Asymmetric diadenosine 5',5''-P1,P4-tetraphosphate (Ap4A) hydrolases play a major role in maintaining homeostasis by cleaving the metabolite diadenosine tetraphosphate (Ap4A) back into ATP and AMP. The NMR solution structures of the 17-kDa human asymmetric Ap4A hydrolase have been solved in both the presence and absence of the product ATP. The adenine moiety of the nucleotide predominantly binds in a ring stacking arrangement equivalent to that observed in the x-ray structure of the homologue from Caenorhabditis elegans. The binding site is, however, markedly divergent to that observed in the plant/pathogenic bacteria class of enzymes, opening avenues for the exploration of specific therapeutics. Binding of ATP induces substantial conformational and dynamic changes that were not observed in the C. elegans structure. In contrast to the C. elegans homologue, important side chains that play a major role in substrate binding do not have to reorient to accommodate the ligand. This may have important implications in the mechanism of substrate recognition in this class of enzymes.

Nudix (nucleoside diphosphate linked to O) hydrolases are a superfamily of enzymes with over 300 identified members (1) that are required for maintaining physiological homeostasis by the metabolism of signaling molecules and potentially toxic substances. This superfamily is subdivided according to substrate specificity, including dinucleotide polyphosphatases (including Ap4A\(^2\) hydrolases), ribo- or deoxyribonucleoside triphosphatases, and pyrophosphatases of nucleotide sugars, NADH, and ADP-ribose. All of the Nudix enzyme structures solved to date display the same “prototypical” scaffold comprising an \(\alpha\)-\(\beta\)-\(\alpha\) sandwich fold in which one of the helices and the loop preceding it make up the catalytic site for metal-mediated hydrolysis of the substrate. This loop-helix motif contains the generalized “Nudix signature,” GX\(\_\)EX,REUXEXGU, in which U is a bulky aliphatic residue usually Ile, Leu, or Val. Although the folds are similar, loop and helix insertions are commonly observed outside of the highly conserved catalytic region. As a consequence, the family displays a range of nucleotide-binding positions even for similar substrates (2). Furthermore, dimeric enzymes are also observed in which the substrate can be found between adjacent monomers. Perhaps the most striking example of this to date is in the x-ray structure of ADP-ribose pyrophosphatase (3), which requires dimerization through extensive domain swapping for substrate recognition and catalytic activity. Hence, throughout evolution, nature has preserved the scaffold of the reaction sub-site and facilitated diverse substrate recognition by employing residue or loop insertions and in some cases adopted contributions from each monomer in the dimeric state.

Nudix Ap4A hydrolases (hereafter referred to as Ap4Aases) have been isolated from all kingdoms of life (1). Phylogenetic analysis (4) further reveals two distinct groups within these kingdoms. Plant and bacterial enzymes fall into one subgroup, and the animal and archaeal enzymes fall into another (Fig. 1). All preferentially cleave Ap4A asymmetrically into ATP and AMP by a mechanism that has been studied in detail with a plant (Lupinus angustifolius) (5) and a bacterial (Bartonella bacilliformis) enzyme (6). In this, a conserved glutamate acts as a catalytic base aiding the “in-line” nucleophilic attack at the P4 phosphate (i.e. fourth from the tightest binding base) by a water molecule. The attack is mediated by up to three magnesium ions and results in inversion of stereochemistry at the P4 center (7).

The substrate, Ap4A, is found in all organisms from Archeae to humans with basal levels up to 5 \(\mu\)M (8) and is predominately a by-product of protein synthesis, specifically by aminoacyl-RNA syntheses (9). Although the biological significance of Ap4A is not fully understood, intracellularly it has been implicated in apoptosis (10) and DNA repair (11), whereas extracellular Ap4A may have important roles as a neurotransmitter and in the cardiovascular system (12, 13). In bacteria Ap4A levels may also play a role in invasion. For example, knocking out the asymmetric and/or the unrelated symmetric Ap4Aase abrogated the invasive capacity of Salmonella typhimurium (14). Intracellular invasion has also been associated with the asymmetric Ap4A hydrolases from Bacilliformis and Escherichia coli (15–17).

