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The eukaryotic tRNA-guanine transglycosylase enzyme inserts queuine into tRNA *via* a sequential bi-bi mechanism

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Table of contents **Page**

1. **Materials and methods……………………………………………………………..……1**

2. **Supplementary figure 1…………………………………………………..………..3**

Materials and Methods

Recombinant protein production

BL21(DE3) *tgt*::Km_r *E. coli* cells[15a] were transformed with pET15b plasmid containing codon optimsed human QTRT1 cDNA to produce an N-terminal His-tag fusion or a pCDF-1b plasmid containing codon optimized human QTRT2 cDNA encoding or a C-terminal Strep-Tag®II fusion. Cells were grown in Terrific broth (4L) in a shaker-incubator at 37 °C until an OD_{600} of 0.8, the temperature decreased to 18 °C, 0.5 mM IPTG added, the cells grown overnight at 18 °C, then collected by centrifugation (3,500 *x* g) for 10 min at 4 °C. Cell pellets were resuspended in ice-cold Binding (BD) Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 7.4) with complete EDTA-free protease inhibitor cocktail (Roche) and lysed using a French-Press. Samples were centrifuged at 17, 500 *x* g for 20 min, the supernatant retained and 1 µg/mL DNase I (Ambion) added. After 30 min at 4 °C, the supernatant was passed through a Nalgene 0.45 µm syringe filter. In the case of hQTRT1, 5 mM imidazole was added to the extract, which was loaded onto a pre-packed 1 mL HisTrap™ HP column (GE Healthcare). The column was washed successively with BD Buffer containing 5 mM, 30 mM, 50 mM, and 80 mM imidazole before protein was eluted with 250 mM imidazole solution. hQTRT2 extract was dialysed in BD Buffer (pH 8), loaded twice through a 2 mL bed volume of Streptactin Superflow Plus resin (Qiagen, 50% *w/v* solution). The resin was washed twice with BD Buffer (30 mL) and the protein eluted in buffer containing 2.5 mM Desthiobiotin. Purified proteins were stored at -20 °C in 50% glycerol: BD buffer solution.

Labelling of human tyrosyl tRNA with radioactive guanine at position 34

Human tyrosyl tRNA ($htRNA^{Tyr}$) was generated by *in vitro* transcription[5c] and the guanine base at position 34 replaced with [8-^{3}H] guanine (specific activity is 21.2 Ci/ mmol; Moravek Inc.) to generate $htRNA^{Tyr}$-G* (4^{tyr}-2_{34}*). The pre-labelling reactions were performed in Ribosyltransferse (RBT) Buffer (50 mM Tris-HCl pH 7.5, 20 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT) containing *in vitro* transcribed $htRNA^{Tyr}$ (10 µM), hQTRT1 (700 nM), hQTRT2 (700 nM), and [^{3}H] guanine (200 nM) to a final volume of 500 µL. The reaction was incubated for 1 h at 37 °C, followed by the addition of the equal volume of (1:1) acid-phenol:chloroform (pH 4.5), the solution was mixed well and spun at 16,000 x g for 5 min. The upper aqueous phase was removed to a new tube and the charged tRNA precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The sample was incubated overnight at -20 °C, before the tRNA was pelleted at 16,000 x g for 20 min. The pellet was washed with ice-cold 70 % ethanol, air-dried, the 4^{tyr}-2_{34}* resuspended in nuclease-free water and the concentration measured at A_{260} spectrophotometrically.

Single turn over assay

Single turnover reactions were set up to contain the indicated combinations of hQTRT1 (10 μM), hQTRT2 (10 μM), 4^{tyr}-2_{34}* (10 μM), guanine (50 μM) or queuine (50 μM) in RBT buffer (100 μL final) for 30 min at 37 °C. To measure the loss of titrated guanine from 4^{tyr}-2_{34}* each reaction was quenched with 2.5 mL ice-cold 10% trichloroacetic acid (TCA, aq.) and incubated on ice for one h to precipitate the tRNA. The RNA precipitate was collected on GF/C 2.4 cm glass fiber filter disks (GE Healthcare Whatman™) using a vacuum manifold and each disk washed with 40 mL ice-cold 5% TCA (aq.) followed by 5 mL of ice-cold 95% ethanol (aq.). Filters were air-dried and placed in 10 mL of Ecoscint A (National Diagnostics) and scintillation counted.

