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Nicholas E. Burgis
Eastern Washington University, nburgis@ewu.edu

Caitlin April
Eastern Washington University, capril@ewu.edu

Kandise VanWormer
Eastern Washington University

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Brief communication

Arginine-178 is an essential residue for ITPA function

Nicholas E. Burgis *, Caitlin April, Kandise VanWormer

Department of Chemistry, Biochemistry & Physics, Eastern Washington University, Cheney, WA, 99004, USA

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ABSTRACT

The inosine triphosphate pyrophosphatase (ITPA) enzyme plays a critical cellular role by removing noncanonical nucleoside triphosphates from nucleotide pools. One of the first pathological ITPA mutants identified is R178C (rs746930990), which causes a fatal infantile encephalopathy, termed developmental and epileptic encephalopathy 35 (DEE 35). The accumulation of noncanonical nucleotides such as inosine triphosphate (ITP), is suspected to affect RNA and/or interfere with normal nucleotide function, leading to development of DEE 35. Molecular dynamics simulations have shown that the very rare R178C mutation does not significantly perturb the overall structure of the protein, but results in a high level of structural flexibility and disrupts active-site hydrogen bond networks, while preliminary biochemical data indicate that ITP hydrolyzing activity is significantly reduced for the R178C mutant. Here we report Michaelis-Menten enzyme kinetics data for the R178C ITPA mutant and three other position 178 ITPA mutants. These data confirm that position 178 is essential for ITPA activity and even conservative mutation at this site (R178K) results in significantly reduced enzyme activity. Our data support that disruption of the active-site hydrogen bond network is a major cause of diminished ITP hydrolyzing activity for the R178C mutation. These results suggest an avenue for developing therapies to address DEE 35.

1. Introduction

The inosine triphosphate pyrophosphatase (ITPA) protein has a critical role in intracellular nucleotide metabolism (see reviews [1–3]). The enzyme hydrolyzes both ribose and deoxyribose nucleoside triphosphates containing noncanonical purines, such as inosine triphosphate (ITP), to their monophosphate form [4]. This activity is thought to protect cells from accumulating high concentrations of ITP, which has potential to be incorporated into nucleic acids or interfere with normal nucleotide metabolism [3,5–7]. At this time, 45 very young patients have been identified with very rare and generally fatal mutations which result in severe ITPA deficiency [5,8–16]. Patients presented with an early onset encephalopathy termed developmental and epileptic encephalopathy 35 (DEE 35) (MIM# 616647) [5]. The molecular mechanism of pathogenesis for these patients is unknown. Interestingly, deoxyinosine was not detected in patient DNA [11], which suggests that the likely cause of pathogenesis is at the RNA or nucleotide level, such as a defect in RNA function or metabolism, or interference of ITP in normal nucleotide biochemistry (see Ref. [7] for further discussion).

One of the first fatal ITPA mutations identified was a point mutation that resulted in an amino acid change from arginine to cysteine at position 178 (R178C, rs746930990) [5]. Arg-178 is located in the substrate specificity pocket of ITPA and its terminal guanidino group makes critical noncovalent interactions with the incoming ITP substrate (Fig. 1), which is thought to contribute to substrate specificity [17]. Molecular dynamics simulations indicate that the R178C protein maintains its overall fold in solution, but that intermonomer flexibility is increased and that active site hydrogen bond networks were disrupted, compared to wild-type protein [18]. Data with wild-type enzyme show Arg-178 interacting with residues on α-helix 2 (Fig. 1). α-helix 2 contains critical active site residues and this interaction has a stabilizing effect on the helix [18].

To better understand the effects of the R178C mutation on enzyme activity we compared kinetic constants of wild-type ITPA and four position 178 ITPA mutants: R178C, R178S, R178K and R178A (see Fig. 2 for amino acid structures). For the R178C mutant, we found that substrate binding was similar to wild-type, but that the rate of catalysis was severely diminished. For the other mutants, substrate binding was diminished overall, however R178K had a rate of catalysis similar to wild-type. Our data imply that the guanidino group of Arg-178 performs an essential function by contributing to both substrate binding and formation of the active site hydrogen bond network, and that a small

* Corresponding author.
E-mail address: nburgis@ewu.edu (N.E. Burgis).

