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Expression, purification, and buffer solubility optimization of the putative human peptidyl-tRNA hydrolase PTRHD1

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ABSTRACT

Performing the essential function of recycling peptidyl-tRNAs, peptidyl-tRNA hydrolases are ubiquitous in all domains of life. The multicomponent eukaryotic Pth system differs greatly from the bacterial system composed predominantly of a single Pth1 enzyme. While bacterial Pth1s are structurally well characterized and promising new targets for antibiotic development, eukaryotic Pths are largely understudied. From amino acid sequence alignment and secondary structure predictions, the human gene product PTRHD1 was classified as a eukaryotic Pth. Herein, we report cloning, recombinant bacterial expression, and weak binding to peptidyl-tRNA for PTRHD1. Additionally, we report binding to tRNA but absence of peptidyl-tRNA hydrolase activity. Thus, PTRHD1 is not a Pth and the functional consequence of nucleotide binding remains undefined.

1. Introduction

With the prevalence of drug resistance in bacteria, the need for new antibiotics and new antibiotic targets is urgent and growing. Bacterial Peptidyl-tRNA hydrolase (Pth1) is emerging as a promising antibiotic target [1–4]. In bacteria, Pth1 performs the essential function of hydrolyzing peptidyl-tRNA generated from premature termination of protein biosynthesis and the expression of minigenes or short ORFs [5–7]. Accumulation of peptidyl-tRNA is lethal [8]. In the absence of Pth function, sequestration of tRNA as peptidyl-tRNA leads to tRNA starvation and inhibition of protein biosynthesis [9,10].

Though Pth activity is ubiquitous in all domains of life, most bacteria contain only one highly conserved Pth1 enzyme. Select strains have acquired a structurally unrelated Pth2 enzyme [11], however functional redundancy has not been established. The bacterial system differs greatly from the multicomponent eukaryotic Pth systems. A Pth1 ortholog is present in eukaryotes, however it is not essential. Similarly, the other well-characterized Pth in eukaryotes, Pth2, is also not essential. Knockout of Pth1, Pth2, or even both simultaneously do not affect viability in yeast [12]. Thus, essential Pth1 activity in bacteria is only part of a more complex and functionally redundant system in eukaryotes. While contributing to the attractiveness of Pth1 as a new target for antibiotic development, this also demonstrates the lack of understanding regarding the eukaryotic Pth system.

Better understanding of the multi-component eukaryotic Pth system is warranted to further validate and advance Pth1 as an antibiotic target. From amino acid sequence alignment, PTRHD1, or C2orf79, was identified as a human peptidyl-tRNA hydrolase. Herein, we report the cloning, recombinant expression in bacteria, purification, buffer solubility optimization, and nucleotide binding of human PTRHD1. Expressed under control of a T7 promoter, over 60 mg of purified protein were produced per liter of minimal media. PTRHD1 was purified to greater than 90% in one chromatography step as determined from Coomassie stained SDS-PAGE. To extend the usable lifetime, storage buffer conditions that optimized long-term solubility were also determined.

Abbreviations: PTRHD1, peptidyl-tRNA hydrolase domain containing 1; Pth1, bacterial peptidyl-tRNA hydrolase 1; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LB, luria broth; DEPC, diethylpyrocarbonate.

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2. Materials and methods

2.1. Synthesis of PTRHD1 with hexahistidine tag

E. coli codon optimized DNA encoding PTRHD1 was commercially synthesized (GenScript, NY, USA). PTRHD1 was subcloned into the pET28b expression vector (Novagen/EMD Millipore, Billerica MA, USA) using BamHI and NdeI restriction sites. The resulting PTRHD1 construct had an N-terminal hexahistidine tag and thrombin protease site directly before the first amino acid. The integrity of the construct was confirmed by DNA sequencing.

2.2. Recombinant expression of PTRHD1

Chemically competent BL21(DE3)pLysS *E. coli* (Novagen/EMD4 Biosciences, Darmtstadt, Germany) were transformed with PTRHD1-pET28b plasmid and grown on LB plates. All bacteria were grown in the presence of 30 μg/mL kanamycin unless otherwise noted. A single colony was selected and grown in 3 mL of LB to an OD_{600} of approximately 1.0. One milliliter aliquots were briefly centrifuged at 14,000 g and the supernatant removed. The cell pellets were resuspended in 500 μL LB and 300 μL of 80% glycerol, then placed at −80 °C to create frozen stocks.

For small scale expression, a 4 mL LB starter culture was inoculated with ~10 μL of PTRHD1 frozen stock and grown at 37 °C overnight. In the morning, the saturated culture was spun down and cells resuspended in 40 mL of M9 minimal media containing 4 g/L of glucose. Minimal media was preferable due to increased solubility of expressed PTRHD1, decreased background after purification, and the potential for future structural studies requiring isotope labeling. Grown at 37 °C to an OD_{600} of approximately 0.6, the culture was then split into 3 mL aliquots and induced with varying concentrations of IPTG in duplicate. Cultures were grown at 14 °C and gel samples were taken. Expression and solubility were analyzed via SDS-PAGE.

