Disease-Associated Mutations Inactivate AMP-Lysine Hydrolase Activity of Aprataxin*

Heather F. Seidle, Pawel Bieganowski, and Charles Brenner¶

From the Departments of Genetics and Biochemistry and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, New Hampshire 03756

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¶To whom correspondence should be addressed: Norris Cotton Cancer Center, Dartmouth Medical School, Rubin 733—HB7937, Lebanon, NH 03756, Tel: 603-653-9922; Fax: 603-653-9923; E-mail: charles.brenner@dartmouth.edu.
Ataxia-oculomotor apraxia syndrome 1 is an early onset cerebellar ataxia that results from loss of function mutations in the APTX gene, encoding Aprataxin, which contains three conserved domains. The forkhead associated domain of Aprataxin mediates protein-protein interactions with molecules that respond to DNA damage but the cellular phenotype of the disease does not appear to be consistent with a major loss in DNA damage responses. Disease-associated mutations in Aprataxin target a histidine triad domain that is similar to Hint, a universally conserved AMP-lysine hydrolase, or truncate the protein N-terminal to a zinc finger. With novel fluorogenic substrates, we demonstrate that Aprataxin possesses an active-site dependent AMP-lysine and GMP-lysine hydrolase activity that depends additionally on the zinc finger for protein stability and on the forkhead associated domain for enzymatic activity. Alleles carrying any of eight recessive mutations associated with ataxia and oculomotor apraxia encode proteins with huge losses in protein stability and enzymatic activity, consistent with a null phenotype. The mild presentation allele, APTX-K197Q, associated with ataxia but not oculomotor apraxia, encodes a protein with a mild defect in stability and activity while enzyme encoded by the atypical presentation allele, APTX-R199H, retained substantial function, consistent with altered and not loss of activity. The data suggest that the essential function of Aprataxin is reversal of nucleotidylated protein modifications, that all three domains contribute to formation of a stable enzyme, and that the in vitro behavior of cloned APTX alleles can score disease-associated mutations.
Ataxia-oculomotor apraxia (AOA) has been characterized as a unique disorder since 1988 (1). Found in many ethnic backgrounds but most frequently diagnosed in Japan and Portugal, AOA is typically an autosomal recessive, early onset cerebellar ataxia with abnormal movements of the head and eye, late neuropathy, long survival, hypercholesterolemia, hypoalbuminemia and hyperlipidemia. Families with a disease now termed ataxia-oculomotor apraxia 1 (AOA1; MIM 208920) have mutations in the APTX gene (MIM 606350) at 9p13.3, which encodes Aprataxin (2,3). Recently, four family members diagnosed with coenzyme Q deficiency and cerebellar ataxia have been shown to harbor Aprataxin mutations (4). AOA2 (MIM 606002) individuals (5) and children with an early onset form of amyotrophic lateral sclerosis (6) have mutations in an unrelated gene termed ALS4/SETX (MIM 608465) at 9q34, which is thought to encode a helicase.

Aprataxin is a ubiquitously expressed nuclear protein (7) translated from several splice variants. The predominant message encodes a primary translation product of 342 amino acids (8), though transcripts encoding forms of Aprataxin of 168 (2) and 356 amino acids (9) have been reported. As shown in Fig. 1, Aprataxin contains three domains: a forkhead associated (FHA) domain that resembles that of the vertebrate polynucleotide kinase, a histidine triad (HIT) domain, and a C-terminal zinc finger domain (2,3). The FHA domain is a phosphoprotein binding motif found in many proteins involved in DNA damage responses (10). Indeed, consistent with a possible role in single-strand break repair, Aprataxin has been reported to associate with Xrcc1, PARP-1, p53 (7) and Xrcc4 (11) and lead to mild sensitivity to hydrogen peroxide (7,11). However, in contrast to the striking defects in DNA damage responses of cultured cells
with *atm* mutations (for a recent review, see (12)), cultured *aptx*-mutant cells do not exhibit defects in ATM signaling or radioresistant DNA synthesis (11). The potential relationships between other biochemical pathways, such as co-enzyme Q deficiency (13) or glycogen synthase 3 signaling (14), and Aprataxin function has not been investigated.