Electromobility shift assay (EMSA)

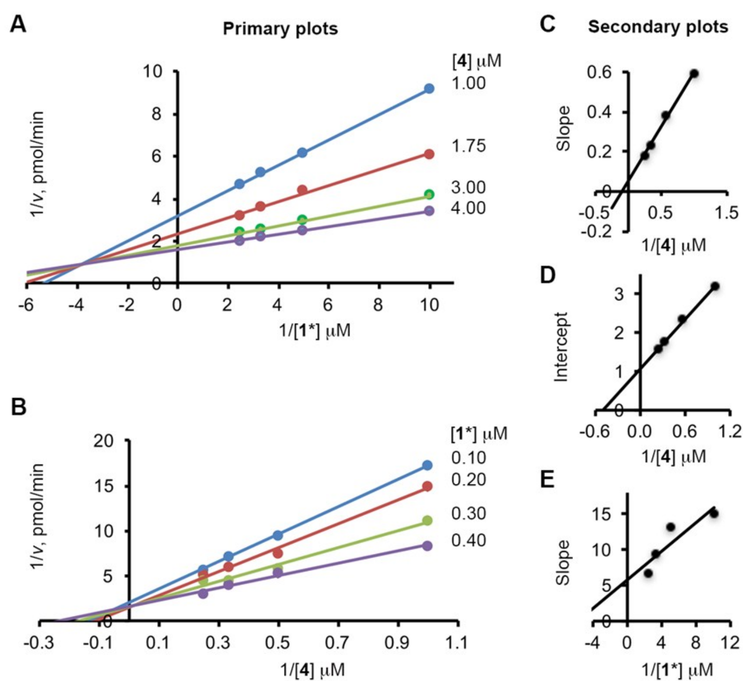
Reactions were set up to contain the indicated combinations of hQTRT1 (100 nM), hQTRT2 (100 nM), *in vitro* transcribed $htRNA^{Tyr}$ (10 μM) and 9-deazguanine (25 μM) in RBT buffer to a final volume of 20 μL and incubated at 37 °C for an h. Samples were mixed with 5 μL of 4X LDS loading dye (Invitrogen) and loaded on a 10% Bis-Tris gel (Invitrogen) and electrophoresed in 1x MOPS buffer (Invitrogen) at 200 volts for one h at 4 °C. The gel was stained with instant Blue Coomassie stain to visualize protein bands.

Kinetics assays of the human tRNA guanine transglycosylase enzyme

To examine the reaction mechanism of the eukaryotic tRNA transglycosylase enzyme with guanine as substrate, the following components were added to reactions in the following order; RBT buffer, $htRNA^{Tyr}$ (1, 2, 3 and 4 μM), 100 nM hQTRT1, 100 nM hQTRT2 and [8-^{3}H] guanine (100, 200, 300 and 400 nM) in a final volume of 100 μL for 2, 7 and 15 min. Reactions were terminated by the addition of ice-cold 5 % trichloroacetic acid and processed for scintillation counting as described above. To study the mechanism with queuine as substrate the guanine was substituted with [methylene-^{3}H] queuine (7 Ci/ mmol; Moravek Inc.).

Microscale equilibrium binding assay

The recombinant hQTRT1 and hQTRT2 proteins were dialysed into 20 mM Tris-HCl, 100 mM NaCl, 10 % glycerol, pH 8.0 at 4 °C. The interaction of the tRNA guanine transglycosylase enzyme with a range of [^{3}H] Queuine or [^{3}H]Guanine nucleobase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1.2, 2.0, 3.0 and 4.0 μM) was performed for 3 h in the absence of the second substrate (i.e. tRNA). The enzyme binding reaction was prepared in RBT buffer in the following order; 100 nM hQTRT1, 100 nM hQTRT2, 10 μM adenine (non-specific blocker), varying concentrations of [^{3}H] Queuine or [^{3}H] Guanine to a final volume of 100 μL with RNase free H_2O. Binding assays were also performed with [^{3}H] Thymidine to evaluate the specificity of the binding reactions. The tRNA guanine transglycosylase enzyme was bound to 1 μL HIS-Select® HC Nickel Magnetic Beads (Sigma; ≥15 mg/mL binding capacity) by mixing the reaction for 20 min on a roller. The protein-bound beads were isolated by magnetic separation and mixed with 10 mL of Ecoscint and the protein-bound radioactivity determined by scintillation counting. In addition, the supernatant (free ligand) was also added to 10 mL of Ecoscint, mixed well, and radioactivity levels evaluated.



Supplementary Fig. 1. Primary and secondary plots for queuine (1) and tRNA (4). (**A**) Primary plots of 1/v against 1/[**1**] and (**B**) of 1/v against 1/[**4**]. Initial velocities were determined by varying the concentration of **1** (0.1-0.4 μM), at several fixed concentrations of **4** and by varying the concentration of 4 (1-4 μM) at several fixed concentrations of **1**, respectively. (**C**) Secondary plot from **A** showing slope against the second substrate **4** (tRNA). (**D**) Intercept replot of the second substrate **4** (tRNA) from the primary plot **A**. (**E**) Secondary plot of slope against **1** from **B**. Data points were generated from nine independent determinations of the initial velocity data.