Published in final edited form as:

Arch Biochem Biophys. 2017 October 15; 632: 4–10. doi:10.1016/j.abb.2017.08.001.

Flavin-N5-oxide: A new, catalytic motif in flavoenzymology

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Abstract

Flavin-N5-oxide is a recently discovered intermediate used by EncM (1,3-diketone oxidation), DszA (sulfone monooxygenase) and RutA (amide monooxygenase). This review describes the mechanism of these enzymes and proposes criteria for the identification of additional Flavin-N5-oxide dependent enzymes.

1. Introduction

Flavoenzymes are ubiquitous in all forms of life and extensive studies in this area demonstrate that the isoalloxazine heterocycle is the most chemically versatile heterocycle in biology [1–3]. The flavin redox states are shown in Fig. 1. Flavin can undergo 1 and 2-electron reversible redox chemistry to form the flavin semiquinone and dihydroflavin respectively. The ability of flavins to undergo single electron transfer chemistry underlies much of the catalytic versatility of this cofactor. Flavin-N5-oxide, a recently discovered flavin oxidation state, is formed by two-electron oxidation of flavin or by four-electron oxidation of reduced flavin. This novel oxidation state was first observed in the EncM-catalyzed 1,3-diketone oxidation in enterocin biosynthesis [3–6]. Since then, two other examples have been reported, one (DszA) is involved in dibenzothiophene sulfone oxidation [7] and the second (RutA) is involved in the oxidative amide cleavage of uracil [8]. In this review, the mechanistic chemistry of these three enzymes will be described and general features of flavin-N5-oxide-mediated reactions will be identified to facilitate future prospecting for additional examples of this chemistry.

2. EncM-catalyzed 1,3-diketone oxidation in enterocin biosynthesis

Enterocin is a polyketide antibiotic produced by several actinomycetes [9]. Its biosynthesis is outlined in Fig. 2 and is covered in detail in the review by Robin Teufel in this issue [10]. Of particular interest here is the EncM-catalyzed 1,3-diketone oxidation of **7** to provide the substrate for a novel Favorskii rearrangement.

The current mechanistic proposal for this oxidation reaction is shown in Fig. 3. Reduced flavin reacts with oxygen by a SET mechanism to give peroxide **12**, which then loses water to give the flavin-N5-oxide. This reacts with the 1,3-diketone **7** to form **14**. Alcohol elimination from the flavin followed by hydride transfer generates the 1,2,3-triketone product **8**. Remarkably, while enol intermediates are ubiquitous in biosynthesis, EncM is the

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only example of a flavoenzyme-catalyzed enol hydroxylation. It is also notable that oxoammonium ions are versatile oxidizing agents in organic synthesis [11] that can participate in similar ketone oxidations [12–14]. This enzyme is the first biochemically characterized example of a flavin-N5-oxide-utilizing enzyme and sets the stage for the discovery of other enzymes that use this new flavin oxidation state.

3. DszA-catalyzed sulfone oxidative ring opening in dibenzothiophene catabolism

Dibenzothiophene **16** is a representative example of a broad class of sulfur-containing compounds found in petroleum. The microbial catabolism of dibenzothiophene has been explored because of the difficulty of removing these compounds using the conventional hydrodesulfurisation process [15,16]. The dibenzothiophene catabolic pathway in *Rhodococcus erythropolis* is shown in Fig. 4. The first two steps (DszC-catalyzed) of this pathway involve flavin mediated thioether oxidation to the sulfone **18** and the last step (DszB-catalyzed) involves bisulfite loss - probably by an electrophilic aromatic substitution reaction. The conversion of sulfone **18** to sulfinic acid **19**, shown in the third step, involves a novel oxidative C-S bond cleavage reaction catalyzed by DszA. Our current mechanistic proposal for this reaction is outlined in Fig. 5. Reaction of reduced flavin with oxygen generates the flavin peroxide **21** which then adds to the sulfone to give the sulfone-stabilized delocalized carbanion **22**. Protonation of this carbanion, followed by elimination of the oxidized flavin, gives **23**. Reaction of the flavin with the resulting substrate hydroperoxide gives the flavin-N5-oxide and hydroxyl sulfone. Ring opening of **24**, with C-S bond cleavage, gives **25** which then tautomerizes to the aromatized product **19**. Reduction of the flavin-N5-oxide with NADH gives **26** and elimination of water regenerates the starting oxidized flavin.

Several experiments support formation of a flavin-N5-oxide in the DszA-catalyzed sulfone oxidation reaction. When the reaction was run in [^{18}O]- H_2O , no ^{18}O incorporation into the biphenyl product was observed. The DszA-catalyzed reaction requires reduced flavin and oxygen supporting the initial formation of the flavin hydroperoxide. When the reaction is run using pre-reduced flavin as the cofactor, a new stable product was formed that comigrated with and had identical UV-visible and mass spectra with a synthesized sample of flavin-N5-oxide. Since flavin-N5-oxide reacts readily with NADH to give oxidized flavin, it is essential to run the enzymatic reaction in the absence of NADH in order to detect this intermediate. When the reaction was run using $^{18}\text{O}_2$ gas, ^{18}O -incorporation into the flavin-N5-oxide and the phenolic hydroxyl of **19** was observed [7].