HIT domains, named for a motif related to His-φ-His-φ-His-φ-φ, where φ is a hydrophobic amino acid, are found in a superfamily of nucleotide hydrolases and nucleotide transferases that includes the universally conserved Hint enzyme, the Fhit tumor suppressor protein, and galactose-1-phosphate uridylyltransferase (15). Contrary to assertion (2,16), the HIT domain of Aprataxin is more similar to the Hint branch of the HIT superfamily than to Fhit (15,17). Whereas Fhit-homologous enzymes hydrolyze diadenosine polyphosphates such as ApppA and AppppA into mononucleotides (18,19), Hint-homologous enzymes have extremely low activity on such substrates and instead hydrolyze model substrates in which AMP is joined by a phosphoramidate linkage to the ε amino group of lysine (20,21). Recently, in work to characterize a potential role for Hint hydrolases in regulating sexual differentiation in birds (22), we developed an adenylylated lysine substrate linked to aminomethylcoumarin that allows Hint enzymatic activity, coupled to trypsin cleavage, to produce a bright fluorescent product (23). In yeast, the enzymatic activity of the Hint homolog, Hnt1, functions as a positive regulator of components of general transcription factor TFIH (20). In *E. coli*, the Hint-homologous *hinT* gene is required for resistance to elevated levels of certain salts (24). Though specific protein targets remain to be identified, it is has been hypothesized that Hint hydrolases may reverse nucleotidylylated protein modifications of lysine (15).

Two specific models linking Aprataxin inactivation to disease have been
proposed. According to the first model, diadenosine polyphosphate hydrolysis is required for DNA repair (2). However, because intact, purified Aprataxin was reported to be devoid of diadenosine polyphosphate hydrolase activity (16) and there is no known connection between diadenosine polyphosphate hydrolysis and DNA repair, this model appears to be unlikely. According to the second model, Aprataxin stabilizes Xrc1 by a physical association between phosphorylated Xrc1 and the FHA domain of Aprataxin (25). This model appears to be undermined by the lack of X-ray sensitivity of aptx fibroblasts (11) and the fact that disease-associated mutations in APTX do not alter the ability of Aprataxin to associate with Xrc1 (25). Here we demonstrate that Aprataxin possesses an intrinsic, active site dependent AMP-lysine and GMP-lysine hydrolase activity. AMP-lysine hydrolase activity on a model substrate was also greatly reduced by expression of Aprataxin without the N-terminal FHA domain and eliminated by truncation of the zinc finger domain, indicating that all three domains contribute to formation of a stable enzyme. Finally, biochemical analysis indicated that eight reported disease-associated alleles are null or nearly null for the Hint active site while a reported mild presentation allele and an atypical presentation allele can be functionally diagnosed by in vitro stability and activity.
EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—E. coli strain BL21* and DH10B were used for cloning. Plasmid pB352 and derivatives were used for expression of human APTX (11). Site directed mutagenesis of plasmid pB352 was used to create plasmids to express APTX alleles K197Q, A198V, R199H, P206L, H260A, V263G, D267G, W279R, W279X, 689insT and 840delT using primers listed in Supplemental Table 1. The 168 amino acid Aprataxin coding sequence was produced by adding an NdeI site by site-directed mutagenesis using primer 7779 and amplification by PCR with primers 0720 and 0721. The construct was recovered from ligation of an NdeI and XhoI digestion of the resulting PCR product. All constructs were confirmed by DNA sequencing.

