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†

*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″-P²,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)-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 kcat markedly, whereas mutations R54Q, E58Q and E122Q had smaller effects. None of the mutations produced a substantial change in the Km 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″-P²,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)-Gly-(Xaa)-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′-GAAGATCTATGGATTCTCCCGAAGG-3′ and 5′-GG-AAGCTTATTAGAGATGTGGAGCAAC-3′ to copy bases 139–625 from clone AH-7 and concurrently introduce recognition sequences for BglII and HindIII 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 BamHI and HindIII 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 EcoR1/XhoI or BamHI/XhoI 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″-P²,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.

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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 "N 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, [θ] 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.

Kₘ 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 I₅₀ values for fluoride inhibition were determined in mixtures containing 100 μM [³H]Ap₄A and different concentrations of NaF. The radioactive products
\[ ^3 \text{H}\text{ATP} \text{ and } ^3 \text{H}\text{AMP} \text{ were separated from } ^3 \text{H}\text{Ap}_4\text{A} \text{ (740 TBq/mol; Moravek Biochemicals, Brea, CA, U.S.A.) by TLC. Silica-gel plates (Merck 5554) were developed in dioxane/ammonia/water (6:1:4, v/v/v) and the radioactivity of ATP counted [13].} \]

Substrate analogue production and hydrolysis

Mono-2′-deoxyadenylated \[ ^3 \text{H}\text{dadienose tetraphosphate} \text{ was synthesized enzymatically by the use of human 2′,5′-oligoadenylate synthetase [14]. Hydrolysis was analyzed by incubating 0.34 mM mono-2′-deoxyadenylated \[ ^3 \text{H}\text{dadienose tetraphosphate} \text{ (total 150 000 c.p.m.) with wild-type and mutant Ap}_4\text{A hydrolases in the presence of 50 mM Tris/HCl, pH 8.0, 5 mM MgCl}_2 \text{ and 0.1 mM dithiothreitol. Products } ^3 \text{H}\text{ATP} \text{ and 2′-deoxyadenylated } ^3 \text{H}\text{ATP} \text{ were separated by TLC on silica-gel plates developed in dioxane/ammonia/water (6:1:6, v/v/v).} \]

RESULTS

Mutational analysis of lupin Ap\textsubscript{4}A hydrolase

The 160 amino acids of lupin Ap\textsubscript{4}A 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}–{Glu}–{Xaa}–Arg-Glu-Uaa-Xaa–{(Glu)}–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 and arginine-54, which are all strongly conserved across sequences of Ap\textsubscript{4}A 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 \textit{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 Ap\textsubscript{4}A hydrolase from lupin and were modified either by substitution of glutamate for glutamine, lysine or arginine residues to eliminate the charge, or by substitution of glutamate with aspartate to alter the position of the charge.

Mutant Ap\textsubscript{4}A 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, \( \beta \)-sheet, \( \beta \)-turn, aperiodic structure elements and \( \alpha \)-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 \(^1\)H 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 Ap\textsubscript{4}A hydrolase expressed and cleaved from a GST fusion protein, produced from wild-type cDNA clone Ap\textsubscript{4}A hydrolase AH-8, matched the apparent \( M_r \) of 17 800–19 000 for native Ap\textsubscript{4}A hydrolase as purified from lupin. As expressed, the enzyme had a \( k_{\text{cat}} \) of 39.2 \( \pm 2.3 \) 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 \( k_{\text{cat}} \) of the lupin Ap\textsubscript{4}A hydrolase, as a consequence of its higher purity and the considerably more gentle procedures used in its preparation.

Table 1 Physical properties of mutant \textit{L. angustifolius} Ap\textsubscript{4}A hydrolases

<table>
<thead>
<tr>
<th>Mutation</th>
<th>( M_r ) (expected)</th>
<th>( M_r ) (determined)</th>
<th>CD (% helix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>19129</td>
<td>19115</td>
<td>34.3</td>
</tr>
<tr>
<td>R54Q</td>
<td>19101</td>
<td>19111</td>
<td>37.6</td>
</tr>
<tr>
<td>E58Q</td>
<td>19116</td>
<td>19102</td>
<td>35.7</td>
</tr>
<tr>
<td>E59Q</td>
<td>19129</td>
<td>19126</td>
<td>32.8</td>
</tr>
<tr>
<td>E59D</td>
<td>19116</td>
<td>19114</td>
<td>35.6</td>
</tr>
<tr>
<td>E59Q</td>
<td>19129</td>
<td>19127</td>
<td>32.6</td>
</tr>
<tr>
<td>*E55Q</td>
<td>18814</td>
<td>18804</td>
<td>32.7</td>
</tr>
<tr>
<td>*E125Q</td>
<td>18814</td>
<td>18815</td>
<td>34.2</td>
</tr>
</tbody>
</table>

* 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.

