Characterization of active-site residues in diadenosine tetraphosphate hydrolase from *Lupinus angustifolius*

Danuta MAKSEL*, Paul R. GOOLEY*, James D. SWARBRICK*, Andrzej GURANOWSKI*†, Christine GANGE*, G. Michael BLACKBURN‡ and Kenwyn R. GAYLER*¹

*Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia, †Department of Biochemistry and Biotechnology, Agricultural University, 35 Wolynska Street, 60-637 Poznan, Poland and ‡Krebs Institute, Department of Chemistry, The University of Sheffield, Sheffield S3 7HF, U.K.

Site-directed mutagenesis has been used to characterize the functions of key amino acid residues in the catalytic site of the 'nudix' hydrolase, (asymmetrical) diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) hydrolase (EC 3.6.1.17) from *Lupinus angustifolius*, the three-dimensional solution structure of which has recently been solved. Residues within the nudix motif, Gly-(Xaa)₅-Glu-(Xaa)₇-Arg-Glu-Uaa-Xaa-(Glu)₂-Xaa-Gly (where Xaa represents unspecified amino acids and Uaa represents the bulky aliphatic amino acids Ile, Leu or Val) conserved in 'nudix enzymes', and residues important for catalysis from elsewhere in the molecule, were mutated and the expressed proteins characterized. The results reveal a high degree of functional conservation between lupin asymmetric Ap₄A hydrolase and the 8-oxo-dGTP hydrolase from *Escherichia coli*. Charged residues in positions equivalent to those that ligate an enzyme-bound metal ion in the

E. coli 8-oxo-dGTP hydrolase [Harris, Wu, Massiah and Mildvan (2000) *Biochemistry* 39, 1655–1674] were shown to contribute to catalysis to similar extents in the lupin enzyme. Mutations E55Q, E59Q and E125Q all reduced *k*_{cat} markedly, whereas mutations R54Q, E58Q and E122Q had smaller effects. None of the mutations produced a substantial change in the *K*_m for Ap₄A, but several extensively modified the pH-dependence and fluoride-sensitivities of the hydrolase. It was concluded that the precisely positioned glutamate residues Glu-55, Glu-59 and Glu-125 are conserved as functionally significant components of the hydrolytic mechanism in both of these members of the nudix family of hydrolases.

Key words: Ap₄A, catalytic site, kinetics, mutagenesis, nudix.

INTRODUCTION

It has recently been suggested [1,2] that enhanced ability for the asymmetric hydrolysis of diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A) into AMP and ATP is a characteristic of some invasive bacteria that increases the chances of their survival. Disruption of Ap₄A hydrolases may, therefore, offer a potential method of therapy of diseases caused by such bacteria. In this context we have been studying the mechanism of action of an asymmetric Ap₄A hydrolase from lupin (*Lupinus angustifolius* L.) [3]. Lupin Ap₄A hydrolase has particularly high sequence identity with the Ap₄A hydrolase encoded by *ialA*, one of two genes associated with the ability of *Bartonella bacilliformis* to invade and survive as a parasite of human erythrocytes [4]. The three-dimensional structure of lupin Ap₄A hydrolase has recently been solved [5,6] and compared with the structure of *Escherichia coli* MutT [7–9] (Figure 1). Asymmetric Ap₄A hydrolases and *E. coli* MutT, an 8-oxo-dGTP hydrolase, both belong to the nucleoside diphosphate linked to another moiety, X (nudix) superfamily of nucleotide hydrolases [10], and are the only members of this family for which three-dimensional structures have been determined.

There are considerable similarities between the structures of these two nudix enzymes, particularly in the region of the proteins containing the conserved nudix motif, Gly-(Xaa)₅-Glu-(Xaa)₇-Arg-Glu-Uaa-Xaa-(Glu)₂-Xaa-Gly [6]. In the present paper, the functions of particular amino acid residues in the lupin Ap₄A hydrolase were investigated using site-directed mutagenesis. The effects of mutation of amino acids both within and outside of the nudix motif of lupin Ap₄A hydrolase were analysed.