Enzyme Expression and Purification—E. coli strain BB2-1 was used for protein expression. BB2-1 was produced by disrupting the E. coli hinT gene in strain BL21* as described (26). Primers 7024 and 7025 were used for PCR amplification of the chloramphenicol resistance marker of plasmid pKD3. Stable chloramphenicol-resistant transformants of BL21* were tested by PCR with primers 7026 and 7027 to confirm correct recombination of the chloramphenicol resistance marker into the hinT locus. E. coli transformants carrying APTX expression plasmids were aerated at 24°C. At an OD_{600} of 0.5, expression was induced with 100 µM isopropyl-β-D-thiogalactopyranoside. Cultures were grown 16 hr and harvested by centrifugation. Frozen (-80°C) and thawed cell pellets (~5g wet weight) were lysed using Bugbuster (Novagen) with DNase I and EDTA-free Complete Protease Inhibitor cocktail (Roche). Clarified lysates were loaded on 1.5 ml Talon columns (Clontech). Aprataxin proteins were eluted with 10 ml of 250
mM imidazole, 10 mM HEPES, pH 7, and concentrated into 100 mM NaCl, 10 mM HEPES, pH 7.2 with 10 kDa cutoff centrifugal concentrators (Amicon). Proteins were transferred to nitrocellulose membranes and detected using an anti-penta-His antibody conjugated to horseradish peroxidase (Qiagen) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Enzyme Assays**—The fluorogenic substrate tBoc-LysAMP-MCA was synthesized as described (23). Synthesis of tBoc-LysGMP-MCA is reported in the Supplemental Material. tBoc-LysAMP-MCA and tBoc-LysGMP-MCA hydrolytic activities were assayed in 25 µl volumes containing 2.5 to 250 µM of substrate, 12 nmol of wild-type or 12-60 nmol mutant enzyme, 100 mM NaCl, 10 mM HEPES, pH 7.2, for 30-60 min at room temperature. Reactions were initiated by the addition of substrate and stopped by addition of 75 µl of 80 mg/ml trypsin. After a 10 min incubation with trypsin, fluorescence (excitation 355 nm, emission 460 nm) was measured with a Wallac 1420 Multilabel counter. pH and divalent cation analyses (Supplemental Material) were completed in 10 mM HEPES, with pH from 6.8 to 8.2. GpppBODIPY hydrolysis was assayed as described previously (19). Assays were initiated by addition of 12 nmol of enzyme and stopped after 30 to 60 min by the addition of Na Citrate, pH 3. GpppBODIPY concentration ranged between 0 and 25 µM. Dinucleoside polyphosphate hydrolysis assays were performed using 60 nmol of wild-type enzyme with 5-200 µM substrate in a 50 µl volume. Assays were incubated at room temperature for 30 min and stopped by addition of 50 µL Na₂CO₃, pH 11.6. 50 µl of the reaction mixture was injected onto a strong anion exchange column (Princeton Chromatography) equilibrated with 10 mM K₂HPO₄, pH 2.58 (Buffer A) on a Varian Prostar HPLC. The elution program was
100% A for 10 min, a 2 min gradient to 100% 750 mM K$_2$HPO$_4$, pH 2.58 (Buffer B), and 100% B for 6 min. Elution times for AMP, ADP, ATP, ApppA, and AppppA were 6.7 min, 15.3, 16.2, 15.7, and 16.3, respectively. Product amounts were determined from the peak areas and a standard curve of AMP using Varian Galaxie Software. Assays to determine divalent cation effects (Supplemental Material) were completed as described above with the addition of 0.5 mM MgCl$_2$. All assays were performed at least in triplicate.
RESULTS

*Aprataxin Exhibits AMP and GMP-Lysine Hydrolase Activity*—The 342 amino acid *APTX* cDNA was fused to an amino-terminal His-tag and the resulting protein was purified to homogeneity by metal chelate affinity chromatography. The resulting enzyme was assayed for activity with tBoc-AMPLys-MCA, a model substrate containing an adenylylated lysine (23), as well as a newly synthesized tBoc-GMPLys-MCA substrate, and the Fhit substrates GpppBODIPY (19), ApppA and AppppA (Table 1). To avoid contamination by the *E. coli* Hint hydrolase, wild-type and all mutant *APTX* constructs were expressed in and purified from *E. coli* strain BB2, which contains a deletion for the Hint-homologous *hinT* gene (24).

Aprataxin exhibits strong *k*\(_{cat}\)-discrimination against hydrolysis of the dinucleoside polyphosphates ApppA and AppppA and the dinucleoside polyphosphate analog GpppBODIPY, with 30-fold, 3-fold and 7-fold lower turnover rates with these compounds, respectively, than the turnover rates against substrates with nucleotidylylated lysine residues. The enzyme exhibited relatively less *K*\(_M\)-discrimination between the nucleotide substrates presented to it and, in fact, displayed a preference for the dinucleoside polyphosphates and their analogs (13 to 39 µM) versus substrates containing nucleotidylylated lysines (47 and 116 µM). The relatively high *K*\(_M\) values for tBoc-AMPLys-MCA and tBoc-GMPLys-MCA did not offset their advantages in the *k*\(_{cat}\) term. As shown in Table 1, on the basis of the specificity constant, *k*\(_{cat}\)/*K*\(_M\), Aprataxin is an AMP-lysine hydrolase whose activity is limited by the relatively high *K*\(_M\) values of the model peptide substrates presented to it.
Metal-Independence, Active Site-Dependence, FHA Domain-Dependence and pH-Dependence of the AMP-Lysine Hydrolase Activity of Aprataxin—Fhit enzymatic activity on dinucleoside polyphosphate substrates is magnesium-dependent (27) but Hint enzymatic activity is metal-independent (23). Consistent with biochemical similarity to Hint, none of the observed activities on nucleotidylylated lysine substrates or dinucleoside polyphosphate substrates were inhibited by EDTA or stimulated by magnesium (Supplemental Fig. 1).

