RutA-Catalyzed Oxidative Cleavage of the Uracil Amide Involves Formation of a Flavin-N5-oxide

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Supporting Information

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Materials

All chemicals were purchased from Sigma-Aldrich unless mentioned otherwise. LB broth (Lennox) was purchased from EMB Millipore. Chloramphenicol and IPTG were obtained from Lab Scientific Inc. Amicon Ultra centrifugal filter devices (10,000 MWCO) were obtained from Millipore. Histrap column was obtained from GE Healthcare. Econo-Pack 10DG desalting columns were purchased from Bio-Rad. 2.5 L baffled ultra yield flasks for protein overexpression were obtained from Thomson Instrument Company. HPLC and LC-MS solvents were purchased from EMD and were used without further purification. ZORBAX Eclipse XDB-C18 column (15 cm x 4.6 mm, 5 μm particles) was purchased from Agilent Technologies. PFP column (150 x 4.6 mm, 2.6 μm particles) was purchased from phenomenex.

Over-expression and purification of RutA and Fre

The RutA gene containing overexpression plasmid (pCA24N)[1] was transformed into *Escherichia coli* BL21 (DE3). For the overexpression of the protein, a starter culture was grown overnight in 15 ml of LB medium containing 20 μg/ml of chloramphenicol at 37 °C. 1.5 liter of LB medium (20 g/L) containing 20 μg/ml of chloramphenicol, was inoculated with this starter culture. The cells were grown at 37 °C with shaking (220 rpm) until the culture reached an OD600 of 0.6. The culture was then incubated at 4°C for ~45min without shaking. Then the culture was induced by adding IPTG to a final concentration of 0.4 mM, the temperature was lowered to 15 °C and the cells were grown with shaking (180 rpm) for a further 15 hours. The cells were then harvested by centrifugation at 10,000g for 10 min at 4 °C and store at -80 ˚C.

The cell pellet was re-suspended in 30 ml of lysis buffer (50 mM KH_2PO_4, 150 mM NaCl, 10 mM imidazole, pH 8.0). Lysozyme (5 mg) was added and the cells were lysed by sonication on ice (Misonix Sonicator 3000, six cycles of 30 s duration during which 1.5 s sonicator pulses at output level 0.8 were followed by 1.5 s pauses). The resulting suspension was centrifuged (18,000 rpm, 30 min) and the supernatant was filtered through a sterile syringe filter (pore size 0.45 μm). The clarified supernatant was loaded onto a 5 mL Ni-NTA-affinity column pre-equilibrated with lysis buffer kept at 4°C. The Ni-NTA-affinity column was then washed with 50 ml wash buffer (50 mM KH_2PO_4, 150 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted from the column with elution buffer (50 mM KH_2PO_4, 150 mM NaCl, 200 mM imidazole, pH 8.0) at 4°C. The fractions containing protein were pooled and concentrated using YM-10 Amicon ultracentrifugal filters at 5000g to a final volume of 3 mL. The concentrated sample was buffer exchanged, using an Econo-Pac 10DG desalting column, into 100 mM phosphate buffer at pH 7.5 containing 100 mM NaCl and glycerol to a final concentration of 15%. Protein concentration was determined from the absorbance at 280 nm (A280) with an Extinction coefficient calculated using the ProtParam tool of the ExPASy proteomics Server (ϵ_{280} = 56380). A typical yield was 20 mg/liter.

The *E.coli* Fre gene[2] was cloned into pTHT vector (derivative of pET28b vector with TEV protease cleavage site after the N-terminal His-tag). The plasmid was transformed into *Escherichia coli* BL21 (DE3). A starter culture was grown overnight in 15 ml of LB medium containing 40 μg/ml of Kanamycin at 37 ˚C. Next, 1.5 L of LB medium containing 40 μg/ml of Kanamycin was inoculated with this starting culture. The cells were grown at 37 ˚C till the OD_{600} reached a value of 0.6. The culture was then incubated at 4 ˚C for 45 min without

shaking and induced with 0.5 mM IPTG followed by incubation at 15 ˚C for 15 hr with shaking (180 rpm). The cells were then harvested by centrifugation and stored at -80 ˚C until further use. The purification procedure was same as that for RutA.