EXPERIMENTAL

Origin of clones

cDNA encoding the asymmetric Ap₄A hydrolase from *L. angustifolius* was retrieved from plasmid pAH-7 [3] using the primers 5'-GAAGATCTATGGATTCTCCTCCCGAAGG-3' and 5'-GG-AAGCTTATTAGAGATGTGGAGCAAAC-3' to copy bases 139–625 from clone AH-7 and concurrently introduce recognition sequences for *Bgl*II and *Hind*III at the 5'- and 3'- ends of the clone respectively. The resultant PCR fragment, AH-8, which encoded amino acids Met¹⁰ to Leu¹⁹⁹ of the asymmetric Ap₄A hydrolase, was directionally cloned between *Bam*HI and *Hind*III sites in vector pMAL-c2 (New England Biolabs) for expression.

As previously described [3], residues Met¹⁰ to Leu¹⁹⁹ of lupin Ap₄A hydrolase AH-7, subcloned as protein AH-8, appear equivalent to the Ap₄A hydrolase as purified from the plant [3]. Met¹⁰ to Leu¹⁹⁹ from protein AH-7 are numbered as residues 1–160 in protein AH-8. Unless otherwise specified, amino acids are numbered with respect to protein AH-8 throughout this study.

Expression and purification of Ap₄A hydrolase

To produce pure Ap₄A hydrolase for the present study, protein AH-8 was expressed either as a fusion protein with maltose-binding protein as previously described [3], or its DNA was cloned into *Eco*RI/*Xho*I or *Bam*HI/*Xho*I sites in pGEX-6P-3 vector for expression as a fusion protein with glutathione S-transferase (GST). Between the GST domain and the multiple

Abbreviations used: Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetraphosphate; GST, glutathione S-transferase; nudix, nucleoside diphosphate linked to another moiety, X; *E. coli* MutT, *E. coli* 8-oxo-dGTP hydrolase; MALDI-TOF-MS, matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry.

¹ To whom correspondence should be addressed (e-mail ken@biochemistry.unimelb.edu.au).

Figure 1 Three-dimensional structures of MutT protein from *E. coli* and Ap₄A hydrolase from lupin

The three-dimensional structures of 8-oxo-dGTP hydrolase from *E. coli* (Brookhaven Protein Data Bank Accession number 1MUT; [28]) and Ap₄A hydrolase from lupin (Research Collaboratory for Structural Bioinformatics Protein Data Bank Accession number 1F3Y; [6]) are compared. The positions of side chains of residues demonstrated to be important for the activity are highlighted. Glutamate-46, whose mutation to glutamine did not affect the activity of lupin Ap₄A hydrolase, is also shown for comparison purposes.

cloning site, pGEX-6P-3 encodes the recognition sequence Leu-Gly-Val-Leu-Phe-Gln-Gly-Pro for site-specific cleavage by PreScission protease (Pharmacia), that itself is a fusion protein of GST and human rhinovirus 3C protease. After affinity purification on GSH-Sepharose 4B (Pharmacia) and cleavage from GST with PreScission protease, Ap₄A hydrolase and its mutants were produced with either five or eight amino acid extensions (Gly-Pro-Leu-Gly-Ser- or Gly-Pro-Leu-Gly-Ser-Pro-Asn-Cys-) on the N-terminal end of sequence AH-8 as indicated in the text.

For NMR analysis, Ap₄A hydrolase was prepared with ¹⁵N labelling, as described in Swarbrick et al. [5,6].

Site-directed mutagenesis

Site-directed mutagenesis was performed using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Pairs of complementary oligonucleotides 35–41 bases in length, with a ‘melting’ temperature of 75–78 °C and containing the desired mutation, were used to prime the replication of circular plasmid DNA by *Pfu* or *Pfu* turbo DNA polymerase. The PCR reaction volume was 50 µl and contained the following: 50–150 ng plasmid DNA, 11 pmol of each mutagenic oligonucleotide 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 BSA and 2.5 units of *Pfu* DNA polymerase. The reaction was subjected to an initial heating step of 30 s at 95 °C, and 20 rounds of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 19 min in a GeneAmp® PCR System 2400 (PerkinElmer). Parental DNA was digested with 10 units of *DpnI* endonuclease to remove the methylated parent strands, and synthesized plasmid DNA was transformed into *E. coli* XL1-Blue cells. The identity of all mutants was confirmed by complete sequencing of both DNA strands by dye terminator cycle sequencing.