We constructed a H260A allele, targeted to the middle His residue of the HIT motif, to determine whether all of the AMP-lysine hydrolase activity is due to the Hint-homologous active site of Aprataxin. As shown in Table 2, the H260A substitution eliminates all detectable activity, demonstrating that the AMP-lysine hydrolase activity of Aprataxin depends on the Hint-homologous active site.

As shown in Fig. 1, an Aprataxin transcript capable of encoding a 168 amino acid form, containing the Hint and zinc finger domains but not the FHA domain is reportedly expressed (2). To test whether the 168 amino acid form might contribute significantly to cellular Aprataxin activity, we expressed and purified this molecule. As shown in Table 2, loss of the FHA domain reduced $k_{cat}/K_{m}$ by 200-fold on the model substrate, tBoc-AMPLys-MCA.

Using the coupled assay initially developed for Hint (23), in which trypsin activity is not limiting, we determined the pH-dependence of $k_{cat}$, $K_s$ and $k_{cat}/K_m$ for the Aprataxin-dependent AMP-lysine hydrolysis step of tBoc-AMPLys-MCA hydrolysis. The data indicate that $k_{cat}$ declines and $K_s$ is elevated with increasing pH. The pH-
dependence of $k_{cat}/K_m$ allowed enzyme activity to be modeled with a single titratable group ($pK_a = 7.6$) that functions in the protonated form (Fig. 2).

**Disease-Associated Mutations Destabilize Aprataxin and Inactivate the Hydrolase**—As a dual test of whether tBoc-AMPLys-MCA hydrolytic activity is important for Aprataxin activity and whether all of the reported disease-associated mutations in Aprataxin have biochemical defects consistent with disease causation, we generated point mutations in the 342 amino acid $APTX$ cDNA and expressed and purified each polypeptide from a hinT mutant strain of *E. coli*. Ten such mutant proteins, alongside the H260A active site mutant, the full-length wild-type and the 168 amino acid forms, were analyzed by SDS-PAGE and Western using reactivity to the N-terminal His-tag (Fig. 3) and for hydrolysis of tBoc-AMPLys-MCA (Table 2). In short, eight $APTX$ mutations associated with ataxia and oculomotor apraxia produced a protein with strikingly lower stability and lower enzymatic activity than did the wild-type polypeptide. The remaining two disease-associated mutant proteins were notable exceptions whose biochemical phenotypes are consistent with atypical disease presentations.

As shown in Fig. 1, all known disease-associated mutations occur in the Hint domain or immediately C-terminal to the Hint domain. Four missense mutations have been reported in a 10 amino acid stretch from K197 to P206 (2,3,28-30). Three missense mutations have been reported in a 17 amino acid stretch from V263 to W279 that begins in the His-ϕ-His-ϕ-His-ϕ-ϕ motif (2,30). Conceptual translation of the frequently identified insertional mutant, 689insT (2,3,28), produces a frameshift and truncation between the two clusters of missense mutations within the Hint domain. The deletion mutant, 840delT (2), introduces a frameshift immediately after the W279R missense
codon (30), which is also the site of truncation in W279X, the Portuguese founder allele (3,29,30) that has been more recently identified in an American family with coenzyme Q deficiency and cerebellar ataxia (4). The Tunisian splice site alteration, IVS7+1, is also expected to truncate the protein N-terminal to the zinc finger (31). Thus, the 689insT allele is a Hint domain truncation while the 840delT, W279X and IVS7+1 alleles are zinc finger domain truncation mutants.

As analyzed by Coomassie-stained SDS-PAGE and Western (Fig. 3), five of seven of the point mutants were grossly destabilized, producing little full-length protein. Proteins expressed from these alleles were predominantly a ~19 kDa form that retained the N-terminal His-tag, which indicates that the stable form of these polypeptides retained the FHA domain but not the Hint domain. Consistent with this interpretation, the purified A198V, P206L, V263G and D267G mutant proteins had no detectable catalytic activity on tBoc-LysAMP-MCA (Table 2). The W279R mutant, which retained some full-length polypeptide, had a barely detectable catalytic activity, reduced 160-fold from that of wild-type. Coupled with the inactivity of the active-site mutant H260A allele, which is physically stable but devoid of activity, the severe presentation of all of these alleles suggests that loss of the Hint domain renders the Aprataxin polypeptide null for biological activity.

