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Catalysis of a flavoenzyme-mediated amide hydrolysis

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A new pyrimidine catabolic pathway (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), two equivalents of ammonia and one equivalent of carbon dioxide[1]. The b1012 operon, containing 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-ureidoacrylate (**3**, Scheme 1).

RutA and RutF were overexpressed in *E. coli* BL-21 and purified by Ni-affinity chromatography. RutA overexpressed well and was purified to homogeneity. RutF also overexpressed well but most of the protein was insoluble and this protein was only partially purified. When uracil (**1**) was incubated at 25 °C in the presence of RutA, RutF, NADH and FMN, the formation of 3-ureidoacrylate (**3**) was detected by HPLC analysis Figure 1(A). Attempts to isolate and characterize the product by ^{1}H-NMR and ESI-MS were unsuccessful because it was unstable to acid and degraded during lyophilization. To characterize the product without isolation, a reaction mixture containing $^{13}C^{15}N$ labeled uracil (**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 1(B)). 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** & **6**) are shown in Scheme 2. Addition of the 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_2O^{18} buffer using $^{13}C^{15}N$ labeled uracil (**1**). NMR analysis showed no isotopic shift at C4 for the product (Figure 1(C)). This rules 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 1(C)). To differentiate 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 the 3-ureidoacrylate (**3**). This was confirmed by NMR and chromatographic identity with a synthesized sample of 3-ureidoacrylate.

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SUPPORTING INFORMATION Detailed experimental procedures for the synthesis 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.

A mechanistic hypothesis for the formation of *(Z)*-3-ureidoacrylic acid (**3**) is outlined in Scheme 3. In this proposal, RutF (or Fre) 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 uracil (**1**) to form (**13**), which undergoes ring opening to (**14**). Elimination of the (*Z*)-3-ureidoacrylic peracid (**15**) followed by reduction of the acylhydroperoxide completes the reaction.

We have not detected 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 because the oxygen labeling experiment (Figure 1(C)) eliminates the possibility of acyl hydroperoxide hydrolysis. Two mechanisms for this reduction were considered: RutA-catalyzed reduction or 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 2(A). 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 enzyme 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 (*Z*)-3-ureidoacrylate (**3**). This compound decomposes over time to form primarily *(Z)*-3-ureidoacrylic acid (**3**) and uracil (**1**) (Figure 2(C)). The half life of **15** is 3 hours at pH 10.0 at 25 °C and much less at lower pH. In the presence of 1.5 equivalents of NADH, a freshly prepared sample of *(Z)*-3-ureidoacrylic peracid (**15**) was completely converted to *(Z)*-3-ureidoacrylic acid (**3**) within five minutes (Figure 2 (D)). A similar rapid reduction was observed using DTT. These results suggested that the *(Z)*-3-ureidoacrylic peracid (**15**) formed during the enzymatic reaction is reduced to the (*Z*)-3-ureidoacrylic acid (**3**) non-enzymatically 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 the 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 precedence[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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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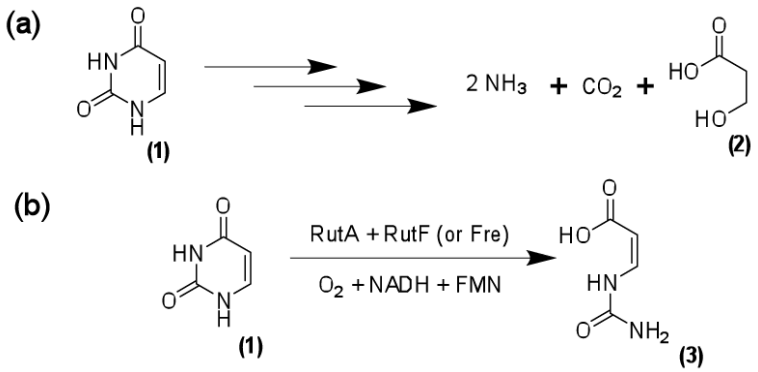
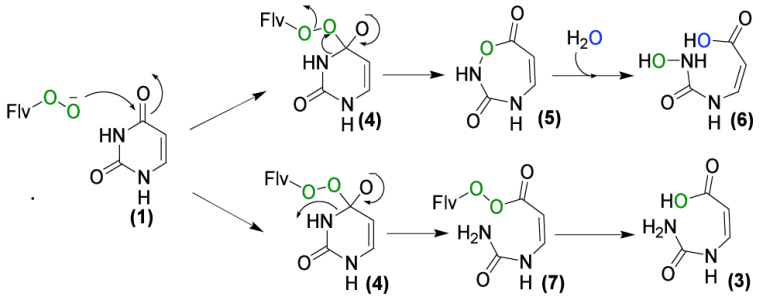


Scheme 1.
(a) The new pyrimidine catabolic pathway in *E. coli* K12. (b) The reaction catalyzed by RutA.