HPLC parameters

An Agilent 1260 HPLC equipped with a quaternary pump was used. The system included a diode array UV-Vis detector and products were detected using absorbance at 280 nm, and 450 nm. Analysis was performed either on a ZORBAX Eclipse XDB-C18 column (15 cm x 4.6 mm, 5 µm particles, Agilent Technologies) or PFP column (150 x 4.6 mm, 2.6 µm particles, Kinetex).

HPLC conditions (for C18 column):
A-Water
B-100 mM potassium phosphate buffer, pH 6.6
C-Methanol

HPLC method:
0 min-100% B, 5 min- 10% A 90% B, 12 min-48%A 40%B 12% C, 14 min-50%A 30%B 20% C, 18 min-30% A 10% B 60% C, 20 min-100% B, 25 min-100% B

HPLC conditions (for PFP column):
A-Water
B-100 mM potassium phosphate buffer, pH 6.6
C-Methanol

HPLC method:
0 min-90% A 10% B, 2 min- 90% A 10% B, 22 min-20%A 10%B 70% C, 27 min-20%A 10%B 70% C, 28 min-90% A 10% B, 36 min-90% A 10% B

LC-MS parameters

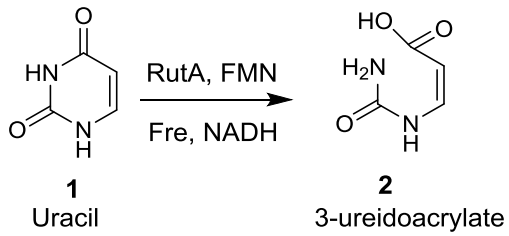
LC-ESI-TOF-MS was performed using an Agilent 1260 HPLC system which is equipped with a binary pump and a 1200 series diode array detector followed by a MicroToF-Q II mass spectrometer (Bruker Daltonics) using an ESI source either in negative mode or positive mode. Analysis was performed on an LC-18-T column (15 cm x 3 mm, 3 µm particles, Supelco).

LC conditions:
A-5 mM Ammonium acetate buffer, pH 6.6
B-75% Methanol and 25 % Water.

LC method: (for positive and negative mode on MS) 0 min-100% A, 2 min-100% A, 12 min-30% A 70% B, 17 min-30% A 70% B, 18 min-100% A, 30 min-100%A

RutA-catalyzed reaction using Fre-NADH as reducing system

The RutA catalyzed reaction was performed in 100 mM phosphate buffer, pH 7.5. A 200 µL aliquot of the reaction mixture containing RutA (50 µM), uracil (500 µM), FMN (20 µM), *E.coli* Fre (400 nM) and NADH (1 mM) was incubated at RT for 3 hr. The protein was heat-denatured and the resulting solution was filtered using an Amicon Ultra centrifugal filter (10kda cut-off). The samples were analyzed by HPLC (using a C-18 column).



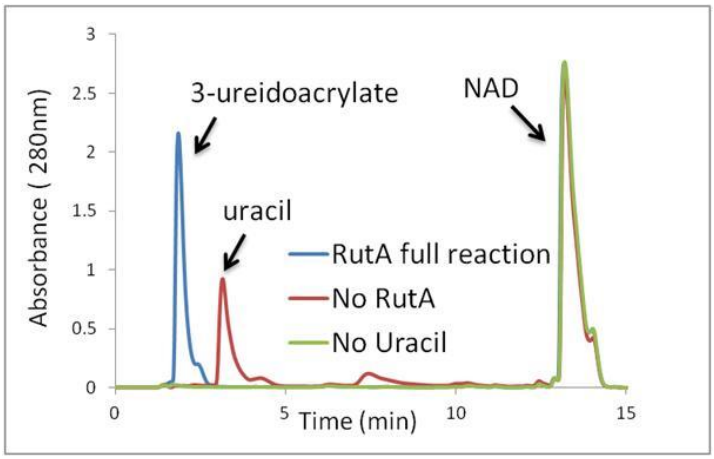


Figure S1: HPLC analysis of the RutA reaction mixture (280 nm) showing the formation of 3-ureidoacrylate (**2**) only when all the components were present.

RutA-catalyzed reaction using photoreduced FMN

In order to photoreduce FMN, a solution containing FMN (500 µM) and EDTA (10 mM) was irradiated, under anaerobic conditions, for 5 min in a glove box with an A21 LED light bulb (100-W, white light). Uracil **(**100 µM**)** and RutA (100 µM) were added to a solution containing photo-reduced FMN (100 µM). The resulting mixture was incubated in the glove-box for 0.5 hr. and then exposed to oxygen for an additional 1 hr at 25 ˚C. The protein was heat-denatured and the resulting solution was filtered using an Amicon Ultra centrifugal filter (10kda cut-off). The samples were analyzed by HPLC (using a PFP column) and LC-MS. The results are shown in Figure **4A-E**.