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 Ap\textsubscript{4}A 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 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 \( k_{\text{cat}} \). 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\(_\gamma\)–C\(_\gamma\)). 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 \( k_{\text{cat}} \) and \( k_{\text{cat}} \). The most deleterious mutation, E59Q, produced a 10-fold reduction in \( k_{\text{cat}} \), virtually eliminating all catalytic activity for the Ap\textsubscript{4}A hydrolase. A similar result has also been obtained for the equivalent residue in a range of other nudix
Figure 2  Effects of single amino acid substitution on the activity of the Ap₄A hydrolase from *L. angustifolius*

(A) Positions of mutations in lupin Ap₄A hydrolase. Consensus sequence for nudix hydrolases [10] is shown above and mutations are depicted below the amino acid sequence of recombinant Ap₄A hydrolase [3]. (B) Effects on $k_{cat}$ (s⁻¹) for Ap₄A hydrolysis determined on independently expressed proteins. Values expressed as means± S.E.M.; n = 3–5.

Table 2  Kinetic parameters for mutant *L. angustifolius* Ap₄A hydrolases

$k_{cat}$, $K_m$ for Ap₄A and $I_{50}$ for fluoride were determined on expressed Ap₄A hydrolases. ‡Values determined on cleaved and purified proteins containing after cleavage N-terminal extensions of Gly-Pro-Leu-Gly-Ser- and Gly-Pro-Leu-Gly-Ser-Pro-Asn-Cys- respectively. †Values determined on fusion proteins prior to cleavage. S.E.M. was determined for 3–5 repetitions.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>$k_{cat}$ (s⁻¹)± S.E.M.</th>
<th>$K_m$ (µM)</th>
<th>$I_{50}$ for fluoride (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>43.5*</td>
<td>2.5*</td>
<td>3</td>
</tr>
<tr>
<td>Wild-type</td>
<td>40.8±2.7†</td>
<td>2.5†</td>
<td>100</td>
</tr>
<tr>
<td>R54Q</td>
<td>4.2±0.4‡</td>
<td>0.5‡</td>
<td>1000</td>
</tr>
<tr>
<td>E55Q</td>
<td>0.005±0.001‡</td>
<td>0.12‡</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>E58D</td>
<td>3.7±0.3‡</td>
<td>0.96‡</td>
<td>50</td>
</tr>
<tr>
<td>E58Q</td>
<td>17.9±0.9†</td>
<td>2†</td>
<td>20</td>
</tr>
<tr>
<td>E59D</td>
<td>0.011±0.001†</td>
<td>10†</td>
<td>6</td>
</tr>
<tr>
<td>E59Q</td>
<td>0.00031±0.00006‡</td>
<td>5.9‡</td>
<td>7</td>
</tr>
<tr>
<td>E122Q</td>
<td>28.4±3.6†</td>
<td>0.5‡</td>
<td>7</td>
</tr>
<tr>
<td>E125Q</td>
<td>0.29±0.02‡</td>
<td>1.4†</td>
<td>250</td>
</tr>
<tr>
<td>AH8 wild-type–GST</td>
<td>39.2±2.3‡</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>AH8 wild-type–MBP</td>
<td>35.6±2.5‡</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

enzymes [7,18,19]. Substitution of aspartate for the highly conserved glutamate-59 reduced the rate of hydrolysis 3 x 10⁴-fold (Table 2), suggesting that not only the charge but also its position are essential.

Catalysis was also dependent on glutamate-55, a second of the highly conserved glutamate residues within the nudix region. Neutralization of the charge at this position caused a 10⁴-fold drop in activity in mutant E55Q. Glutamate-55 in the lupin Ap₄A hydrolase structure is the equivalent of glutamate-53 in MutT [9] and of glutamate-66 in diphosphoinositol polyphosphate phosphohydrolase [20], where catalysis in each case is similarly lost by mutation of these residues.

Mutations at other conserved positions, arginine-54 and glutamate-58 within the nudix motif, had significant but much less dramatic effects. Removal of the charge at arginine-54 (R54Q) decreased $k_{cat}$ 8-fold. Repositioning of the negative charge at position-58 in mutant E58D disabled catalysis more than by removal of the charge, as in mutant E58Q; mutation E58D lowered $k_{cat}$ 10-fold, while mutation E58Q lowered $k_{cat}$ only by 50%.