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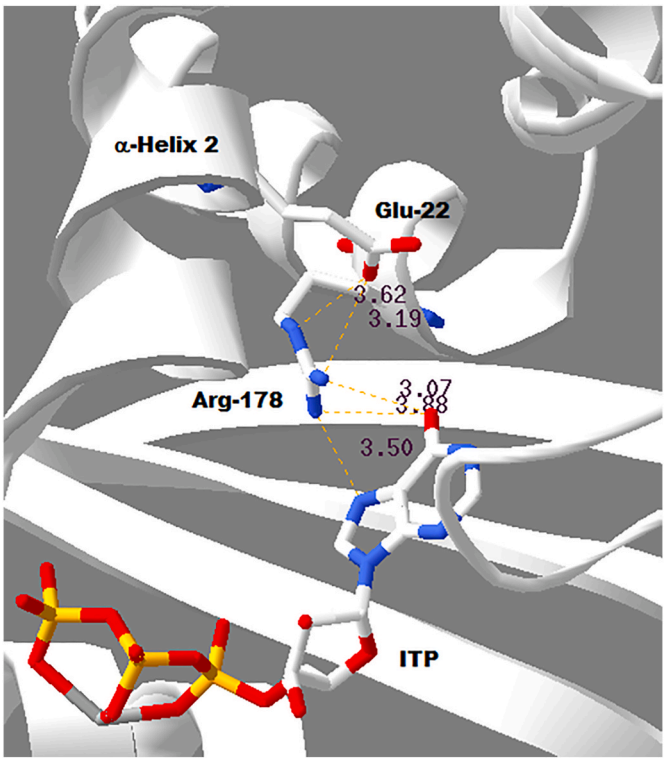

Fig. 1. Model of wild-type ITPA showing Arg-178 involved in substrate binding and stabilization of α-helix 2. Image based on pdb file 2J4E [17] rendered in Deep View (https://spdbv.unil.ch/). Residues of interest and ITP substrate are in CPK coloring. Potential hydrogen bonds are represented as yellow dashed-lines with distances in Angstroms noted.

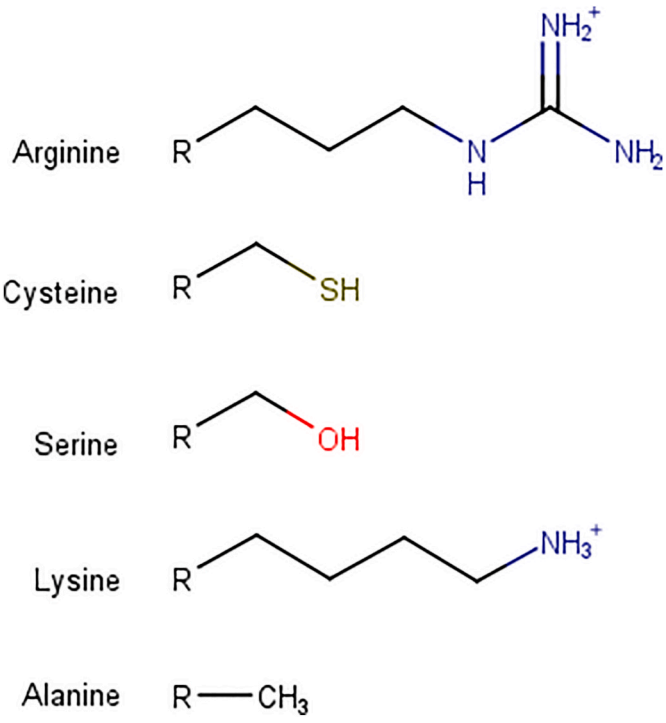

Fig. 2. Structure of R-groups for position-178 mutants studied. Structures are drawn with MarvinSketch using CPK coloring.

molecule or aptamer could be developed to address ITPA deficiency for R178C patients.

2. Materials and methods

2.1. Plasmid construction

pET28a-based mutant plasmids were constructed as described previously [19] using pET28a-ITPA plasmid as a template [20]. Briefly, an Agilent Technology QuikChange II Site-Directed Mutagenesis Kit was used with PAGE-purified primers designed using the manufacturer's website following the manufacturer's protocol. Proper construction of each mutant plasmid was confirmed by DNA sequencing (MCLAB, https://www.mclab.com/).