RutA reaction in the presence of $^{18}O_2$ using photoreduced FMN

FMN was photo-reduced in a glove-box using EDTA as described above. Substrate and enzyme were added. Final concentrations of reduced FMN, uracil and RutA were 100 μM each. The resulting mixture was incubated in a glove-box for 0.5 hr, exposed to $^{18}O_2$ and incubated for an additional 1 hr at 25 ˚C. The protein was heat-denatured and the resulting solution was filtered using an Amicon Ultra centrifugal filter (10kda cut-off). Finally the samples were analyzed by LC-MS. The result is shown in Figure **4F**.

Quantification of the FMN-N5-oxide formed in the RutA-catalysed reaction

The RutA-catalyzed reaction, using photoreduced FMN, was carried out as described above. Final concentrations of reduced FMN, uracil and RutA were 100 μM. Based on calculated peak areas, it was determined that 54 μM FMN was consumed in the full reaction. Consumption of uracil was 52 μM. Thus, the ratio of FMN consumed to uracil consumed was 1:0.9 consistent with the stoichiometric conversion of FMN to FMN-N5-oxide during the course of the RutA catalyzed reaction.

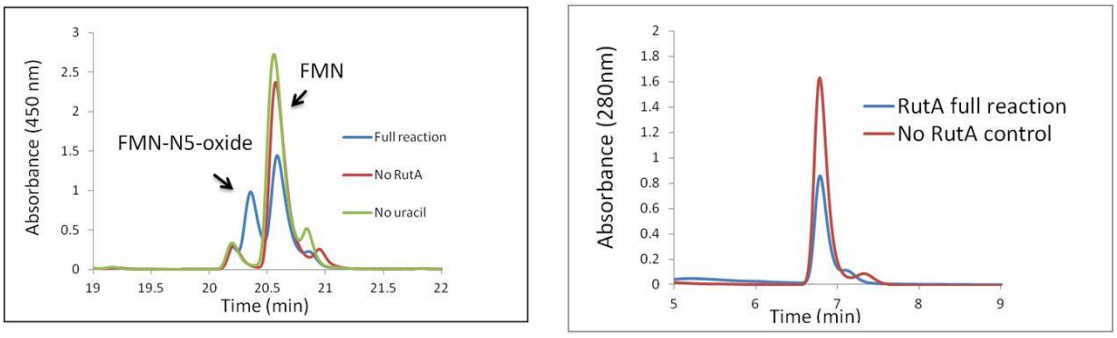


Figure S2: Estimation of FMN-N5-oxide formed by calculating the amount of FMN and uracil consumed in the RutA catalyzed reaction. Panel A shows the consumption of FMN (54 μM) in the full reaction. Panel B shows consumption of uracil (52 μM) in the full reaction.

General mechanism for flavin-N5-oxide formation involving hydroperoxide intermediate

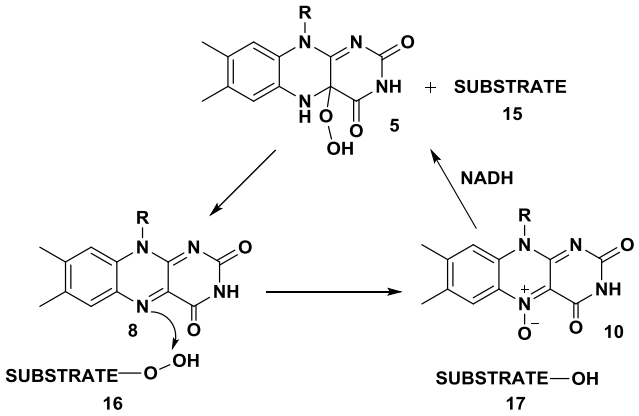


Figure S3: Proposed catalytic motif for the formation of a flavin-N5-oxide in the reduction of hydroperoxide intermediates (16).

References

(1) Mukherjee, T.; Zhang, Y.; Abdelwahed, S.; Ealick, S. E.; Begley, T. P. (2010) *J Am Chem Soc 132*, 5550-5551.
(2) Xun, L.; Sandvik, E. R. (2000) *Appl. Environ. Microbiol 66*, 481-486.