There is a fundamental interest in understanding this “plant-bacteria” and “animal” grouping on the molecular level, and in addition the knowledge is desirable for the design of therapeutic inhibitory compounds that would selectively bind to the plant/bacteria group. Toward these goals,
there have been two structures of the asymmetric Ap4A hydrolases from both the plant-bacteria and animal groups solved to date: the NMR solution structure from the narrow-leaved blue lupin, *L. angustifolius*, both free (18) and as a complex with a novel MgF₃ leafed blue lupin, *L. angustifolius* enzymes, respectively. Secondary structure elements are depicted either above or below the sequences for the human or *L. angustifolius* enzymes, respectively. Numbering is as observed in the coordinates deposited in the Protein Data Bank.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Human Ap4Aase was expressed as described previously (21). The protein was expressed as a glutathione S-transferase fusion from pGEX-6P-3 in a 2-liter fermentor, and was isotopically enriched with [¹³C]ammonium chloride and [¹³C]glucose in *E. coli* BL21(DE3) grown in M9 medium following established protocols. The fusion protein was purified on glutathione-Sepharose, cleaved with PreScission protease, followed by removal of established protocols. The fusion protein was purified on glutathione-Sepharose and anion exchange chromatography using a Mono-Q column.

**NMR Spectroscopy**—Samples of human Ap4Aase (0.3–0.45 ml, 0.2–1.1 mM) were prepared in 20 mM imidazole, 20 mM MgCl₂, 10 mM dithiothreitol, 1 mM EDTA, 0.02% sodium azide, and either 100% D₂O or 90/10% H₂O/D₂O at pH 6.5 and sealed under nitrogen. For the determination of the complex structure, samples included the addition of 1.5 equivalence of [¹³C,¹⁵N]labeled ATP, Stocks of 80 mM AMP, ADP, and adenosine, were made up in NMR buffer (AMP and ADP) or in 50% Me₂SO/NMR buffer (adenosine) for the titration experiments. Experiments were carried out at 20 °C on Varian Inova 500 and Inova 600 spectrometers, and assignment details have been published elsewhere (21). Distance constants were obtained from three-dimensional [¹³C,¹⁵N]-NOESY-HSQC, three-dimensional [¹³C,¹⁵N]-HSQC-NOESY, and three-dimensional [¹³C,¹⁵N]-NOESY-HSQC experiments. In the ATP complex, three-dimensional [¹³C,¹⁵N]-NOESY-HSQC and three-dimensional HCCH-TOCSY spectra over the aromatic/anomeric regions were further recorded. Stereospecific methyl assignments for leucine and valine were made via a 10% [¹³C,¹⁵N]-labeled sample (22). Limited α, angles for aromatic residues were determined by using quantitative J correlation using a pair of spin-echo difference [¹³C,¹⁵N]-HSQC-based experiments (23). [¹⁵N] steady-state NOE data were acquired using a total relaxation delay of 5 s, with on-resonance presaturation during the final 5 s for the NOE spectrum. Data were processed using NMRPipe (24) and subsequently analyzed using XEASY (25). Relaxation and titration data were analyzed using the program SPARKY. Dissociation rate constants were fitted using a simple two-state model (27).

**Structure Determination**—Structures were initially calculated using the CANDID (28) macro within the program CYANA. Torsion angle constraints (φ and ψ) were generated from an analysis of chemical shifts using TALOS (29). Explicit hydrogen bonds were included as upper and lower distance bounds in φ-helices only for residues observed to have significantly exchanging amide protons. Calibration of the NOE data was handled by CANDID and at all stages of this phase of structure calculations. The best 20 from 100 trial structures were selected as judged by the lowest target function and were used to represent the structure ensemble. At no stage was the structure from the *C. elegans* homologue used to expedite the calculations.

Structures were further refined using Xplor-NIH 2.9.1 (30) using a torsion angle dynamics protocol modified to incorporate data base potentials (31). 100 trial structures were calculated starting from a minimized extended conformation with random initial velocities. Structures were selected on the basis of lowest residual target function and total energy. ATP-bound structures were further calculated in CNS version 1.0 (32) with the heating temperature lowered from 50,000 to 20,000 K. To improve calculation stability, the structures from the previous calculations in Xplor-NIH were used as starting structures, and the integration step size was reduced from 15 to 5 fs. The ATP was positioned ~20 Å away from the binding cleft. Ring current calculations were done within the program MOLMOL 2K.1 (33) using the Heigh-Mallion model (34) with a 5-Å cut-off distance. In this, the ATP was replaced with an adenosine residue with identical atom positions, and default parameters were used. The calculated ring shift was obtained from the arithmetic average of the calculated individual ring shifts for each prochiral atom within a given degenerate pair. The observed shielding was measured from a
Mechanism of Ap₄A Hydrolase

The lowest energy conformers were obtained from 100 starting structures.

