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Deazaflavins as photocatalysts for the direct reductive regeneration of flavoenzymes

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ABSTRACT

Deazaflavins are potentially useful redox mediators for the direct, nicotinamide-independent regeneration of oxidoreductases. Especially the O_2-stability of their reduced forms have attracted significant interest for the regeneration of monooxygenases.

In this contribution we further investigate the photochemical properties of deazaflavins and investigate the scope and limitations of deazaflavin-based photoenzymatic reaction systems.

Introduction

Oxidoreductases are amongst the most promising catalysts for preparative organic synthesis for selective reduction, oxidation and oxyfunctionalisation reactions. Amongst them, flavin-dependent oxidoreductases are of particular interest due to the versatility of the flavin-prosthetic group for selective reduction [1–5], and oxyfunctionalisation reactions (Scheme 1) [6–9]. For most of these reactions, the catalytic mechanism entails reductive activation of the enzyme-bound flavin cofactor. The reduced flavin cofactor then either reduces a substrate molecule (as in case of the so-called old yellow enzymes or ene reductases) or it reacts with molecular oxygen forming a peroxoflavin capable of selective oxyfunctionalisation reactions such as epoxidation [10–16], Baeyer-Villiger oxidations [17–20], or aromatic hydroxylation reactions [13,21–32].

As mentioned above, all these structurally and mechanistically diverse enzymes have a reductive activation of the enzyme-bound flavin in common. Generally, the reducing equivalents required for this reaction are obtained from the natural nicotinamide cofactors (NAD(P)H). For practical and economic reasons stoichiometric use of NAD(P)H is not feasible, which is why in the past decades a myriad of different *in situ* regeneration approaches have been developed allowing for the use of NAD(P)H in catalytic amounts only [33–35].

Despite the success of these methodologies, a more direct approach to regenerate the enzyme-bound flavin group could offer some advantages such as simplified reaction schemes. For this, we and others have developed a range of chemical [36–42], electrochemical [43–45], and photochemical [46–51] approaches to target the flavin prosthetic group directly while circumventing the natural nicotinamide cofactor (together with the enzymatic regeneration system).

The mediators of choice for such NAD(P)H-independent regeneration systems are flavins themselves. However, while flavin-based regeneration systems perform well with O_2-independent enzymes such as the OYEs, their performance with O_2-dependent monooxygenases is rather poor; the major limitation being the poor O_2-stability of reduced flavins. One possibility to circumvent this *Oxygen Dilemma* [52] was suggested by Reetz and coworkers [49], i.e. to utilise deazaflavins instead of the 'normal' ones (Scheme 2) to promote a P450BM3-catalysed aerobic hydroxylation of lauric acid. Compared to using 'normal' flavins, significantly higher productivities were observed, which was attributed to the higher oxidative stability of reduced deazaflavins. These findings are in line with much earlier findings by Massey and coworkers who demonstrated that fully reduced deazaflavins, in contrast to their 'normal' analogues, exhibit a high stability against O_2 [53].

Already in the 1970s, deazaflavins have been subject of extensive research efforts. Especially Massey, Hemmerich and coworkers have worked out the reactivity of deazaflavins revealing that the deaza-semiquinone radical is significantly less stable compared to the 'normal' semiquinone [53–60]. This favours disproportionation and dimerization reactions leading to non-radical products exhibiting low(er) O_2 reactivity. Furthermore, reduction of deazaflavins by exclusive 1 e-donors like dithionite proceeds relatively slow compared to natural

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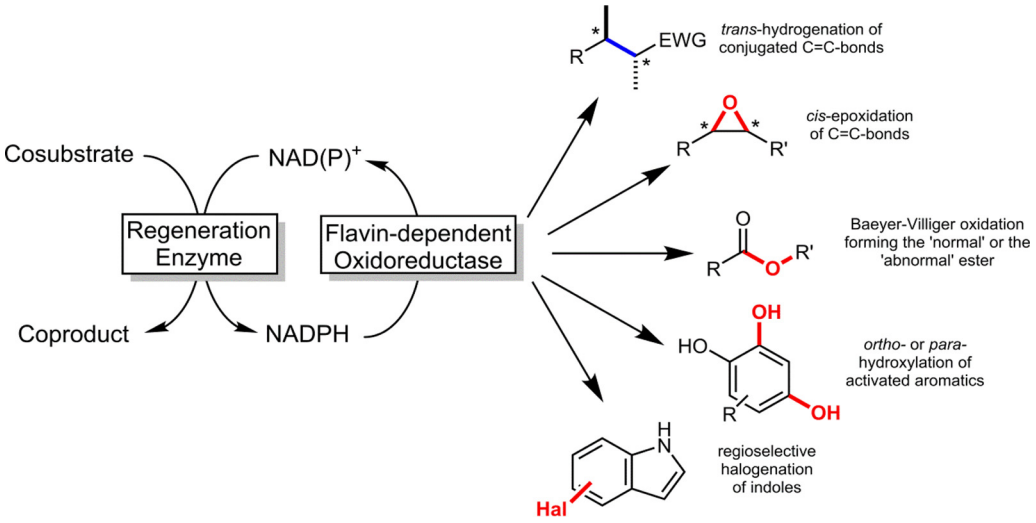
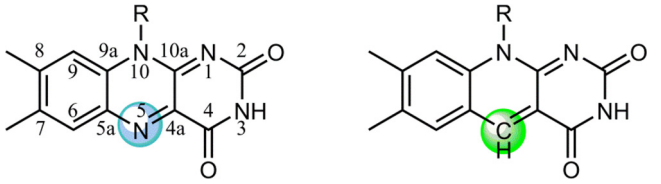