Determination of molecular mass

Molecular masses were determined by linear matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF–MS) with 2,5-dihydroxybenzoic acid as a matrix.

CD

CD spectra were obtained on an AVIV CD spectrometer (model 62 DS). Protein samples were prepared in 20 mM potassium phosphate, pH 7.4. Spectra were recorded at room temperature over the range 190–250 nm at 0.2 nm intervals, with a time constant of 1 s. The baseline spectrum obtained for the buffer alone was subtracted. CD data were converted to the mean residue ellipticity, $[\theta]$ in deg cm²/dmol. Secondary structure predictions were made using the computer program PROSEC, based on the method of Yang et al. [11].

Ap₄A hydrolase activity assay

Formation of AMP and ATP from Ap₄A was assayed by HPLC, as described by Guranowski et al. [12]. Reaction mixtures containing 50 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 % (w/v) BSA and 0.5 mM Ap₄A were incubated at 25 °C with limiting amounts of enzyme to ensure linear rates of reaction. Products of hydrolysis were separated by HPLC using a 1 ml Mono Q column (Pharmacia) and a linear gradient from 0.05 to 0.5 M NH₄HCO₃. Nucleotide levels were determined by integration of peak areas detected at 254 nm.

K_m values were determined from the rates of hydrolysis of different concentrations of [³H]Ap₄A. For example, between 0.15 µM and 2.5 µM for mutant E55Q and between 1.5 µM and 50 µM for mutant E59D. The *I*₅₀ values for fluoride inhibition were determined in mixtures containing 100 µM [³H]Ap₄A and different concentrations of NaF. The radioactive products

[^3H]ATP and [^3H]AMP were separated from [^3H]Ap₄A (740 TBq/mol; Moravsek Biochemicals, Brea, CA, U.S.A.) by TLC. Silica-gel plates (Merck 5554) were developed in dioxane/ammonia/water (6:1:4, v/v/v) and the radioactivity of ATP counted [13].

Substrate analogue production and hydrolysis

Mono-2'-deoxyadenylated [^3H]diadenosine tetraphosphate was synthesized enzymatically by the use of human 2',5'-oligoadenylate synthetase [14]. Hydrolysis was analyzed by incubating 0.34 mM mono-2'-deoxyadenylated [^3H]diadenosine tetraphosphate (total 150000 c.p.m.) with wild-type and mutant Ap₄A hydrolases in the presence of 50 mM Tris/HCl, pH 8.0, 5 mM MgCl₂ and 0.1 mM dithiothreitol. Products [^3H]ATP and 2'-deoxyadenylated [^3H]ATP were separated by TLC on silica-gel plates developed in dioxane/ammonia/water (6:1:6, v/v/v).

RESULTS

Mutational analysis of lupin Ap₄A hydrolase

The 160 amino acids of lupin Ap₄A hydrolase contain a high proportion of polar or charged residues: 27 acidic residues that could interact with bound metal ions and 19 basic residues that could interact with phosphate groups. In this protein the conserved nudix motif Gly-(Xaa)₅-Glu-(Xaa)₇-Arg-Glu-Uaa-Xaa-(Glu)₂-Xaa-Gly, which is characteristic of 'nudix enzymes' [10], is located between glycine-40 and glycine-61. Ten of the charged residues are located within this motif, including glutamate-46, glutamate-55, glutamate-58, glutamate-59 and arginine-54, which are all strongly conserved across sequences of Ap₄A hydrolases from plants, animals and bacteria [15]. The location of these residues on helix 1 within the three-dimensional structure of the protein is shown in Figure 1 [6].

Previously, mutagenesis studies of the homologous MutT protein from *E. coli* showed that the charged residues, particularly glutamate residues within the nudix motif, were essential for the catalytic activity of MutT as an 8-oxo-dGTP hydrolase [7,9,16]. These, and a range of charged residues outside of the nudix motif, were targeted within the Ap₄A hydrolase from lupin and were modified either by substitution of glutamine for glutamate, lysine or arginine residues to eliminate the charge, or by

substitution of glutamate with aspartate to alter the position of the charge.

