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# Catalysis of a Flavoenzyme-Mediated Amide Hydrolysis

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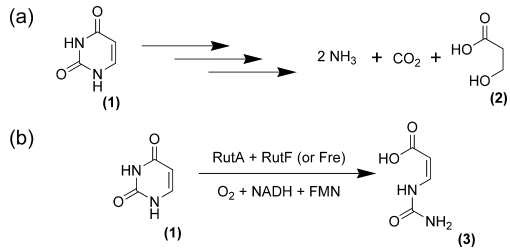
A new pyrimidine catabolic pathway (the Rut pathway) was recently discovered in *Escherichia coli* K12.[1,2] In this pathway, uracil (**1**) (or thymine) is converted to 3-hydroxypropionate (**2**) (or 2-methyl-3-hydroxypropionate), 2 equiv of ammonia, and 1 equiv of carbon dioxide.[1] The b1012 operon, which contains seven genes (*RutA-G*), is required for this conversion. Here we demonstrate that the flavoenzyme RutA, along with the flavin reductase RutF, catalyzes the initial uracil ring opening by an unprecedented "oxidative" hydrolysis reaction to give 3-ureidoacrylic acid (**3**, Scheme 1).

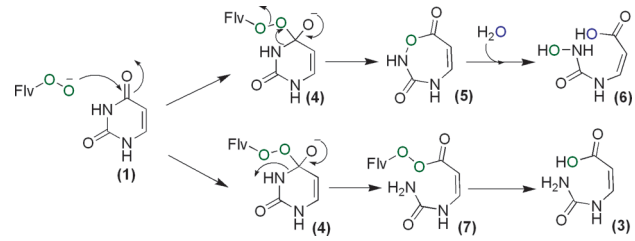
***Scheme 1.*** (a) New Pyrimidine Catabolic Pathway in *E. coli* K12; (b) Reaction Catalyzed by RutA




RutA and RutF were overexpressed in *E. coli* BL21(DE3) and purified by Ni-affinity chromatography. RutA overexpressed well and was purified to homogeneity. RutF also overexpressed well; however, most of the protein was insoluble, and this protein was only partially purified. When **1** was incubated at 25 °C in the presence of RutA, RutF, NADH, and FMN, the formation of 3-ureidoacrylate was detected by HPLC analysis (Figure 1A). Attempts to isolate and characterize the product by $^{1}$H NMR and ESI-MS were unsuccessful because it was unstable under acidic conditions and degraded during lyophilization. To characterize the product without isolation, a reaction mixture containing $^{13}$C,$^{15}$N-labeled **1**, RutA, RutF, NADH, and FMN in phosphate buffer at pH 8.0 was incubated at room temperature overnight and analyzed by $^{13}$C NMR. The spectrum of the product showed no coupling between N3 and C4, demonstrating that RutA catalyzed the cleavage of the N3−C4 bond as the first step of the Rut pathway (Figure 1B). An identical reaction product was obtained by substituting RutF with homogeneous Fre, a flavin reductase.[3]

Three plausible structures for the N3−C4 bond-cleaved product (**3**, **5**, and **6**) are shown in Scheme 2. Addition of flavin hydroperoxide (generated by reaction of molecular oxygen with reduced flavin) to C4 of uracil would give **4**, which could then undergo a Baeyer−Villiger-like rearrangement to give **5** or **7**. Hydrolysis of **5** would give **6**, and reduction of **7** would give **3**. To differentiate between **3**/**5** and **6**, the RutAF reaction was run in 50% $H_2{}^{18}O$ buffer using $^{13}$C,$^{15}$N-labeled **1**. NMR analysis showed no isotopic shift at C4 of the product (Figure 1C). This ruled out **6** as the enzymatic product. In further support of this, when the reaction was run using $^{18}O_2/^{16}O_2$, a clear isotopic shift at C4 of the product was observed (Figure 1C). To differentiate

***Scheme 2.*** Three Plausible Structures for the RutA Product




between compounds **3** and **5**, the reaction product was hydrolyzed with 10% trifluoroacetic acid: **5** should generate hydroxyurea, and **3** should yield urea. In the event, $^{13}$C NMR analysis of the reaction mixture showed a triplet (coupling to N1 and N3) at 162.7 ppm, consistent with the formation of urea. Thus, the RutAF product is likely to be **3**. This was confirmed by NMR and chromatographic identity with a synthesized sample of 3-ureidoacrylic acid.