Atypical Disease-Associated Mutations Retain Substantial Stability and Activity—
The two point mutants, K197Q and R199H, which were least destabilized on the basis of SDS-PAGE and Western, also retained the greatest enzymatic activities, 27% and 45%, respectively. The K197Q allele has not been found as a homozygous mutation in any
patient with a severe AOA1 presentation but rather in a brother and sister who also carry the normally severe W279X allele but have a late onset ataxia, lacking oculomotor apraxia and mental retardation (29). The partial stability of the K197Q polypeptide and the partial activity of the resulting enzyme clearly indicate that this allele is not a null for Aprataxin activity. Thus, the data explain how the K197Q allele masks the appearance of the early onset phenotype associated with W279X, which our analysis indicates is essentially a null.

As shown in Fig. 3, apart from the non-disease associated active-site mutant, H260A, which encodes an extremely stable but inactive protein, the Portuguese R199H substitution mutant encodes the most stable disease-associated mutant protein. However, unlike the active-site mutant protein, the R199H protein retains substantial enzymatic activity on the model substrate (Table 2). Consistent with the lack of a null biochemical phenotype, the R199H allele is associated with an atypical presentation that includes dystonia and masklike faces (3). Though more specific substrates will be required to catalog novel enzymology of the R199H protein, the substantial stability and activity of the R199H enzyme distinguish this allele from the biochemically hypomorphic or null features of all other disease-associated proteins.

As shown in Fig. 3 and Table 2, the three alleles that result in Hint domain or zinc finger domain truncations were each proteolyzed to a stable ~19 kDa N-terminal fragment lacking the Hint domain, which resulted in a complete loss of enzymatic activity. By these criteria, the zinc finger domain is essential for formation of a stable polypeptide such that the C-terminal truncations depicted in Fig. 1 may all be considered nulls. By contrast, expression of the 168 amino acid form of Aprataxin produced a stable
polypeptide retaining some activity. These data indicate that the FHA domain is not required for folding or stability of the Hint domain but may function in targeting substrates.
DISCUSSION

We suggest that because Aprataxin coding sequences emerged in cells that already possessed canonical Hint hydrolases, which have much higher activity on model nucleotidylylated lysine substrates (20,21,23), the Hint domain of Aprataxin may have evolved to reduce specificity on generic Hint substrates while its FHA domain may have evolved to target the active site to specific protein substrates. Incorporation of peptides targeted toward the FHA domain of Aprataxin into substrates such as tBoc-AMPLys-MCA and analysis of Aprataxin-interacting proteins as potential Aprataxin substrates will be necessary to test this hypothesis.

The fact that all disease-associated mutations of APTX are targeted to the Hint hydrolase domain or the zinc finger domain, which we determine to be required for stability of the Hint hydrolase domain, is strong evidence that enzymatic activity is required for function of Aprataxin. Moreover, by our criteria, the A198V (30), P206L (2,3,28), V263G (2), D267G (30), 689insT (2,3), W279X (3,4,29,30) and 840delT (2) alleles are functionally null; the W279R (30) allele is severely hypomorphic; and the K197Q allele, which was reported to present mildly (29), is partially functional.

Moreover, the substantial function and stability of the R199H enzyme suggest that the dystonia and masklike face presentations associated with aptx-R199H do not arise from simple loss of Aprataxin function (3).