Scheme 2.
Three plausible structures for the RutA product.

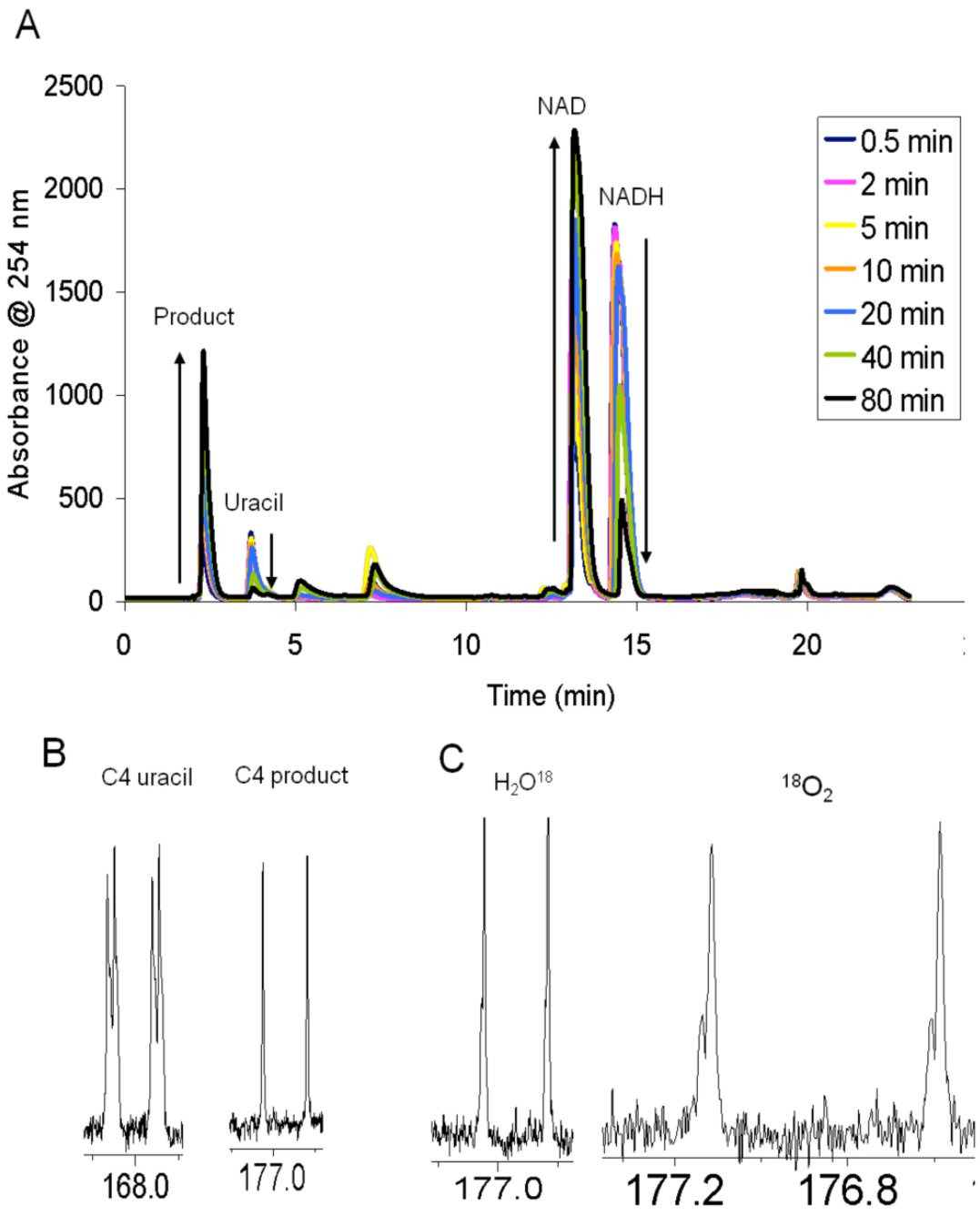
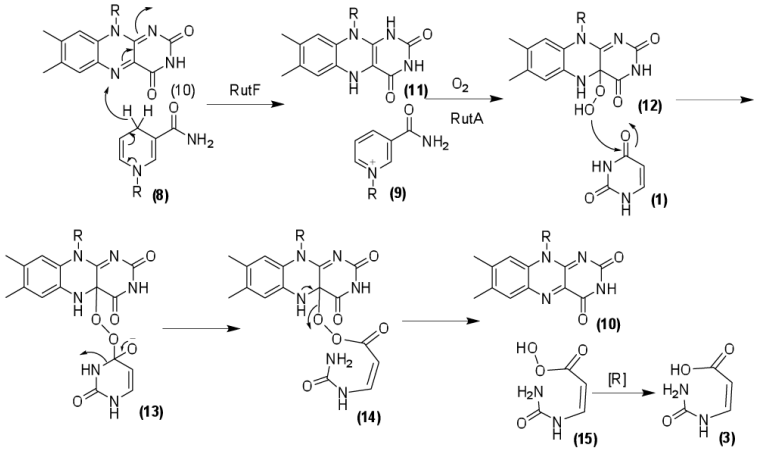