4. RutA-catalyzed oxidative amide cleavage in uracil catabolism

In the Rut pathway, uracil **27** is catabolized to 3-hydroxypropionate **32**, ammonia and carbon dioxide (Fig. 6) [17,18]. RutA and the flavin reductase RutF catalyze the first step of this pathway in which uracil **27** is converted to 3-ureidoacrylic acid **28** [19]. Our current mechanistic proposal for this reaction is outlined in Fig. 7. Reaction of reduced flavin with oxygen generates the flavin peroxide **21** which adds to uracil **27** to give **33**. Elimination

gives the peracid **34** which then oxidizes the flavin to give the flavin-N5-oxide **4** and the 3-ureidoacrylic acid **28**. Reduction of the flavin-N5-oxide with NADH completes the reaction.

Several experiments are consistent with this mechanism for the RutA-catalyzed uracil amide cleavage. No ^{18}O incorporation into 3-ureidoacrylic acid product was observed when the reaction was run in $[\text{}^{18}\text{O}]\text{-H}_2\text{O}$. The reaction requires reduced flavin and oxygen, supporting the initial formation of the flavin hydroperoxide. When the reaction is run in the absence of NADH, using pre-reduced flavin as the cofactor, a new stable product was detected by HPLC analysis of the reaction mixture. This product was identical in its UV-visible spectra and chromatographic properties to the corresponding product isolated from the DszA catalyzed reaction. When the reaction was run using $^{18}\text{O}_2$ gas, ^{18}O -incorporation into the flavin-N5-oxide and the carboxy group of the 3-ureidoacrylic acid was observed. These observations strongly support formation of flavin-N5-oxide in the RutA-catalyzed uracil degradation and identify RutA as the first example of a flavin hydroperoxide mediated oxidative amide cleavage.

5. Criteria for the identification of new flavin-N5-oxide-utilizing enzymes

While flavin-N5-oxide was initially considered as a possible intermediate in flavin-mediated oxidation reactions, early research established the central role of the flavin hydroperoxide in such reactions and flavin-N5-oxide disappeared from consideration [20,21]. Here we will analyze the EncM, DszA and RutA-catalyzed reactions in an attempt to develop criteria to facilitate the identification of additional examples of flavin-N5-oxide-utilizing enzymes. As the amino acid sequences of EncM, DszA and RutA do not yet enable us to identify characteristic sequence motifs, our analysis will focus on mechanistic features of the three known enzymes.

It is well known that the terminal oxygen of the flavin C4a-peroxide has both nucleophilic and electrophilic character depending on its protonation state. For example, it can react with ketones to give products of the Baeyer-Villiger reaction [22–24] and it can also react with nucleophilic thioethers to give sulfoxides and sulfones. In the EncM-catalyzed 1,3 diketone oxidation, a flavin hydroperoxide is likely to generate a mixture of products arising from competing C2 oxidation and C1 and C3 Baeyer-Villiger oxidation (Fig. 8). To improve the selectivity of this reaction, the nucleophilicity of the peroxide is reduced in EncM by replacing it with the flavin-N5-oxide. This replacement is analogous to the use in organic synthesis of oxoammonium ion based oxidants, rather than peracids, for the oxidation at C α of ketones [12–14]. The chemical versatility of oxoammonium oxidants [11] suggests that additional examples of the EncM motif will be found in other flavin mediated oxidation reactions where the nucleophilicity of the flavin hydroperoxide needs to be attenuated to avoid competing addition to an adjacent electrophilic center (e.g. α -hydroxylation of ketones and related compounds).

The reactions catalyzed by RutA and DszA involve formation of an intermediate hydroperoxide by nucleophilic attack of the flavin peroxide anion on the substrate. This hydroperoxide is then reduced to the corresponding alcohol, by oxidized flavin, to produce

the flavin-N5-oxide. Based on this, we propose that the RutA/DszA motif may be found in other flavoenzymes that proceed via substrate hydroperoxide intermediates.

Acknowledgement

This research was supported by the Robert A. Welch Foundation (A0034).

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Fig. 1.

The four oxidation states of flavin showing the recently discovered flavin-N5-oxide (in red) found in the EncM (1,3-diketone oxidation), DszA (sulfone monooxygenase) and RutA (amide monooxygenase) - catalyzed reactions (R = ribityl phosphate or ribityl-ADP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Enterocin biosynthetic pathway. The flavin-N5-oxide mediated 1,3-diketone oxidation is shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Mechanistic proposal for the flavin-N5-oxide-mediated 1,3-diketone oxidation in the reaction catalyzed by EncM (R = ribityl-ADP). Structures **13/4** represent the resting state of the enzyme. The EncM flavin is covalently attached to His78 of the enzyme.

Fig. 4.
The dibenzothiophene catabolic pathway in *Rhodococcus erythropolis*.

Fig. 5. Mechanistic proposal for the DszA-catalyzed reaction involving a FMN-N5-oxide intermediate. In contrast to EncM, the DszA flavin is not covalently attached to the enzyme.

Fig. 6.

The Rut pathway for uracil catabolism showing the RutA-catalyzed hydrolysis of uracil in the first step. RutB and RutE are the hydrolase and the reductase involved in the last two steps of the catabolic pathway.

Fig. 7.
Mechanistic proposal for the RutA-catalyzed reaction involving a FMN-N5-oxide intermediate. In contrast to EncM, the RutA flavin is not covalently attached to the enzyme.

Fig. 8.

A chemical rationale for the use of flavin-N5-oxide rather than a flavin hydroperoxide for the α -hydroxylation of ketones.