Mutant Ap₄A hydrolases > 90% pure as judged by SDS/PAGE were produced by chromatography on GSH-Sepharose, with concurrent cleavage of the fusion protein with PreScission protease, and the molecular masses of mutant proteins confirmed by MALDI-TOF-MS (Table 1). CD spectra for the seven most inhibited mutants could be superimposed upon that of the wild-type enzyme, and the proportions of secondary structure, β -sheet, β -turn, aperiodic structure elements and α -helix (Table 1) calculated from the CD spectra were the same in all expressed proteins. The structural integrity of the most inhibited mutant, E59Q, was also qualitatively assessed by ^1H NMR spectroscopy. Comparison of NOESY spectra of wild-type and E59Q showed no significant changes to the position of most of the resonances (results not shown), indicating that the fold of the protein is maintained. It was concluded that there had been no major disruption to the structure of the enzyme as a consequence of any of these mutations.

As noted in Table 1, the M_r of Ap₄A hydrolase expressed and cleaved from a GST fusion protein, produced from wild-type cDNA clone Ap₄A hydrolase AH-8, matched the apparent M_r 18500–19000 for native Ap₄A hydrolase as purified from lupin. As expressed, the enzyme had a k_{cat} $39.2 \pm 2.3 \text{ s}^{-1}$, well above that of 1.3 s^{-1} calculated for the native enzyme purified from lupin seeds [17]. It is likely that the higher values obtained with the recombinant enzyme more accurately reflects the true k_{cat} of the lupin Ap₄A hydrolase, as a consequence of its higher purity and the considerably more gentle procedures used in its preparation.

Mutations affecting catalysis rate

Preliminary studies showed that elimination of the charge on a number of the amino acids outside of the nudix motif, and even some within the nudix motif, did not alter the catalytic activity of lupin Ap₄A hydrolase. Neither glutamate-46, a residue highly conserved in nudix enzymes [15], nor a number of other glutamate, lysine and arginine residues picked at random throughout the molecule appeared to be essential, as elimination of their charges had no effect on catalytic activity (Figure 2). Nevertheless, residues both within and outside of the conserved nudix motif were identified, where modification of the charge reduced the catalytic activity of the enzyme substantially.

Charged residues which were necessary for maximum catalytic activity were identified in two distinct regions of the enzyme. Mutation of residues arginine-54, glutamate-55, glutamate-58 and glutamate-59 within the nudix motif, led in each case to a significant reduction in k_{cat} . Amongst the 11 other residues mutated in the present study, only two residues, glutamate-122 and glutamate-125, from outside of the conserved nudix motif, were detected as important for catalysis. As shown in Figure 1, folding of the protein brings residue glutamate-125 close to glutamate-59 in the nudix motif on helix 1, even though these residues are 66 amino acids apart in the linear sequence. Although no constraints to metal ions were used in determining the structure [6], glutamate-125 was calculated to be approx. 10 Å from glutamate-59 (C γ -C γ). Glutamate-125 is, therefore, in the vicinity of the glutamate-rich patch provided by the conserved helix in the nudix motif [7].

Effects of mutation on catalysis were analysed by determining both k_{cat} and K_m . The most deleterious mutation, E59Q, produced a 10³-fold reduction in k_{cat} , virtually eliminating all catalytic activity for the Ap₄A hydrolase. A similar result has also been obtained for the equivalent residue in a range of other nudix

Table 1 Physical properties of mutant *L. angustifolius* Ap₄A hydrolases

Expected M_r values of recombinant Ap₄A hydrolases were calculated from the amino acid sequence translated from the DNA sequence. The M_r of expressed and cleaved proteins were determined by linear MALDI MS. The proportions of α -helix in wild-type and mutant Ap₄A hydrolases were estimated using the software package PROSEC, based on the methods of Yang et al. [11].

Mutation	M_r (expected)	M_r (determined)	CD (% helix)
Wild-type	19129	19115	34.3
R54Q	19101	19113	37.6
E58D	19116	19102	35.7
E58Q	19129	19126	32.8
E59D	19116	19114	35.6
E59Q	19129	19127	32.6
*E55Q	18814	18804	32.7
*E125Q	18814	18815	34.2

* Mutants E55Q and E125Q contain five extra amino acids (Gly-Pro-Leu-Gly-Ser-) at the N-terminus. Wild-type and all other mutants contain eight extra amino acids (Gly-Pro-Leu-Gly-Ser-Pro-Asn-Cys-) at the N-terminus.