A mechanistic hypothesis for the formation of (*Z*)-3-ureidoacrylic acid (**3**) is outlined in Scheme 3. In this proposal, RutF (or Fre)

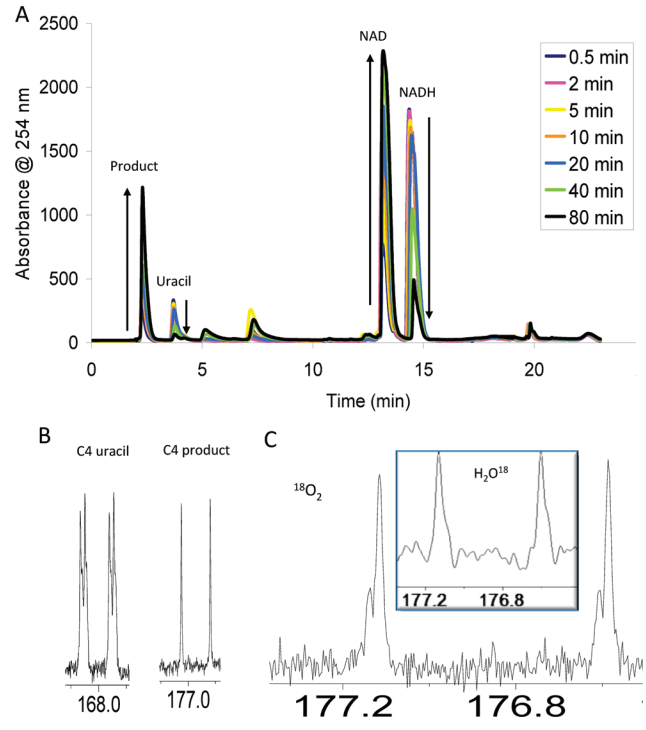



***Figure 1.*** (A) HPLC chromatogram of a time course for the RutA/RutF-catalyzed ring opening of uracil. (B) Partial $^{13}$C NMR spectra of the C4 resonance of $^{13}$C,$^{15}$N-labeled uracil showing the C4−N3 coupling. This coupling is absent in the reaction product. (C) $^{13}$C NMR spectra showing the isotopic shift at C4 of the product when the reaction was run in the presence of $^{18}O_2$. No isotopic shift was observed when the reaction was run in $H_2{}^{18}O$ (box).

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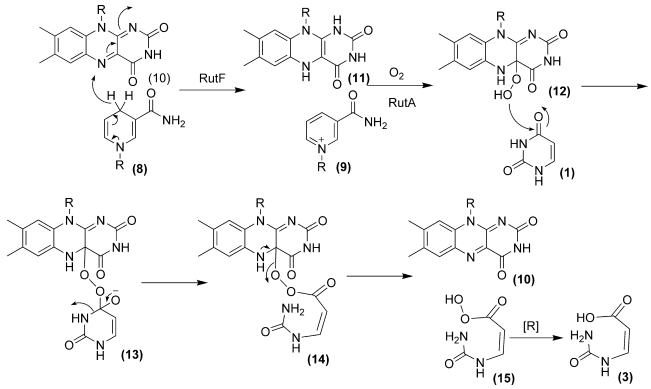
***Scheme 3.*** Mechanistic Proposal for the Formation of (*Z*)-3-Ureidoacrylic Acid (**3**)




catalyzes the formation of dihydroflavin (**11**), which then reacts with molecular oxygen to form the flavin hydroperoxide (**12**).[4] This then adds to the C4 carbonyl of **1** to form **13**, which undergoes ring opening to give **14**. Elimination of (*Z*)-3-ureidoacrylic peracid (**15**) followed by reduction of the acylhydroperoxide completes the reaction.

We did not detect the formation of **15** in our reaction mixtures either by NMR or by HPLC using a reference sample of **15** (see below). This suggests that it is rapidly reduced to **3** under the reaction conditions, as the oxygen-labeling experiment (Figure 1C) eliminates the possibility of acyl hydroperoxide hydrolysis. Two mechanisms for this reduction were considered: RutA-catalyzed reduction and reduction by excess NADH or DTT in the reaction buffer.

Flavoenzyme-catalyzed hydroperoxide reduction is a well-characterized process that occurs by oxidation of an active-site cysteine followed by flavin-mediated reduction of the resulting sulfenic acid.[6] To address the possibility of enzymatic reduction of **15**, the X-ray crystal structure of RutA was solved at 1.8 Å. The active site is shown in Figure 2A. While we have not yet been able to obtain a structure of the enzyme−substrate complex, we can identify a substrate binding site above the *Si* face of the isoalloxazine ring. Since there are no cysteine residues located in this region of the protein, we conclude that reduction of **15** is not RutA-catalyzed.