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## Table 1

**Substrate Specificity of Aprataxin**

| Substrate            | $k_{cat}$ (s$^{-1}$) | $K_M$ (µM) | $k_{cat}/K_M$ (s$^{-1}$M$^{-1}$) |
|----------------------|----------------------|------------|----------------------------------|
| tBoc-LysAMP-MCA      | 0.0028 ± 0.0002      | 47 ± 9     | 60 ± 22                          |
| tBoc-LysGMP-MCA      | 0.003 ± 0.0004       | 116 ± 24   | 26 ± 17                          |
| GpppBODIPY           | 0.0004 ± 0.00005     | 13.1 ± 4.7 | 31 ± 11                          |
| ApppA                | 0.00008 ± 0.00002    | 21 ± 1.6   | 3.8 ± 1.3                        |
| AppppA               | 0.0009 ± 0.00005     | 39 ± 6     | 23 ± 8.3                         |
### Table 2

Enzymatic Activity of Expressed APTX Transcripts and Alleles with tBoc-LysAMP-MCA

| Enzyme     | $k_{cat}$ (sec$^{-1}$) | $K_M$ (µM) | $k_{cat}/K_M$ (sec$^{-1}$ M$^{-1}$) | Transcript or Allele Reference |
|------------|------------------------|------------|-----------------------------------|--------------------------------|
| Aptx-342   | 0.003 ± 0.0002         | 47 ± 9     | 64 ± 22                           | (2,3)                          |
| Aptx-168   | 0.00006 ± 0.000005     | 187 ± 52   | 0.3 ± 0.1                         | (2)                            |
| K197Q      | 0.004 ± 0.0002         | 241 ± 40   | 17 ± 5                            | (29)                           |
| A198V      | < 0.00002              |            |                                   |                                 |
| R199H      | 0.004 ± 0.0002         | 139 ± 18   | 29 ± 11                           | (3)                            |
| P206L      | < 0.00002              |            |                                   |                                 |
| H260A      | < 0.00002              |            |                                   |                                 |
| V263G      | < 0.00002              |            |                                   |                                 |
| D267G      | < 0.00002              |            |                                   |                                 |
| W279R      | 0.000007 ± 0.000002    | 194 ± 156  | 0.4 ± 0.1                         | (30)                           |
| 689insT    | < 0.00002              |            |                                   |                                 |
| W279X      | < 0.00002              |            |                                   | (3,4,29,30)                    |
| 840delT    | < 0.00002              |            |                                   |                                 |
Fig. 1
Fig. 2

- **Figure 1**: Graph showing $k_{cat} (S^{-1} \times 10^3)$ vs. pH. The data points are spread across a pH range from 6.5 to 8.5 with a trend indicating a decrease in $k_{cat}$ as pH increases.

- **Figure 2**: Graph showing $K_m (\mu M)$ vs. pH. The data points are spread across a pH range from 6.5 to 8.5 with a trend indicating an increase in $K_m$ as pH increases.

- **Figure 3**: Graph showing $k_{cat}/K_m$ (S$^{-1}$ M$^{-1}$) vs. pH. The data points are spread across a pH range from 6.5 to 8.5 with a trend indicating a decrease in $k_{cat}/K_m$ as pH increases.
Fig. 3

A

| 10 | 25 | 35 | 75 | 120 |
|----|----|----|----|-----|
| Aptx-342 |
| Aptx-168 |
| K197Q |
| A198V |
| R199H |
| P206L |
| H260A |
| V263G |
| D267G |
| W279R |
| 689insT |
| W279X |
| 840delT |

B

| 15 | 30 | 50 | 75 |
|----|----|----|----|
| Aptx-342 |
| Aptx-168 |
| K197Q |
| A198V |
| R199H |
| P206L |
| H260A |
| V263G |
| D267G |
| W279R |
| 689insT |
| W279X |
| 840delT |
Legends to Figures

Fig 1. Domain structure and disease-associated alleles of Aprataxin

Important features are indicated: forkhead associated domain (FHA); nuclear localization signal (NLS); Hint domain; and zinc finger. Disease-associated frameshifts, truncations, splice site alterations and amino acid substitutions are indicated at their approximate positions in the amino acid sequence.

Fig. 2. pH profiles of Aprataxin hydrolysis of tBoc-LysAMP-MCA

tBoc-LysAMP-MCA hydrolysis activity was assayed in 25 μl volumes containing 12 nmol of enzyme, 10 mM HEPES pH 6.8-8.2, 100 mM NaCl, for 30-60 min at room temperature. Kinetic values were determined from Michaelis-Menten plots using non-linear regression. pH profiles for A) $k_{cat}$, B) $K_M$ and C) $k_{cat}/K_M$ are provided.