| TABLE 1 |
| --- |
| Structural statistics and mean pairwise deviations |
| --- |
| Experimental restraints | APO (33 accepted) | ATP complex (39, 25 accepted) |
| Distance | | |
| Total | 1950 | 2022 |
| Sequential (| | |
| Medium range (1 < | | |
| Intra-residue ( | | |
| Hydrogen bonds | 32 | 32 |
| ATP constraints | | |
| r.m.s.d. from experimental distance restraints | 0.0323 ± 0.0058 | 0.0272 ± 0.0005 |
| r.m.s.d. from experimental dihedral restraints | 0.2672 ± 0.0504 | 0.2661 ± 0.0484 |
| r.m.s.d. from idealized covalent geometry | | |
| Bonds (Å) | 0.0026 ± 0.00023 | 0.0025 ± 0.00032 |
| Angles (°) | 0.4247 ± 0.0199 | 0.3943 ± 0.0231 |
| Improper (°) | 0.3940 ± 0.0184 | 0.3877 ± 0.0185 |
| Energies (kcal mol⁻¹) | | |
| Lennard-Jones | -291.16 ± 39.92 | -375.44 ± 41.85 |
| Ramachandran (°) | -472.02 ± 54.48 | |
| F, G, A, D | 82, 11, 5, 2 | 86, 10, 3, 1 |
| r.m.s.d. | | |
| Non-mobile 7–21,27–150 | | |
| Backbone atoms | 0.35 ± 0.06 | 0.31 ± 0.006 |
| Heavy atoms | 0.8 ± 0.06 | 0.81 ± 0.06 |
| Secondary structure | | |
| Ramachandran (°) | | |
| F, G, A, D | 82, 11, 5, 2 | 86, 10, 3, 1 |
| r.m.s.d. to the mean | | |
| Non-mobile 7–21,27–150 | | |
| Backbone atoms | 0.25 ± 0.05 | 0.20 ± 0.03 |
| Heavy | 0.71 ± 0.07 | 0.67 ± 0.06 |
| Adenosine ("open" and "closed") | 0.24 ± 0.06 | 0.26 ± 0.17 |

- Maximum distance/dihedral violations: 0.35 Å/3° (apo) and 0.28 Å/3° (ATP complex).
- Idealized covalent geometry as implemented in CNS.
- Lennard-Jones van der Waals energy was calculated in CNS.
- Most favored, generously allowed, allowed, and disallowed.
- Residues 8–18, 29–36, 43–44, 47–49, 56–66, 75–77, 83–88, 93–103, 116–130, and 134–147.

RESULTS

Structure Determination—Preparations of the wild type human Ap₄Aase showed poor solubility and typically precipitated within a few days. During screening of a range of catalytic residue mutants, the construct E63A displayed increased resistance to precipitation, and this enabled the NMR structures of the wild type human Ap₄Aase to be determined to high resolution. Glu-63 is one of the essential glutamates involved in catalysis (5). A comparison of the amide resonances of wild type and a quarter of the residues are of good quality as judged by low deviations from idealized geometry and Lennard-Jones potentials, indicating that the structures have favorable nonbonded contacts. Furthermore, 82 and 86% of the residues in the apo- and ATP enzyme, respectively, lie in the most favored region of the Ramachandran plot.

Description of the Free Structure—Overall, the structure of human Ap₄Aase (Fig. 2) exhibits the general αβα Sudix scaffold observed in all Nudix hydrolases solved to date (reviewed in Ref. 36). The scaffold consists of a central four-stranded sheet sandwiched between two anti-parallel helices and a third helix orthogonal to these. Helices αII and αIII are anti-parallel and have an inter-helical angle of ~170° to each other and angles of 90° and 110° to the catalytic helix αI, respectively.

The surface of the protein (Fig. 2E) shows a large cleft running perpendicular to this catalytic helix and, by comparison with the two other Ap₄Aase, houses the ligand-binding site. The walls of this cleft are constructed from the 40-, 90-, and 130-s loops, the latter two forming an overhang characterized by NOE interactions between Ala-89 and the side chain of Pro-133. As expected the catalytic helix is negatively charged to facilitate the binding of several magnesium ions required for hydrolysis (6). In contrast, the cleft is positively charged as a result of the side chains of His-42, Lys-49, Lys-94, and His-116 presumably facilitating phosphate binding of the substrate. At the top of the cleft, the side chains of Tyr-87 and Phe-133 are orientated with their rings roughly in a parallel face-on arrangement (Fig. 2D).