Figure 1.

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 $^{13}C^{15}N$ uracil showing the C4-N3 coupling. This coupling is absent in the reaction product. C: ^{13}C NMR showing isotopic shift at C4 of the product when the reaction was run in the presence of $^{18}O_2$ (right). No isotopic shift was observed when the reaction was run in H_2O^{18} (left).



Scheme 3.
Mechanistic proposal for the formation of *(Z)*-3-ureidoacrylic acid (**3**).

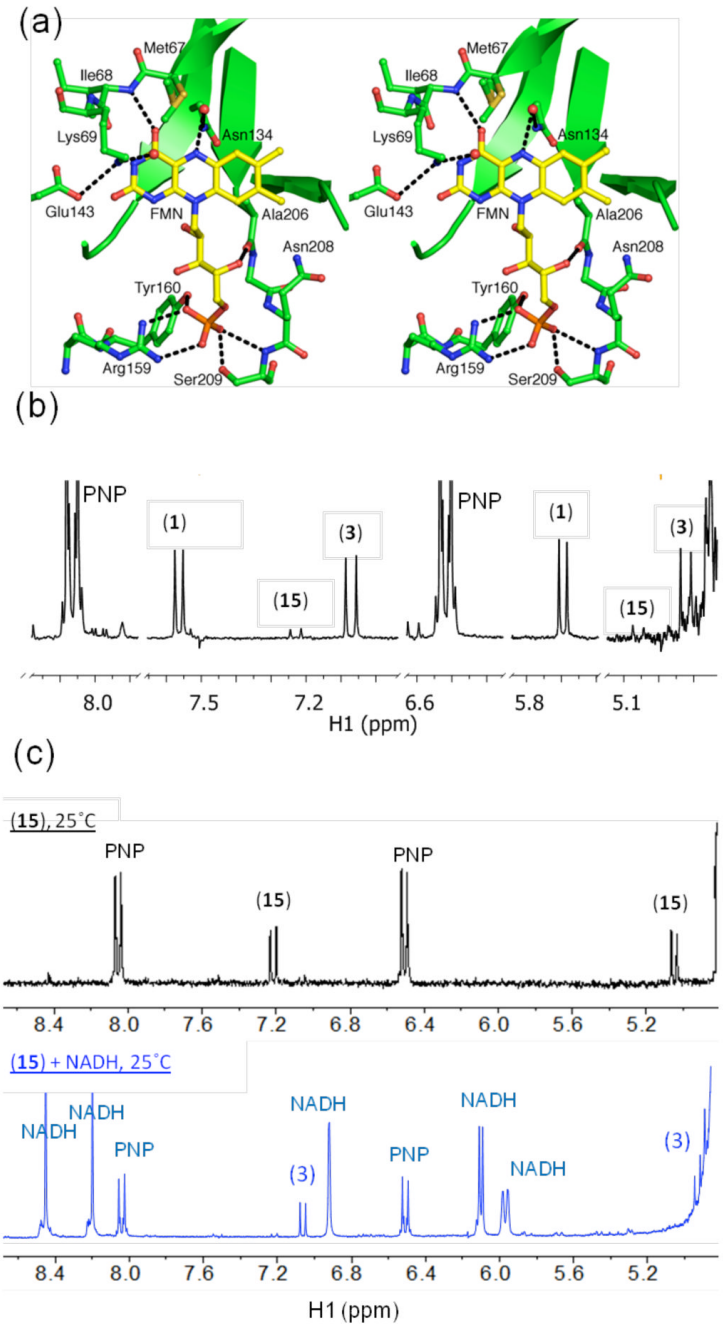


Figure 2.

(a) Stereo view active site of RutA. (b) ^{1}H NMR (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 (300 MHz, D_2O) of the *(Z)*-3-ureidoacrylic peracid (**15**) in presence and absence of NADH at 25 °C.