Figure 3 pH effects on k_{cat} (s^{-1}) of the lupin Ap_4A hydrolase mutants

pH dependence of k_{cat} (s^{-1}) of wild-type Ap_4A hydrolase WT ($\blacklozenge$) and the following mutants: E122Q ($\bullet$), E58Q ($\times$), E58D ($\circ$), R54Q ($\blacksquare$), E125Q ($+$), E59D ($\circ$), E55Q ($\blacktriangle$) and E59Q ($*$).

Lupin Ap_4A hydrolase is also a magnesium-dependent enzyme. In sequence it shows significant identity with the Ap_4A hydrolase from *B. bacilliformis* that requires two enzyme-bound metal ions for activity [24]. Based on the model proposed for inorganic pyrophosphatases, we suggest that in the wild-type lupin Ap_4A hydrolase, residues R54, E55, E58, E59 and E125, and attendant bound magnesium ions, could all co-operate to modify the pK_a of an attacking water to permit hydrolysis at pH 8.0. Perturbation of these charges near the metal as a consequence of the mutations could then lead to the observed need for higher pH to achieve deprotonation and catalysis by the mutants. Neither E59Q nor E122Q activities were enhanced by high pH. Whereas the function of glutamate-122 may be unrelated to metal binding, it appears that mutant E59Q that modified a likely key metal ligand may have led to disruption too great to be reversed by high pH.

Effects on K_m and substrate specificity

The values of K_m for Ap_4A for the mutated Ap_4A hydrolases are presented in Table 2. Although recombinant proteins contained either five or eight amino acid remnants of the protease cleavage site as N-terminal extensions, the K_m s of 2.5 μM for each of the expressed wild-type sequences matched closely the estimate of K_m of 1 μM previously determined for the native Ap_4A hydrolase from *Lupinus luteus* [17]. It was concluded that the five and eight amino acid extensions at the N-terminus had little, if any, effect on access of Ap_4A to the catalytic site.

Despite the dramatic effects on catalytic activity of the above mutations, only relatively small changes were observed in K_m for Ap_4A in most cases. As shown in Table 2, although k_{cat} was lowered by three to five orders of magnitude by mutation of glutamate-59 to aspartate or glutamine, the K_m for Ap_4A was

Table 3 Preference of cleavage of the asymmetric substrate (2'-pdA)-AppppA by variants of the Ap_4A hydrolase from *L. angustifolius*

Products of hydrolysis of (2'-pdA)[^3H]AppppA by expressed *L. angustifolius* Ap_4A hydrolases estimated from the proportions of [^3H]ATP and (2'-pdA)[^3H]ATP produced during incubation. Values are means $\pm$ S.E.M. determined for 4–6 repetitions.

Mutant	Hydrolysis products (%)	
	Type I	Type II
WT	ATP + 2'-deoxyadenylylated AMP	2'-deoxyadenylylated ATP + AMP
R54Q	70.5 $\pm$ 0.6	29.5 $\pm$ 0.6
E58D	70.4 $\pm$ 0.9	29.6 $\pm$ 0.9
E58Q	55.3 $\pm$ 0.2	44.7 $\pm$ 0.2
E125Q	70.5 $\pm$ 0.8	29.5 $\pm$ 0.8
Wild-type	60.7 $\pm$ 0.4	39.3 $\pm$ 0.4

increased only 2–4-fold. This result suggested that alteration of charge at this critical residue within the nudix motif slightly increased the difficulty of binding of substrate. For mutations R54Q, E55Q and E122Q decreases in K_m for Ap_4A between 5–20-fold were observed. In these cases it appeared that Ap_4A bound more tightly to the mutants. Nevertheless, with the possible exceptions of mutations R54Q and E122Q, the scale of the changes in K_m for Ap_4A produced by the above mutations suggests that the accompanying losses of catalytic activity were not due simply to changes in binding of substrates or dissociation of products.