For mid-scale expression in minimal media, a 3 mL LB starter culture was inoculated with ~10 μL of the PTRHD1 frozen cell stock and grown overnight at 37 °C. The next day the culture was spun down and cells resuspended in 150 mL of M9 containing 4 g/L glucose to give a starting OD_{600} of approximately 0.1. The culture was grown at 37 °C to an OD_{600} of approximately 0.6, then split into five 30 mL cultures with the culture volume no more than one-fourth of the total Erlenmeyer flask volume. Cultures were cooled to 14 °C, induced with varying concentrations of IPTG, and grown overnight at 14 °C. The cultures were subsequently harvested and cell pellets stored at −80 °C. Each cell pellet was resuspended in 750 μL of 50 mM sodium phosphate, 300 mM NaCl, pH 7.4 and lysed by sonication (Branson Sonifier 250, Branson Ultrasonics, Danbury, CT, USA) using a 10 repeat schedule of 20 s sonication at 50% power followed by 20 s of rest on ice until the sample had the consistency of water. The lysate was separated from cell debris by centrifugation (16,000 g for 15 min) and soluble protein analyzed via SDS-PAGE.

For large scale expression in minimal media, it was found that starting from cells scraped from plates improved yields compared to overnight starter cultures. To that end, cells were grown from PTRHD1 frozen stocks in 1 mL of LB media for 1.5 h at 37 °C. Roughly 200 μL was spread onto LB agar plates. Incubated overnight at 37 °C, cells from two plates were scraped into 1 L of M9 minimal media containing 4 g/L glucose to yield a starting OD_{600} of approximately 0.1. Cells were grown at 37 °C in Erlenmeyer flasks of volume greater than four times the culture media volume (e.g. no more than 500 mL of media in a 2 L flask) to an OD_{600} of 0.6. The culture was then cooled to 14 °C and overexpression of PTRHD1 was induced with 250 μM IPTG. PTRHD1 was expressed overnight at 14 °C before the cells were harvested by centrifugation and cell pellets stored at −80 °C. Verification of PTRHD1 overexpression and solubility was determined via SDS-PAGE.

2.3. Purification of PTRHD1

PTRHD1 was purified by metal chelation chromatography. A cell pellet obtained from 1 L of M9 minimal media was resuspended in 20 mL of lysis buffer consisting of 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4. One milliliter of Protease Inhibitor Cocktail (P8465, SigmaAldrich, St. Louis, MO, USA) was added. The resuspended cells were sonicated on ice as above for 18 cycles. The soluble fraction was separated by centrifugation (45 min, 24,000 g, 4 °C) and loaded onto a pre-packed 5-mL His-Trap FastFlow nickel column (GE Healthcare, Uppsala, Sweden) previously equilibrated with two column volumes of lysis buffer. After the supernatant was loaded, the column was washed with lysis buffer containing 75 mM imidazole. PTRHD1 was eluted with 250 mM imidazole in the same lysis buffer. Fractions containing PTRHD1 were determined by SDS-PAGE, pooled, and then dialyzed against 25 mM MES, 150 mM NaCl, pH 6.5. Following dialysis, purified PTRHD1 was concentrated using ultrafiltration. The concentration of PTRHD1 was determined from UV absorbance at 280 nm using the extinction coefficient 17,085 M^{-1} cm^{-1} calculated from the primary sequence using ProtParam [13].

2.4. Buffer solubility optimization

The hanging drop method [14] was implemented to test multiple conditions for buffer solubility. All solubility studies were conducted at room temperature, 22 °C. Twenty-four well, pre-greased Hampton Research VD Plates and plain siliconized glass cover slides were used to test fifty-four conditions comprised of both inorganic and organic buffers with varied pH and sodium chloride concentrations. Each well reservoir contained 1 mL of the 100 mM test buffer. For the initial round of pH solubility studies, PTRHD1 was concentrated to 10 mg/mL in a starting buffer of 5 mM sodium phosphate, 150 mM sodium chloride, pH 7.4. For subsequent osmolarity testing, PTRHD1 was concentrated to 10 mg/mL in the most favorable buffer from the first stage, 20 mM MES, 10 mM sodium chloride, pH 6.5. PTRHD1 was combined with test buffers on the cover slide in a ratio of 5 μl of protein to 1 μl of buffer. Vacuum grease sealed the cover slides to the plate and the hanging drops were permitted to equilibrate by vapor diffusion. The solubility of PTRHD1 was scored empirically on a scale of 1–5 based on precipitate formation (5 being completely clear and 1 being completely covered in precipitate). The hanging drops were assessed every 2–3 days for a period of 3 weeks.

2.5. $Pth1^{Ts}$ complementation

C600 *pth*(Ts) [9] *E. coli* cells were transformed with pKQV4 vector containing *S. typhimurium* Pth1 under the control of a Taq promoter or TargeTron vector pAR1219 (Sigma-Aldrich, St. Louis, MO, USA), which expresses T7 polymerase under control of a lac UV5 promoter. Both transformants were grown on LB plates with 20 μg/mL carbenicillin. The C600 *pth*(Ts) cells transformed with pAR1219 were made chemically competent and transformed a second time with pET28b-PTRHD1 and grown on LB plates with 10 μg/mL carbenicillin and 15 μg/mL kanamycin. Three colonies of cells containing *S. typhimurium* Pth1 [15] or PTRHD1 were selected and grown in 4 mL LB cultures containing the appropriate antibiotics. Multiple single colonies were individually grown to an OD_{600} of approximately 0.4 and plated onto LB agar plates with the appropriate antibiotics and 1 mM IPTG. Plates were divided into