To evaluate the chemical stability of **15** in the reaction buffer, an authentic sample of **15** was synthesized by the reaction of $Na_2O_2$ with the *p*-nitrophenolate (PNP) ester of **3**. This compound decomposes over time to form primarily **3** and **1** (Figure 2B). The half-life of **15** is 3 h at 25 °C and pH 10.0 and much shorter at lower pH. In the presence of 1.5 equiv of NADH, a freshly prepared sample of **15** was completely converted to **3** within 5 min (Figure 2C). A similar rapid reduction was observed using DTT. These results suggested that the **15** formed during the enzymatic reaction is reduced to **3** nonenzymatically under our reaction conditions. However, the possibility remains that one of the other enzymes in the Rut pathway catalyzes this reduction.

We have demonstrated that the first step in the Rut pyrimidine catabolic pathway involves ring opening of uracil at the C4 carbonyl. This reaction, while formally a hydrolysis reaction, proceeds by an oxidative mechanism initiated by the addition of a flavin hydroperoxide to the C4 carbonyl. While peroxide-catalyzed amide hydrolysis has chemical precedent,[5] we are not aware of a prior example of analogous chemistry catalyzed by flavin hydroperoxides. This study further illustrates the extraordinary catalytic versatility of the flavin cofactor.

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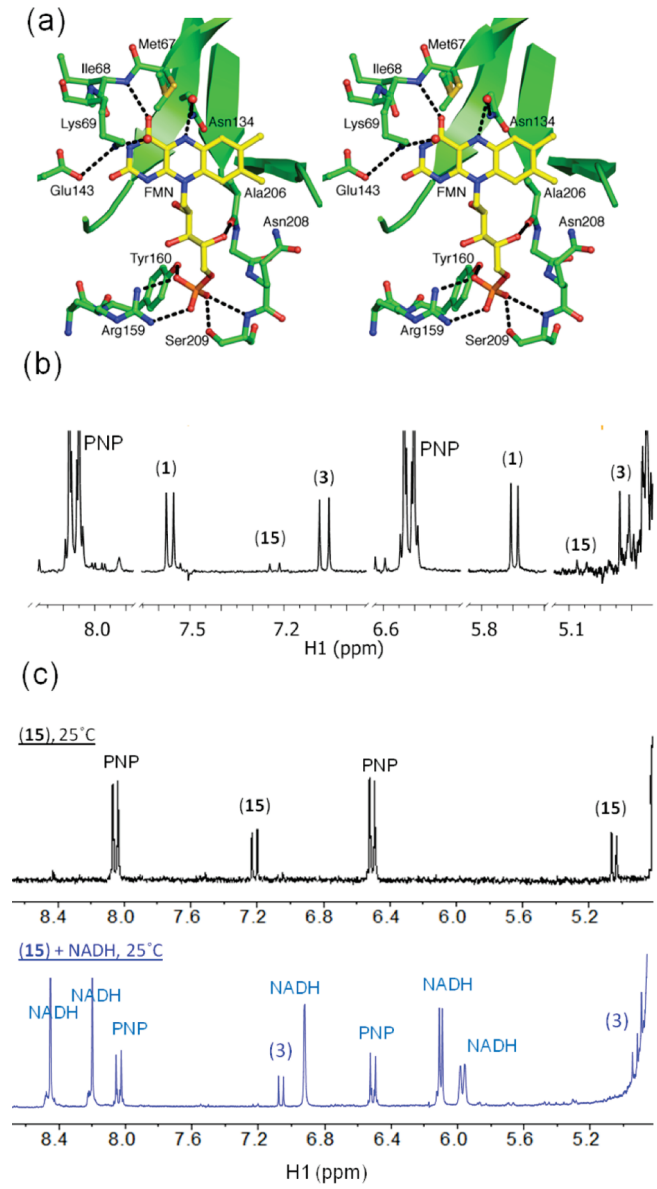


***Figure 2.*** (a) Stereo view of the active site of RutA. (b) $^1$H NMR spectrum (300 MHz, $D_2O$) of decomposed (*Z*)-3-ureidoacrylic peracid (**15**) at 25 °C showing a mixture of **1**, **3**, and **15**. (c) $^1$H NMR spectra (300 MHz, $D_2O$) of **15** in the presence and absence of NADH at 25 °C.

**Supporting Information Available:** Detailed experimental procedures for the syntheses of **3** and **15**, protein purification, enzymatic assays, and NMR and LC-EMS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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