A quarter of the residues are located in nonregular secondary structural elements. It is clear from 15N relaxation data (Fig. 3) that some of these regions are mobile in solution. In particular,
the 20-s loop is mobile and displays $^{15}$N steady-state NOE values of $\sim 0.5$. Furthermore, doubling of resonances is observed in the spectra most likely because of the slow exchange of cis-trans isomerization of Pro-22 as often observed for small peptides. The termini (1–6 and 150–153) are highly mobile, characterized by $^{15}$N steady-state NOE values less than 0.3, sharp resonances, and the observation of only weak sequential NOEs. The amides from 11 other residues located in the substrate binding 40-, 90-, and 130-s loops (Fig. 2A) were not detected and presumed to exhibit microsecond to millisecond motion. Consequently, the assignments of these resonances were obtained from initial structures and side chain spin typing.

The ATP-binding Cleft and Substrate Binding—To gain insight into the effects of nucleotide binding, the product ATP was titrated into samples of either $^{15}$N- or $^{13}$C/$^{15}$N-labeled human Ap$_4$Aase, and the perturbation of the resonances in the $^{15}$N- and $^{13}$C-HSQC spectra was followed. The effects of a variety of exchange regimens were observed (Fig. 4, A and B). Approximately a third of amide resonances displayed moderately fast exchange, 11 were in slow exchange, and 11 new resonances appeared. Fitting the data to a simple fast exchange two-site model for a resonance (Trp-43 H$\beta$) proximal to the binding cleft demonstrates that ATP binds with a $K_d \sim 0.05$ mM. Maximum chemical shift changes of 50 ($^{15}$N) and 100 Hz ($^1$H) were observed yielding a lower limit of 314–618 s$^{-1}$ for the dissociation rate (assuming fast exchange).

As many of the amides located around the binding subsite were not observed, it was necessary to follow side chain resonances, particularly those of aromatic side chains as a more direct probe for the substrate-binding position. The aromatic side chains of His-42, Tyr-87 and Phe-133 project into the
binding cleft (Fig. 4B) and have been implicated in phosphate binding (His-42) or ring stacking with the adenine (Tyr-87 and Phe-133) (20). Large chemical shift perturbations of the side chains of Tyr-87 (H^2 and H^5) and Phe-133 (H^3) were observed to be in slow intermediate exchange and may indicate ring stacking on either side of the adenosine. The ring protons of His-42 were also in slow intermediate exchange, and in this case may indicate phosphate interactions.

Structural and Dynamic Changes Are Observed in the Substrate-binding Loops upon ATP Binding—The structure has also been solved in the presence of the product ATP, and although the structure of the ATP-bound enzyme (Fig. 5A) is globally similar to the apo-structure, structural and dynamic differences are clearly observed particularly within the loop regions around the binding subsite. These differences are also observed in calculations without the inclusion of the ligand constraints. As these differences are much greater than the conformational variability within each ensemble of structures (Fig. 5B), they are significant within the context of the NMR structures and are not due to a lack of data.

The 40-s loop has undergone a small translation of about 3 Å inward toward the center of the cleft and upward toward the 130-s loop. This shift is characterized by contacts observed between the side chains of Ile-40, His-42, and Phe-133 that are present in the ATP structure, indicating a cleft widening on the phenolic ring of Tyr-87). Calculations using the lowest energy conformers with the open state result in minimal overlap of the anomeric and Phe-87 (H^7/H^6) ring resonances (5.87 and 5.93 ppm, respectively). Furthermore, the H-2 resonance of ATP was not observed, and no NOEs were seen from the broadened NH_2 of the ATP. Two different conformations of the adenine moiety (Fig. 5C) are identified consistent with the NOE data. One orientation (80% of calculated structures) displays the adenine sandwiched and stacked between the aromatic rings of Tyr-87 and Phe-133, whereas the other reveals a more open and less buried conformation. In this position, the ring is located higher and lies across the surface at the top of the cleft. In all structures, the adenine moiety of the ATP was observed to adopt an anti conformation with respect to the ribose. No NOEs were observed between the rings of Tyr-87 and Phe-133 in either the apo or in the ATP-complexed spectra, implying that the observed differences in aromatic chemical shifts between the two are mainly due to shielding effects from the nearby adenine ring. Ring current calculations (34) favor the stacked conformation as very little shielding contributions result from the adenine ring to the side chain of Tyr-87 in the "open" state due to the larger inter-annular distance (7 Å for the open versus 3.6 Å for the stacked between C-5 on the adenine and the center of the phenolic ring of Tyr-87). Calculations using the lowest energy conformers with the open state result in minimal averaged shielding values of the Tyr-87 ring resonances: 0.09, 0.18, 0.04, 0.07 ppm, in contrast to those observed 0.54, 0.66, 1.55, 0.40 ppm and calculated for the ring-stacked state.