2.2. Protein purification

Wild-type and mutant proteins were overexpressed and purified as described previously (Supplemental Fig. S1) [18,19]. Briefly, ITPA proteins were overexpressed in *E. coli* and pelleted cells were resuspended in 8 M urea, 20 mM phosphate pH 7.4, 0.5 M NaCl (Buffer A) and then sonicated. Cleared lysates were loaded onto a 1 mL Ni^{2+} charged HisTrap HP affinity chromatography column, washed and eluted with Buffer A containing 500 mM imidazole. Eluant fractions were analyzed by SDS-PAGE. The fractions with the highest level of target protein were collected and dialyzed on ice against 20 mM Tris–HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM 1,4-dithiothreitol (Buffer B) containing 8 M urea using constant flow dialysis where the concentration of urea was reduced to zero in a stepwise manner over a 32-h period. Samples were stored in 50% glycerol at −20 °C and clarified after 48 h before concentration determination (NanoDrop 2000, www.thermofisher.com).

2.3. Enzyme kinetics

Michaelis-Menten parameters were determined with an assay similar to the specific activity assay described in Ref. [18]. Wild-type and the four mutant enzymes were assayed side by side in 100 μl volumes using eight different substrate concentrations ranging from 5 to 100 μM ITP. Each reaction was pre-incubated at 37 °C for 10 min and the reaction proceeded for 10 min with the addition of enzyme. Each reaction contained either 0.5 pmol wild-type enzyme, 2 pmol R178K enzyme, or 100 pmol R178C, R178S or R178A enzyme and was stopped by the addition of an equal volume of 2% sodium dodecyl sulfate followed by mixing and centrifugation. An aliquot of the supernatant was used for IMP quantification by HPLC. Reaction products were separated on a Nucleogen 60-7 DEAE column (www.mn-net.com) using a ThermoFisher UltiMate 3000 HPLC system with a flow rate of 0.6 ml/min and buffer containing 75 mM sodium phosphate, pH 6.4, 5% acetonitrile and 0.4 mM EDTA. IMP was quantified by UV absorption at 248 nm using an UltiMate 3000 VWD-3400RS UV detector. The Enzyme Kinetics module of Sigma Plot software was used to determine kinetic parameters. Kinetic parameters are reported as average values ± standard error.

3. Results

The results of the Michaelis-Menten enzyme kinetics experiments for wild-type ITPA and the four position-178 mutants are reported in Table 1. For wild-type enzyme, the value of the Michaelis constant (*KM*) is in the range of previously reported values [20](unpublished results), but the rate of catalysis (k_{cat}) and specificity constant (k_{cat}/K_M) are decreased. This decrease is proportional to the decrease observed previously for specific activity measurements when the urea based refolded protein purification scheme was employed (as it is here), compared to the standard protein prep [18]. This is likely due to the lower level of enzyme prep purity that is consistently observed with the refolded protein purification scheme which was required to recover soluble ITPA

Table 1
Kinetic constants for position-178 ITPA mutants.

Enzyme	$[E]_T$ (pmol)	Substrate	KM (μM)	Vmax (pmol/min)	k_{cat} (s^{-1})	kcat/KM ($mM^{-1}s^{-1}$)
wild-type	0.5	ITP	11.7 ± 4.0	141 ± 13.9	4.7 ± 0.46	401 ± 143
R178C	100	ITP	18.4 ± 8.5	226 ± 35.6	0.038 ± 0.006	2.1 ± 1.0
R178S	100	ITP	116 ± 55.8	433 ± 134	0.072 ± 0.02	0.62 ± 0.35
R178K	2	ITP	216 ± 111	395 ± 152	3.3 ± 1.3	15.2 ± 9.7
R178A	100	ITP	115 ± 56.9	134 ± 43.2	0.022 ± 0.007	0.20 ± 0.12

R178C enzyme (Supplemental Fig. S1).