The pattern of cleavage of Ap_4A was not affected by any of the mutations, and all mutants continued to cleave Ap_4A asymmetrically and produced ATP and AMP in equal amounts. That small differences were present at the active site of some mutants, however, was implied by measurements of the hydrolysis of an asymmetric substrate analogue (2'-pdA)-[^3H]AppppA, i.e. [^3H]diadenosine tetraphosphate monodeoxyadenylylated at one of the two riboses. As shown in Table 3, hydrolysis of (2'-pdA)AppppA by Ap_4A hydrolase produces two distinct sets of cleavage products, type I and type II, as a consequence of the two possible orientations of the asymmetric substrate within the enzyme binding site. Cleavage to produce ATP and 2'-deoxyadenylylated AMP (type I), rather than 2'-deoxyadenylylated ATP and AMP (type II), occurred reproducibly with a ratio of 60:40 with the wild-type enzyme, indicating that the end of the substrate with an unmodified ATP was somewhat preferred in the substrate binding site to the more bulky 2'-deoxyadenylylated ATP. Mutants R54Q, E58D and E125Q, that show decreases in K_m for Ap_4A consistent with tighter binding of Ap_4A , also showed greater preference for the unmodified ATP end of the asymmetric substrate (Table 3). With mutations R54Q, E58D and E125Q, releasing ATP (type I cleavage) was increased to 70 % of total hydrolysis. Activity of mutants E55Q, E59D and E59Q with this substrate was too low to be detected. Nevertheless such results confirmed the conclusions drawn from measurements of K_m for Ap_4A : that changes in the structure of the enzyme were restricted to relatively small changes in the substrate binding site, and that there was no major disruption to the secondary and tertiary structures overall.

Inhibition by fluoride

Asymmetrical Ap_4A hydrolases from lupins are strongly inhibited by fluoride ions ($I_{50} = 3 \mu\text{M}$) [3,25]. Several of the mutant proteins no longer exhibited such hypersensitivity to fluoride

(Table 2). For mutation R54Q, which retained approx. 10% activity after the removal of the conserved positive charge within the nudix site, the I_{50} for fluoride was 1 mM. The small amount of activity remaining after mutation of the adjacent glutamate-55 to glutamine (E55Q) was even more resistant to fluoride inhibition, and showed no inhibition at fluoride concentrations up to 1 mM. The sensitivity of Ap_4A hydrolase to fluoride was also decreased by the mutation E125Q. Other mutations showed either no reduction or at most a 10-fold reduction in sensitivity to inhibition by fluoride (Table 2).

DISCUSSION

The most obvious outcome of these mutational studies is the striking parallel shown between the catalytic residues in the higher plant Ap_4A hydrolase and those in the *E. coli* 8-oxo-dGTP hydrolase, MutT. The catalytic activity of both enzymes is extremely dependent on two glutamates in the nudix motif (in the lupin these are glutamate-55 and glutamate-59) and on a third glutamate distant from the motif (glutamate-125). Mutation of glutamate-59 to either glutamine or asparagine decreased catalysis by three to five orders of magnitude. Drastic effects of mutation of residues equivalent to glutamate-59 have also been observed in several other MutT enzymes [7,18,19]. Mutation also implicated conserved residues arginine-54 and glutamate-58 in catalysis, although these effects were considerably less significant. In contrast, mutation of glutamate-46, which is a residue that is also conserved in all nudix enzymes [15], did not affect catalysis.

Table 4 shows the individual contributions to catalysis of the residues mutated in lupin Ap_4A hydrolase and of the corresponding residues in *E. coli* MutT [9] calculated from the extents to which mutation of particular residues reduce k_{cat} . As shown in Table 4, residues from corresponding positions were all contributing to catalysis, despite differences in substrate specificity. Residues corresponding to lupin glutamate-55 and glutamate-59, in particular, both made major contributions to catalysis, and glutamate-125 was also a major contributor in the lupin. Together with structural similarities, these data suggest that the role of these residues in the mechanism of hydrolysis described for MutT [9,16,26] is conserved in lupin Ap_4A hydrolase. We therefore expect that glutamate-55, glutamate-59 and glutamate-125 would play similar roles in metal and/or water ligation.

