small to make contact, the conclusion is that there are few, if any, structurally or mechanistically important binding contacts for the P$^2$- and P$^3$-phosphates. This interesting point is discussed further below.

Finally, Trp32 is a completely conserved residue among Ap4A hydrolases of the Nudix family and is commonly found in other family members. This residue does not form interactions with the substrate but appears to stabilize the protein fold through interactions with Leu29 and Gln24 in the βB strand and with
Ile\textsuperscript{118} in the dII helix (27). Consistent with this essential structural role is the fact that the W32G mutant was completely insoluble and inactive when expressed.

**Discussion**

The results of this analysis confirm the importance of residues previously implicated by structural analysis in binding and catalysis at the P\textsuperscript{4}-phosphate and binding of the P\textsuperscript{3}-phosphate of Ap\textsubscript{4}A by the C. elegans Ap\textsubscript{4}A hydrolase. It also provides important information on two further aspects of substrate binding by this enzyme that have implications for substrate recognition in the Nudix hydrolase family as a whole. First, there appear to be few, if any, important binding contacts for the P\textsuperscript{2}- and P\textsuperscript{3}-phosphates. Our previous structural analysis was unable to provide this information. It is possible that a nucleotide-bound metal ion is more important for stabilization of the negative charge during catalysis than any individual nucleotide-bound metal ion is more important for stabilization of the adenine ring between the structurally equivalent residues Tyr\textsubscript{77} and Phe\textsubscript{144}, as 5-phosphoribosyl 1-pyrophosphate and diphosphoinositol polyphosphates and the flexibility of substrate binding noted above suggest that the substrate range and function of Nudix hydrolases may be much wider than previously believed.

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