FIG. 3. Steady-state 1H-15N NOE for human Ap_4Aase. Values for free enzyme (black) and in the presence of 1.5 molar eq of ATP (gray) are plotted. Secondary structure elements are displayed.
0.57, 0.35, 0.98, 0.72 ppm for C, H, C, and H, respectively. It can further be argued that the ring-stacked state would be energetically more favorable based on the difference in buried surface area of the adenine ring 53.6% (open) versus 73.7% (stacked).

"Fragmentation" Assay—To deconvolute the contributions to the binding affinity from the phosphates, the sample has been titrated with AMP, ADP, and adenosine, and the aromatic side chain resonances were followed as a probe for authentic binding (Fig. 4, C and D). Remarkably, over 40 times the molar equivalents of either AMP or ADP was required to observe similar chemical shift changes to the aromatic resonances of Tyr-87 and Phe-133 as observed with the 1.5 molar eq of ATP, reflecting the higher apparent $K_d$ of both.
FIG. 5. Solution structures of human Ap₄Aase in complex with the product ATP. A, stereo view showing superposition of the 36 NMR-derived structures of human Ap₄Aase in the presence of 1.5 molar eq of ATP. \( \alpha \)-Helices are depicted in red and the mobile 20s loop in blue. B, overlay of both apo- (magenta) and ATP-bound (black) ensembles of calculated structures showing conformational changes within the loop regions in response to ATP binding. The first five disordered non-native N-terminal residues are excluded for clarity. C, ensemble of 25 structures showing the location of the ATP ligand. The adenine ring is either stacked (green) or in an open (red) conformation with respect to the side chains of Tyr-87 and Phe-133 (blue). The ribose is colored cyan, and as no constraints were imposed, the phosphate chain is not shown for clarity. The ensemble is super-positioned on a ribbon representation of the lowest energy conformer.
AMP and ADP as compared with ATP. There was no detectable change after the addition of an 80-fold excess of adenine. Notably, His-42 is perturbed identically in both AMP and ADP titrations as expected from the specific interaction with the P1 phosphate. In contrast to the ATP titration, however, the His-49 (H29) resonance is in fast rather than in slow exchange and is only slightly perturbed by ADP and not by AMP.

**DISCUSSION**

Human Ap4Aase can bind a wide range of ligands including various adenosine-5'-phosphorylated and adenosine-5'-phosphorothioylated polyols (37) and in addition can hydrolyze AZT-pA (3'-azido-3'-deoxythymidine-5'tetraphospho-5')-adenosine (38) and 5-phosphoribosyl 1-pyrophosphate (39). By comparing the structure of human Ap4Aase to the structure of the animal and plant counterparts from *C. elegans* and *L. angustifolius*, respectively, together with the results from the fragmentation assay, mechanistic and evolutionary insights into unraveling the binding characteristics of these enzymes can be established.

**Comparison with the X-ray Structure from *C. elegans*—**As the sequence identity between *H. sapiens* and *C. elegans* Ap4Aases is relatively high (46%), it is not surprising that the structures are globally similar (Fig. 6A) with an r.m.s.d. of 1.85 Å over nonloop regions (64 Cα atoms). Most important, both the orientation and conformation of the adenosine moieties are equivalent when the “stacked” state from the NMR ensemble is compared. As observed in the x-ray structure of the *C. elegans* enzyme and in the NMR structure of the cap-binding protein, elf4E bound to m7GDP (40), the base stacks between two aromatic side chains, the conserved Tyr-87 and Phe-133 in human Ap4Aase, and results in favorable π-π ring packing interactions (41). This equivalence in substrate-binding position in the two animal homologues substantiates the distinction between the animal and plant/bacteria-binding modes in solution. A comparison of the worm and human enzymes, however, reveals differences in loop regions in both backbone and side chain conformations.