In general, the position-178 mutants tested all had severely diminished efficiency in hydrolyzing ITP (Table 1). Under our experimental conditions (low rate of catalysis), *KM* measurements are reflective of substrate binding [21]. Therefore, Table 1 indicates that the clinically relevant R178C enzyme binds substrate roughly equivalent to wild-type, but the rate of catalysis is diminished over 100-fold. For the other three enzymes, *KM* values are about 10- to 20-fold greater than wild-type, suggesting that substrate binding is substantially diminished for these mutants. Interestingly, the k_{cat} value for the R178K mutant is close to the wild-type value, suggesting that a positively charged R-group at position 178 is required to support wild-type levels of catalysis with ITP. The k_{cat} values for R178S and R178A are severely diminished, this is consistent with previous specific activity measurements for R178A [19]. Consequently, none of the position-178 mutants tested have both wild-type substrate binding ability and wild-type rates of catalysis, hence the specificity constants are in the range of 25- to 2000-fold less than wild-type, with the most conservative substitution, R178K, having the highest k_{cat}/KM value (Table 1).

4. Discussion

Overall, our results give insight into the role of Arg-178 in enzyme function and the consequences of the R178C mutation on ITPA activity. Our data illustrate that Arg-178 has a critical role in substrate binding and catalysis and that no other amino acid at this position can perform both functions (substrate specificity and α-helix 2 stabilization) further supporting that Arg-178 is essential for ITPA function [18,19].

Because we had difficulty recovering a sufficient amount of R178C mutant to perform enzyme kinetics using our standard protein prep, we used the refolded protein purification scheme described in Materials and Methods. This methodology resulted in contaminating proteins being recovered along with target protein after the immobilized metal affinity chromatography step and variation in the relative amount of target protein recovered. Fig. S1 shows that the three proteins preps most critical to this study (wild-type (lane 2), R178C (lane 4), and R178K (lane 5)) all have the highest level of purity and the intensity of the ITPA bands are at similar levels both between the three preps and relative to the contaminating proteins. While error in protein concentration measurements may have occurred due to contaminating proteins, which could affect k_{cat} and k_{cat}/KM calculations, we believe this is minimal and would only alter the calculated values slightly. The differences in kinetic constants observed are stark so we do not expect protein purity to impact our conclusions.

Concerning substrate binding, x-ray crystallography data [17] shows the terminal amine groups of Arg-178 to be near the inosine base and within hydrogen bonding distance of the 6-position carbonyl and 7-position nitrogen of the purine ring (Fig. 1). This interaction is crucial as the conservative R178K mutant has severely diminished substrate binding ability, even though the terminal amine of lysine is only one bond length shorter than that of arginine. These data underscore that the noncovalent interaction between incoming substrate and arginine in the wild-type enzyme is hydrogen bonding as any electrostatic effects of a positively charged amine group in substrate binding would have likely been observed in the R178K mutant as well due to the longer distances of electrostatic interactions [22].

Considering catalysis, the guanidino group of Arg-178 is thought to participate in a hydrogen bond network that stabilizes α-helix 2, which contains critical active site residues, and this stabilization is thought to lead to wild-type levels of catalysis [18]. Molecular dynamics experiments have demonstrated that destabilization of α-helix 2 occurs with the two clinically relevant R178C and P32T mutants, underscoring the importance of these interactions. The fact that the R178K mutant has a k_{cat} value similar to wild-type, strengthens the idea that a positively charged amine at position-178 is key to maintaining this hydrogen bond network, likely due to critical interaction with Glu-22 (see Ref. [18], Fig. 1).

It is tantalizing to think that the ITP hydrolyzing activity of the R178C mutant may be enhanced by the addition of a molecule that helps stabilize α-helix 2. Because the R178C mutant retains wild-type levels of substrate binding, only an enhancement in catalysis would be required to improve ITP hydrolysis rates. Therefore, it is possible that a small molecule or aptamer could be developed to provide additional stabilization of α-helix 2. The R178K data suggest that a positively charged molecule would be required at this position to form the noncovalent interactions that help stabilize α-helix 2. Additionally, it may be possible to develop a molecule that chemically reacts with the sulfhydryl group of R178C in a way that a stabilizing positive charge is added, but substrate binding is not affected.

In conclusion, our results support that Arg-178 has an essential role in substrate binding and catalysis. For substrate binding the terminal amine groups are required to make critical contacts with electronegative atoms on the substrate base. For catalysis, the guanidino amine groups are critical for proper formation of the hydrogen bond network that stabilizes α-helix 2, which contains key active site residues. Our results indicate that the R178C ITPA mutant can bind substrate sufficiently, but that the catalysis is severely diminished, thus demonstrating that both Arg-178 activities are required for life in humans. Altogether, our results indicate that an aptamer or small molecule could be developed to improve R178C activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2023.109700.

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