There are also likely to be differences between the enzymes. Recent metal-binding studies [24] on the Ap_4A hydrolase from *B.*

bacilliformis shows that in contrast to MutT, which binds a single metal ion, the Ap_4A hydrolase binds two metal ions. Preliminary metal-binding studies by us (P. R. Gooley and A. J. Perry, unpublished work) support this finding and show that lupin Ap_4A hydrolase also binds two metal ions. As these ions are near each other in the *B. bacilliformis* homologue, comparison and discussion of the mechanism of MutT and Ap_4A hydrolase becomes complicated. Conyers et al. [24] suggest that the acidic residues in the loop of the nudix motif at the N-terminal end of helix 1 of Ap_4A hydrolase may be additional metal ligands. However, mutation of glutamate-44 and glutamate-46 in lupin Ap_4A hydrolase shows that neither residue contributes to the catalysis carried out by this enzyme.

In *E. coli* MutT, it is proposed that glutamate-53 acts as one of the ligands for the enzyme-bound magnesium in the absence of bound substrate, and that in the presence of substrate, glutamate-98 replaces glutamate-53 as that ligand for magnesium, freeing glutamate-53 to interact with the catalytic water molecule [9]. Notably, as glutamate-53 orients the attacking water, the glutamate is, in turn, oriented by the charge on the adjacent arginine-52 [9]. These functions appear to be conserved in lupin Ap_4A hydrolase in residues glutamate-55 and arginine-54, and stand to be confirmed by structure analysis. The combined mutational and structural data suggest that glutamate-125 and glutamate-55 are positioned in the lupin Ap_4A hydrolase to act as alternative ligands for Mg^{2+} that function during catalysis in the same way as the model predicts for glutamate-53 and glutamate-98 in the MutT enzyme. Even though the calculated contribution of arginine-54 to catalysis is much less than its equivalent (arginine-52) in MutT (Table 4), alternative evidence that arginine-54, glutamate-55 and glutamate-125 co-operate during catalysis in the lupin Ap_4A hydrolase was provided by analysis of the effects of fluoride on the hydrolytic capacity of mutant Ap_4A hydrolases. Mutation of any one of these residues eliminated the fluoride sensitivity which is characteristic of Ap_4A hydrolases [1,25]. Mutation of glutamate-55 in the lupin Ap_4A hydrolase to glutamine not only completely eliminated sensitivity to fluoride, but also lowered catalysis 10000-fold, whereas mutation R54Q lowered catalysis only 10-fold. Mutation of glutamate-53 to glutamine in MutT made this enzyme insensitive to pH below 8.0, supporting its role in ligation and orientation of the attacking water molecule [9]. Mutation of glutamate-55 to glutamine in the lupin Ap_4A hydrolase was also the only mutation that rendered the enzyme insensitive to pH changes below pH 8.0, further supporting the idea that the role for glutamate-55 is also the catalytic base in lupin Ap_4A hydrolase.

Neither our mutational studies on Ap_4A hydrolase nor those on enzymes hydrolysing 8-oxo-dGTP [7], capped mRNA [19] or diphosphoinositol polyphosphate [18,20] suggest that any of the substrate specificity depends upon the nudix residues. Rather, we conclude that the common functional residues shared by the lupin Ap_4A hydrolase and the *E. coli* MutT enzyme form a catalytic domain that is distinct from the substrate binding domains in the different enzymes and will be conserved in the wide range of nudix hydrolases currently being identified throughout animals, plants and bacteria [15,27].

Table 4 Contributions of active-site residues to catalysis of lupin Ap_4A hydrolase and MutT protein

Contributions to catalysis for lupin Ap_4A hydrolase calculated from the effects of the mutations on k_{cat} (s^{-1}) (see Table 2). The values for corresponding residues of MutT protein are as presented by Harris et al. [9]. According to the authors [9], such contributions to catalysis are not additive, but rather indications of the relative significance of each residue.

Ap ₄ A hydrolase from lupin		MutT protein from <i>E. coli</i>	
Residue	Contribution	Residue	Contribution
Arg-54	10 ^{0.9}	Arg-52	10 ^{3.0}
Glu-55	10 ^{3.9}	Glu-53	10 ^{4.7}
Glu-58	10 ^{1.0}	Glu-56	10 ^{1.4}
Glu-59	10 ^{5.1}	Glu-57	10 ^{5.0}
Glu-125	10 ^{2.1}	Glu-98	10 ^{0.7}

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