First, there are clear differences in the backbone conformations particularly in the apo forms. In human Ap4Aase, the 80-s loop is drawn inward in the free state and opens in the presence of ATP, whereas in the *C. elegans* structure, there is much less observed change (Fig. 6, A and B). Similarly, the N-terminal residues differ only slightly in the x-ray structures of *C. elegans*, but in the human enzyme under solution conditions these residues have undergone a dramatic move outward from the cleft. This conformational change in the three native N-terminal residues may contribute to an energetically favorable stabilization of the binding loop, in the presence of ATP, as shown by the formation of an extension of the strand under the loop. This extension is manifested by the appearance of previously broadened amide resonances in the connecting strand. As this “stabilizing” terminus is partially dynamic in solution, it is proposed that it could facilitate a triggering interaction to destabilize the binding loop and could hence aid in product release. Triggering loops have been described recently (42) and play an important mechanistic role in the initiation of larger loop motions for binding and release of ligands.

Second, significant changes in the side chain conformations lining the substrate-binding cleft were observed in the *C. elegans* structures but were not observed in the current NMR structures of the human form (Fig. 6, A and B). Particularly noted in the x-ray structure is the rotation about χ1 of the aromatic ring from the base stacking pair (Tyr-121). The equivalent residue (Phe-133) in the current structure displays no such rotation. NOEs are observed from Ala-131 and Gln-132 to the ring of Phe-133 and not from Glu-135, suggesting a dominant and observed gauche- χ1 conformation, rather than trans as observed in the free state of the x-ray structure. Similarly, the equivalent side chain of His-42 rotates through 90° in the crystal structure upon substrate binding. Although the amide of His-42 is not observed in the apo form, intense NOE patterns are observed in both free and complex, specifically between the side chain of His-42 to both Thr-44 and Ala-38 and preclude a dominant trans-χ1 in the apo ensembles. In contrast, the side chain of Lys-94 corresponding to Lys-83 in *C. elegans* is not observed in the apo-structure, suggesting resonances are broadened due to intermediate exchange.

Third, a comparison of the two free structures reveals a dynamic difference in the irregular structure prior to and including the 110-s loop of the human counterpart, which is thought to bind one of several magnesium ions (20). No interpretable electron density is observed for residues in this loop in the x-ray structure of the worm homologue and was presumed disordered in the absence of ligand. Both the ensemble and the 15N steady-state NOE values (around 0.75) for this region indicate that it is clearly not significantly mobile in solution either in the apo or ATP-bound state. In the presence of ligand, a conserved Glu-103 in *C. elegans* (equivalent to Glu-115 in human) was observed to bind to one of a cluster of four metals that were postulated to be the driving force for the observed rigidity in the complex state. Hence, this may suggest a difference in the metal roles between human and *C. elegans* Ap4Aases or a reorganization of metal binding has occurred given that the E63A mutant was necessary for the solution study.

Finally, a larger 20-s loop is present in the human counterpart as a result of a six-residue insertion in the sequence (Fig. 1). This is flexible and does not appear to have any clear function but nevertheless is conserved in higher animals.

A recent detailed mutagenesis study (43) of the catalytic and binding residues has highlighted several important residues within the binding site in *C. elegans*. Mutations H31V (equivalent to His-42 in human) and K83M (equivalent to Lys-94 in human) had the largest effect on *Kₐ*, with corresponding increases to 110 and 140 μM from the observed value of 8.8 μM in the wild type enzyme. The *C. elegans* structure shows His-31 and Lys-83 positioned to hydrogen-bond to the P1 and P2 phosphate of the substrate, respectively. Chemical shift perturbation of His-42 in combination with the observed higher binding affinity of ATP and the weaker but equal binding of AMP and ADP is supportive of a P1′-His-42 hydrogen bond with little contribution to the affinity from the P2 phosphate. However, it is interesting to note the difference in side chain behavior of His-42 upon binding in the two enzymes because this appears to have a large role in binding affinity. Such side chain behavior infers differences in the mechanism of ligand binding between the *C. elegans* and human enzymes.

In the *C. elegans* x-ray structure, a number of residues in the substrate-binding site that are in direct contact with the ligand show distinct rotamer differences between the free and inhibitor complex. Hence a “trigger” event is proposed, but not identified, that would initiate such side chain rotations. Our alternative proposal is that this recognition trigger is not required, as critical side chains required for specificity (Phe-133) and affinity (His-42) are on average predisposed with the correct orientation to unite key interactions for binding.