Scheme 1. Simplified scope of flavin-dependent oxidoreductases for selective reduction of C=C-double bonds, epoxidations, Baeyer-Villiger oxidations, aromatic hydroxylations and halogenations. All reactions depend on the reduced nicotinamide cofactor, which for economic reasons has to be regenerated in situ using an (enzymatic) regeneration system.



Scheme 2. Simplified representation of flavins (left) and their deazaflavin analogues (right). For reasons of clarity the 5 position wherein both differ are marked.

flavins and via a covalent adduct intermediate.

These findings motivated us to further evaluate the applicability of deazaflavins as photocatalysts/mediators to promote flavoenzyme catalysed reactions.

Materials and methods

Chemicals

Chemicals were purchased from Sigma-Aldrich, Fluka, Acros or Alfa-Aesar with the highest purity available and used as received.

5-deazariboflavin (dRf) was synthesized following a literature method (SI for more detailed information) [61]. Stock solutions (0.2 mM in 100 mM phosphate buffer, pH 6) of the deazariboflavin were prepared freshly every day.

The old yellow enzyme homologue from *Bacillus subtilis* (YqjM) was produced and purified using a protocol recently established in our group (SI for more detailed information) [36].

The NADH mimic N-benzyl nicotinamide (BNAH) was synthesized following published procedures [39,62].

Experimental setup

Photoreduction reactions were performed in 1 ml reaction mixtures in 1.5 ml glass vials. The reaction mixtures were illuminated from all sides from a distance of 10 cm by a LED light source (SI for more detail). The intensity spectrum of the LED light source was determined by a calibrated spectrophotometer. Samples were gently stirred using Teflon coated magnetic bars. Anaerobic experiments were performed in an anaerobic chamber (on average 98% N_2, 2% H_2) with oxygen levels below ppm levels. UV/Vis spectra were recorded using an Avantes DH-2000 UV–vis-NIR light source and an Avispec 3648 spectrophotometer.

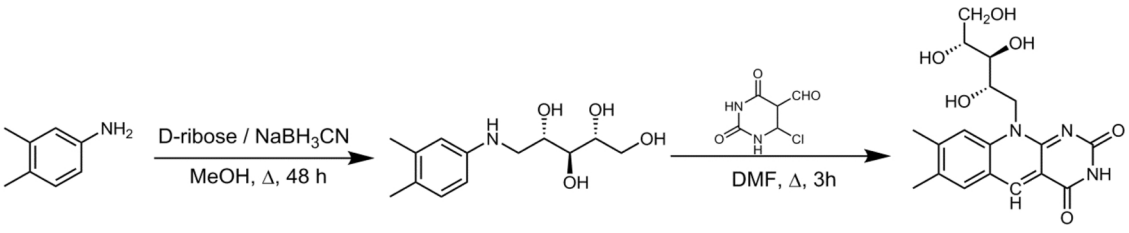
In a typical experiment 100 µM dRf were reduced by 10 mM sacrificial single electron donor or 1 mM hydride donor in a 100 mM KPi buffer at pH 6.0. The absorbance at 400 nm ($\varepsilon = 12500\ M^{-1}\ cm^{-1}$) was followed to determine the oxidation state of dRf over time (Fig. S4.12). Due to interference of absorption when using either hydride donors, the oxidation state was determined by following the absorbance at 430 nm ($\varepsilon = 5700\ M^{-1}\ cm^{-1}$).

Due to the FMN, the YqjM has a typical absorbance spectrum with a peak extinction coefficient at 455 nm which decreases as the FMN in the active site is reduced. At 400 nm the extinction coefficient does not change significantly with the redox-state of the enzyme, which made it possible to determine the redox-state of the dRf and the YqjM simultaneously. For the reduction of YqjM, a dRf solution was first photoreduced by five equivalents of EDTA. Thereafter, 20 µM of YqjM was mixed with 100 µM of the reduced dRf.

Photoenzymatic syntheses were performed using 5 µM YqjM in the presence of 1 mM of 2-methyl cyclohexenone and 10 mM of EDTA in a 100 mM KPi buffer pH 6.0. The 2.5 ml reaction mixtures were extracted with 0,5 ml ethyl acetate and analysed on a CP-wax 52 CB GC column (50 m x 0,53 m x 2 µm)(GC method: 70 °C for 2 min. 2 °C/min–80 °C. 80 °C for 2 min. 2 °C/min–90 °C. 90 °C for 3 min. 25 °C/min–150 °C. 150 °C for 1 min. 25 °C/min–225 °C. 225 °C for 1 min). 5 mM Dodecane was used as internal standard.

Results and discussion

5-deazariboflavin (dRf) was synthesized from 3,4-dimethylaniline in



Scheme 3. Synthetic route for 5-deaza riboflavin (dRf).