Fig. 3. Analysis of expressed and purified APTX transcripts and alleles

Expressed and purified proteins were analyzed by A) SDS-PAGE and B) Western. Predicted sizes of recombinant His-tagged Aprataxin-342, Aprataxin-168, 689insT, W279X and 840delT alleles are 40.9, 21.7, 32.1, 33.4, and 34.4 kDa, respectively.
Supplemental Material

Analysis of tBoc-LysGMP-MCA Synthesis

$^1$NMR (500mHz, D$_2$O, 40°C) $\delta$ 7.83 (d, $J$=2.0 Hz, 1H), 7.95 (s, 1H), 7.77 (d, $J$=8.6 Hz, 1H), 7.53 (d, $J$=8.8 Hz, 1H), 7.29 (d, $J$=7.5 Hz, 1H), 6.26 (d, $J$=1.3 Hz, 1H), 5.78 (d, $J$=4.9 Hz, 1H), 4.57-4.61 (m, 1H), 4.34-4.38 (m, 1H), 4.15-4.19 (m, 1H), 3.85-4.01 (m, 4H), 2.61-2.73 (m, 2H), 1.78 (s, 3H), 1.58-1.69 (m, 2H), 1.51-1.55 (m, 2H), 1.35-1.45 (m, 9H), $^{31}$P NMR (300mHz, DMSO-$d_6$) $\delta$ = -0.062

Negative polarity MALDI: peak at 749.01, calculated peak 750.

Supplementary Table 1. Primers used in this study.

| Primer Name | Primer Sequence (Primers are 5'-3') | Mutation |
|-------------|-------------------------------------|----------|
| 7024        | GCGCGGCGCTAGCATGGCGCAACGCATGGCTATTTGAA AAAGGAATGGCTGGAGTCGGCTGCTGC |          |
| 7025        | ATGATCAGACGATAGCCATATCCCTCG | | |
| 7026        | GATGGTGTGAAACCTTGTC | | |
| 7027        | CTCGAACATCATCTATAGTGCG | | |
| 7779        | CAAGGCTTGAAGATTTATATGCGAGGCACCCAAA | NdeI site |
| 0720        | GATTCATATGCAGACC | Aptx-168 |
| 0721        | GGCTCGAGTCATGTG | Aptx-168 |
| 0730        | GATAAACATTACCCAGGGCCG | K197Q |
| 0731        | CGGGCCTGTGCTGTATTTATC | K197Q |
| 0722        | CCCTAAGGTCCTGTAC | A198V |
| 0723        | GCTAAGGACCTTTGGG | A189V |
| 0732        | CAAAGGCCATTCCCATGTC | R199H |
| 0733        | GCCATGTGATATGGGACCTTGTG | R199H |
| 7780        | CATTGGCTGCTCTCCTGACCTCCATTTCC | P206L |
| 7773        | AGTATGAGCCATGATGCTCTCATGTCAGC | H260A |
| 0734        | CATCTTCATGGGATCGCCAG | V263G |
| 0735        | CTGGCTGATCCCATGAAGATG | V263G |
| 0728        | CAGCCAGGTTTGTAGTC | D267G |
| 0729        | GAATCAAAAACCCTGGGCTG | D267G |
| 7781        | AAAAACAAAAAACATAGGAATTCTTCAATAACA | W279R |
| 7782        | AAAAACAAAAAACATAGGAATTCTTCAATAACA | W279X |
| 0724        | GCACACTGTTGGGGGAAAAG | 689insT |
| 0725        | CTTTCCCCACAGCTGTGC | 689insT |
| 0726        | CATTGGAATCTTTCAATAAC | 840delT |
| 0726        | GTATTGGAAGATCTCATTAC | 840delT |
Supplemental Figure 1. Effects of metals on Aprataxin hydrolysis of tBoc-LysAMP-MCA and AppppA. tBoc-LysAMP-MCA hydrolysis was assayed in 25 μl volumes containing 12 nmol of enzyme, 10 mM HEPES pH 7.2, 100 mM NaCl and 3 mM EDTA, 1 mM MgCl₂ or 1 mM MnCl₂ for 30 min at room temperature. Fluorescence was counted following a 10 min trypsin digestion. AppppA hydrolysis was assayed using 60 nmol of wild-type enzyme with 5-200 μM substrate with or without 0.5 mM MgCl₂ in a 50 μL volume. Assays were incubated at room temperature for 30 min and stopped by addition of 50 μL Na₂CO₃, pH 11.6. 50 μL of the reaction was analyzed by HPLC.
Disease-associated mutations inactivate AMP-lysine hydrolase activity of aprataxin
Heather F. Seidle, Pawel Bieganowski and Charles Brenner

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