A characteristic of all Nudix ApA hydrodases is their sensitivity to inhibition by fluoride (44). The structures of ApAase from *L. angustifolius* and the x-ray structure of RhoA-GDP bound to MgF₃ (45) imply that this is due to inhibition by a tightly bound NTP-MgF₃ complex in which the metallofluoride moiety adopts a trigonal planar arrangement. As this transi-
tion state complex is not observed with the addition of fluoride, this finding may implicate the Glu-63 either as the catalytic base or as a metal ligand. Notably, in the mutagenesis work on the Ap₄Aase homologue from *L. angustifolius* (46), the equivalent glutamate to glutamine mutation completely eliminated the sensitivity to fluoride inhibition, indicating that the formation of the transition state complex is prevented. In the structure of the RhoA-GDP-MgF₃ complex, the ligand coordinated to the catalytic water was not a glutamate but a glutamine, further suggesting that Glu-63 is not acting as the catalytic base.

**FIG. 6. Comparison of the other asymmetric Ap₄A hydrolases.** Overlay of the free (A) and complex (B) structures of Ap₄Aase from *C. elegans* (green) and human (magenta). Loop differences are highlighted in blue in the human enzyme. The adenosine moiety is displayed in yellow (*C. elegans*) and red (human). C, superposition of ribbon representations of the “free” (yellow) and complex (red) NMR structures of Ap₄Aase from *L. angustifolius* and the NMR structure from *H. sapiens*. The adenosine moiety is displayed in green and magenta for the plant and human enzymes, respectively. Selected side chains are labeled for the plant enzyme and colored either yellow (apo) or green (ATP-MgF₃ complexed) and are colored magenta for the human enzyme in complex with ATP.
but likely a magnesium ligand. By reference to the RhoA-GDP-MgF$_3$ complex, this glutamate would coordinate a second magnesium that is coordinated to the P2 phosphate and a fluoride ion but not directly to the MgF$_3$ moiety. These data support the notion that the mutation may have caused a structural change to metal ligation thus giving rise to the observed differences in the metal-binding 110-s loop upon ligand binding between the human E63A enzyme and the C. elegans enzyme.

**Comparison with the Solution Structure of Ap$_4$A Hydrolase from L. angustifolius**—A comparison of the plant and human enzymes is shown in Fig. 6C. As evident from the superposition of the structures, there are clearly two modes of binding. The adenine ring stacks in the human enzyme but in L. angustifolius is displaced almost 8 Å to the left and the inter-base angle is ~90°. The structures overlay quite well over 64 C$_\alpha$ atoms in the sheet and helices with an r.m.s.d. of 2.2 and 2.0 Å for the apo and complex structures, respectively.

Sequence conservation outside of the Nudix signature is low, but several residues that have been identified (1), including the aromatic residues of Tyr-87 and Phe-133 (in human), are conserved either as Tyr or Phe in all asymmetric Ap$_4$Aases. From the structures they clearly have different roles as Tyr-87 closely interacts with the substrate only in the animal type. Furthermore, a 13-residue insertion is observed in the 90-s loop of human that is common to plant and bacteria species (Fig. 1). This comprises a “frayed” helix in the apo-structure from L. angustifolius (18) that becomes well defined on complex formation (19). This helix may represent a necessary “structural motif” that is the source of the different binding modes. In addition, there is a conserved lysine between the last two helices that is found only in the plant-bacteria class and appears to be important for substrate binding (19). In the structure of apo Ap$_4$Aase from L. angustifolius, the rings Tyr-82 and Phe-149 are reasonably positioned ~7.8 Å apart to adopt a role equivalent to the animal binding model. By comparing the animal and plant structures, however, several factors can be proposed that disfavor this mode over that which is observed.

First, the conserved Lys-150 in the plant/bacteria class seems to be a key residue (Fig. 6C). It is part of a three-residue loop insertion and is structurally equivalent to the ring stacking Phe-133 in human Ap$_4$Aase. As a consequence, it displaces the equivalent and conserved Phe-149 ring outward by ~4 Å, facilitating edge-on packing against the adenine and disfavoring ring stacking. It is notable that the longer final helix in plant has a distinct bend at this position, in comparison with the human counterpart (a φ angle of ~130° is observed). In the Ap$_4$Aase from L. angustifolius the side chain of Lys-150 favorably packs against one edge of the adenine and could provide both significant cation-π interaction (47) with the N7 side of the adenine and further hydrogen-bond to the P1 phosphate via the side chain NH$_3$. In the latter, it would play the important role equivalent to His-42 in the human enzyme. In addition, the residue equivalent to His-42 in plant is not charged (Ala or Leu), and instead the loop is oriented rather to form a hydrophobic face that packs against the adenosine, again without substantial movement. Structural differences are observed around this region, and the 40-s loop is more open in the apo form of L. angustifolius. This loop difference may originate back to the conserved and geometrically restrictive Pro-45–Pro-46 pair in the animal sequences (replaced by hydrophobic Pro in plant). The second proline is thought to play a role in the general bending of the strand (36), and the Pro-Pro combination could account for the increase in the bend of the strand leading out from the 40-s loop. As the above favorable interactions between the lysine and the ATP could conceivably occur in the human enzyme, the unfavorable reorientation of the ring stacking Phe-133 is likely the dominant factor. Furthermore, although this lysine is conserved in the majority of Ap$_4$Aases, it is an alanine in C. elegans. Second, the packing environment of the external helix of the plant enzyme would be significantly altered if substrate binding similar to the animal enzyme were to occur. Hydrophobic side chain burial would be reduced between the 40-s loop, which was previously mediated by the bridging adenine ring. When the ring stacking mode of binding is modeled onto the apo plant structure, the buried surface area is in the order of 150 Å$^2$ (similar to that observed in C. elegans ~107 Å$^2$) in comparison to that observed in the plant complex where it is 485 Å$^2$. Furthermore, the downstream bulky Phe-84 (generally conserved aromatic or bulky hydrophobic in the plant/bacteria enzymes, Ala-89 in the human) would have steric clashes with the ribose moiety in this configuration and would further hinder cleft closure.