All assays were initially conducted at pH 8.0, the pH optimum of the wild-type enzyme. Intriguingly, significant reversal of the inhibitory effects of many mutations was achieved by increasing the pH of the reaction. Mutations R54Q, E55Q, E58Q and E58D, E59D and E125Q all showed enhanced activity at pH values up to 10 (Figure 3). Similar shifts in pH optimum towards more alkaline values have been observed in mutants of inorganic pyrophosphatases from *E. coli* and yeast, where they were attributed to the increase of $pK_a$ of the essential basic group [21–23]. In the *E. coli* inorganic pyrophosphatase, it was proposed that a hydroxy group of an active water molecule, stabilized by one or two Mg⁺ ions, was the essential basic group involved in the hydrolysis. It was further proposed that distortion of the active site that disrupted such interaction between metal ions and active water would result in the increase of $pK_a$ of this water [21].
Lupin Ap₄A hydrolase is also a magnesium-dependent enzyme. In sequence it shows significant identity with the Ap₄A 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 Ap₄A hydrolase, residues R54, E55, E58, E59 and E125, and attendant bound magnesium ions, could all co-operate to modify the pKᵢ of an attacking water to permit hydrolysis at pH 8.0. Perturbation of these charges near the metal as a consequence of the substrate binding site to the more bulky 2'-deoxyadénylade AMP (dAMP)–Ap₄A by Ap₄A 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 AMP (type II), occurred reproducibly with a ratio of 60:40 with the wild-type enzyme, indicating that the end of the asymmetric substrate analogue (2'-deoxyadenylated AMP–Ap₄A) is more preferred. The pattern of cleavage of Ap₄A was not affected by any of the mutations, and all mutants continued to cleave Ap₄A symmetrically 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'-deoxyadenylated AMP–Ap₄A) (Table 3). As shown in Table 3, hydrolysis of (2'-deoxyadenylated Ap₄A) by Ap₄A 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'-deoxyadenylated AMP (type I), rather than 2'-deoxyadenylated 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 end of the asymmetric substrate analogue (2'-deoxyadenylated Ap₄A) was more preferred in the substrate binding site to the more bulky 2'-deoxyadenylated ATP. Mutants R54Q, E58D and E125Q, that show greater preference for the unmodified ATP end of the asymmetric substrate analogue (2'-deoxyadenylated Ap₄A) were observed in these cases it appeared that Ap₄A bound more tightly to the mutants. Nevertheless, with the possible exceptions of mutations R54Q and E122Q, the scale of the changes in Kᵢₘ for Ap₄A 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.

### Effects on Kᵢₘ and substrate specificity

The values of Kᵢₘ for Ap₄A for the mutated Ap₄A 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 Kᵢₘ of 2.5 μM for each of the expressed wild-type sequences matched closely the estimate of Kᵢₘ of 1 μM previously determined for the native Ap₄A 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 Ap₄A to the catalytic site.

Despite the dramatic effects on catalytic activity of the above mutations, only relatively small changes were observed in Kᵢₘ for Ap₄A in most cases. As shown in Table 2, although kₗₑₐₛₗₜ was lowered by three to five orders of magnitude by mutation of glutamate-59 to aspartate or glutamine, the Kᵢₘ for Ap₄A was 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 Kᵢₘ for Ap₄A between 5–20-fold were observed. In these cases it appeared that Ap₄A bound more tightly to the mutants. Nevertheless, with the possible exceptions of mutations R54Q and E122Q, the scale of the changes in Kᵢₘ for Ap₄A 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.

### Inhibition by fluoride

Asymmetrical Ap₄A hydrolases from lupins are strongly inhibited by fluoride ions (Iₕ₋ₒ = 3 μM) [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 nixid site, the $k_{cat}$ 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$_A$ 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$_A$ 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 nixid 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$_A$ 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$_A$ 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$_A$ hydrolase from *B. bacilliformis* shows that in contrast to MutT, which binds a single metal ion, the Ap$_A$ 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$_A$ 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$_A$ hydrolase becomes complicated. Conyers et al. [24] suggest that the acidic residues in the loop of the nixid motif at the N-terminal end of helix 1 of Ap$_A$ hydrolase may be additional metal ligands. However, mutation of glutamate-44 and glutamate-46 in lupin Ap$_A$ 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$_A$ 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$_A$ 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$_A$ hydrolase was provided by analysis of the effects of fluoride on the hydrolytic capacity of mutant Ap$_A$ hydrolases. Mutation of any one of these residues eliminated the fluoride sensitivity which is characteristic of Ap$_A$ hydrolases [1,25]. Mutation of glutamate-55 in the lupin Ap$_A$ 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$_A$ 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$_A$ hydrolase.

Neither our mutational studies on Ap$_A$ hydrolase nor those on enzymes hydrolysing 8-oxo-dGTP [7], capped mRNA [19] or diphosphinositol polyphosphate [18,20] suggest that any of the substrate specificity depends upon the nixid residues. Rather, we conclude that the common functional residues shared by the lupin Ap$_A$ 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 nixid hydrolases currently being identified throughout animals, plants and bacteria [15,27].

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**Table 4. Contributions of active-site residues to catalysis of lupin Ap$_A$ hydrolase and MutT protein**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Contribution to catalysis of Ap$_A$ hydrolase from lupin</th>
<th>Contribution to catalysis of MutT protein from <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-54</td>
<td>$10^0$</td>
<td>$10^0$</td>
</tr>
<tr>
<td>Glu-55</td>
<td>$10^3$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Glu-58</td>
<td>$10^0$</td>
<td>$10^1$</td>
</tr>
<tr>
<td>Glu-59</td>
<td>$10^0$</td>
<td>$10^0$</td>
</tr>
<tr>
<td>Glu-125</td>
<td>$10^2$</td>
<td>$10^2$</td>
</tr>
</tbody>
</table>

*Note: Values are in units of *k*₅₀ (s⁻¹) as measured for the effects of mutations with respect to the wild type.*
REFERENCES


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