The differing binding modes can be attributed to a subtle insertion in the loop between the last two helices, a large helix insertion and a bulky aromatic residue, all of which are unique to the plant/bacteria class. Clearly, the different mode of binding is exclusive to each class, and this finding is identified by the current structural evidence and is unequivocally supported by characteristic ring shift data established herein. Accordingly, there are no ring current shifts observed for either of the two potential stacking rings in the enzyme from L. angustifolius (48) compared with the large shifts observed in the human structure upon ATP binding. Furthermore, both the ATP and ATP-MgF$_3$ complexes gave very similar amide chemical shifts in the structures from L. angustifolius (19), indicating that both ATP and ATP-MgF$_3$ bind with equivalent structures, but the ATP-MgF$_3$ gave a tighter complex required for detailed structural studies. Hence, it is valid to have compared not only the aromatic chemical shifts but also the ATP-MgF$_3$ structure from L. angustifolius with the ATP-bound structure from H. sapiens.

**Conclusion**—The structures of both free and ATP-bound human Ap$_4$Aase have been determined by NMR spectroscopy to high resolution. A critical aspect of this work was the preparation of a mutant construct that gave soluble monomeric protein that was amenable to NMR spectroscopy. The inability to form an ATP-MgF$_3$ transition state complex supports the notion that the enzyme has a requirement to bind at least two magnesium ions in which Glu-63 would coordinate a second magnesium but not the MgF$_3$ moiety directly. The free and ATP-bound enzymes show major differences in conformation around the cleft implying a partially plastic binding subsite that is further supported by the absence of 11 amide resonances in the spectra of the free structure. Although the substrate position is equivalent to that observed in the counterpart from C. elegans, in solution key side chains are not observed to reorient upon ligand binding. Furthermore, titration of adenosine, AMP, and ADP implies that both the P1 and P3 but not the P2 phosphate contribute to the binding affinity and that the P1 hydrogen-bond partner is the side chain of His-42. Most importantly, the orientation of this residue and the adenine ring stacking pair (Tyr-87 and Phe-133) are all favorably predisposed on average to facilitate binding in the free structure without the need for reorientation as observed in the x-ray structure. This duality of a plastic binding site in combination with a propensity for a significant population of conformers within the equilibrium ensemble to possess key side chains directed for substrate recognition would be functionally favorable. It explains how human Ap$_4$Aase can bind a range of substrates and furthermore provides an alternative mechanism for substrate recog-
nition to that inferred from the x-ray counterpart. The current mechanism does not support the need for a trigger event to bring about important side chain reorientations required for recognition as postulated in the C. elegans study. Indeed, it is proposed that the motion observed in the N-terminal residues in the ATP-bound state may rather be a mechanism that could trigger product release by destabilizing the binding loop and interactions therein to the product.

Comparing structures of the enzymes from the animal and plant classes unambiguously verifies a genuine distinction in the mode of substrate binding. The origin of this distinction is proposed based on the roles of a conserved lysine (Lys-150), a bulky hydrophobic residue (Phe-84), and an external helix motif (helix II) that are conserved only in the plant/bacteria class. This distinction in conjunction with the detailed knowledge of structure, mechanism, and contributions to ligand affinity presented herein provide invaluable information for the design of therapeutics such as anti-invasives selectively against the plant/bacterial enzymes.

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