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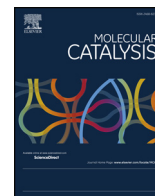
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Deazaflavins as photocatalysts for the direct reductive regeneration of flavoenzymes

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ABSTRACT

Deazaflavins are potentially useful redox mediators for the direct, nicotinamide-independent regeneration of oxidoreductases. Especially the O₂-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

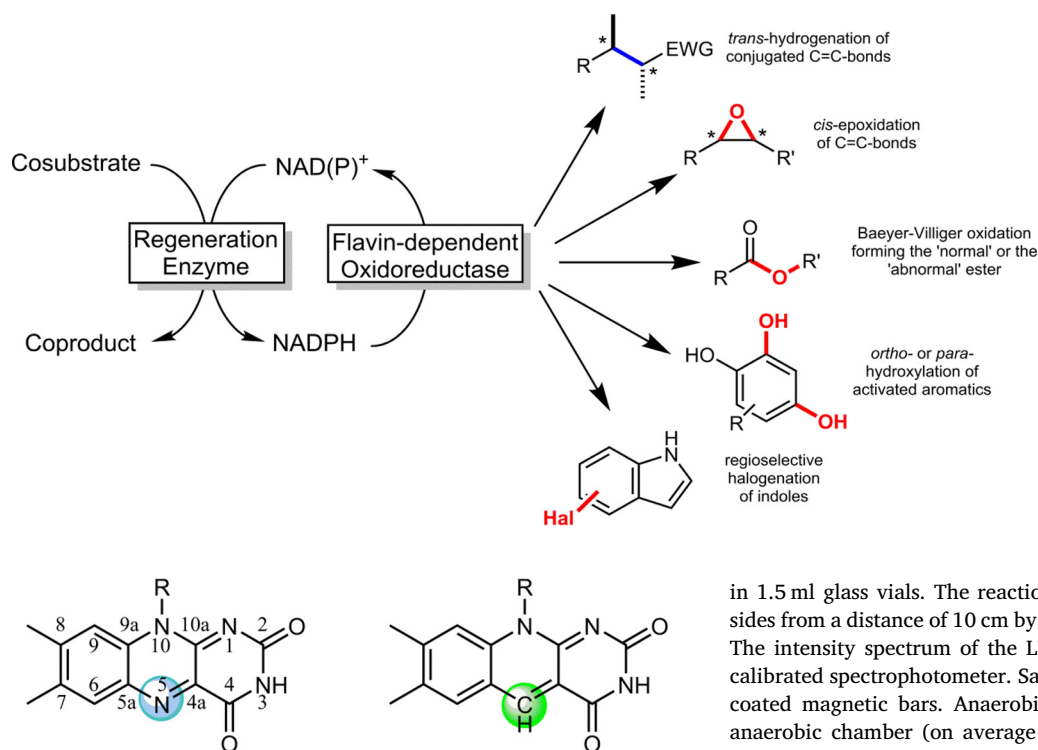
have developed a range of chemical [36–42], electrochemical [43–45], and photochemical [46–51] approaches to target the flavin prosthetic group directly while circumventing the natural nicotinamide cofactor (together with the enzymatic regeneration system).

The mediators of choice for such NAD(P)H-independent regeneration systems are flavins themselves. However, while flavin-based regeneration systems perform well with O₂-independent enzymes such as the OYEs, their performance with O₂-dependent monooxygenases is rather poor; the major limitation being the poor O₂-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 O₂ [53].

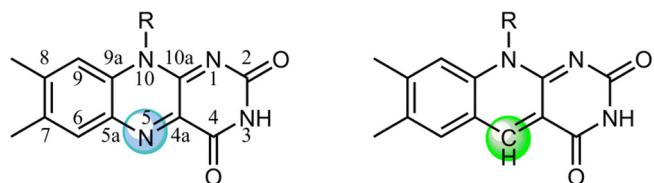
Already in the 1970s, deazaflavins have been subject of extensive research efforts. Especially Massey, Hemmerich and coworkers have worked out the reactivity of deazaflavins revealing that the deaza-semiquinone radical is significantly less stable compared to the ‘normal’ semiquinone [53–60]. This favours disproportionation and dimerization reactions leading to non-radical products exhibiting low(er) O₂ 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% N₂, 2% H₂) 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.

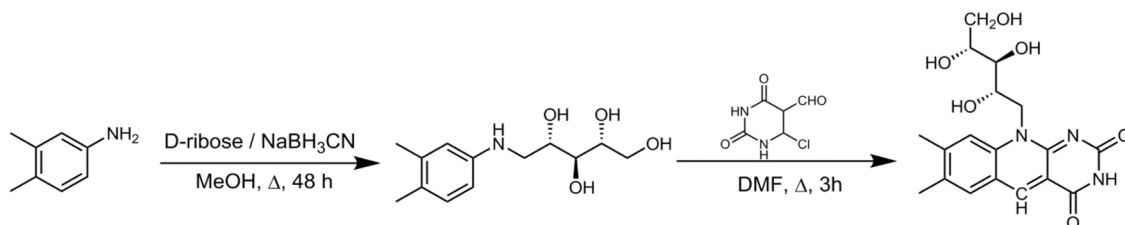
In a typical experiment 100 μM dRf were reduced by 10 mM sacrificial single electron donor or 1 mM hydride donor in a 100 mM KPi buffer at pH 6.0. The absorbance at 400 nm ($\epsilon = 12500 \text{ M}^{-1} \text{ cm}^{-1}$) was followed to determine the oxidation state of dRf over time (Fig. S4.12).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

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 photo-reduced 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 mm 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).

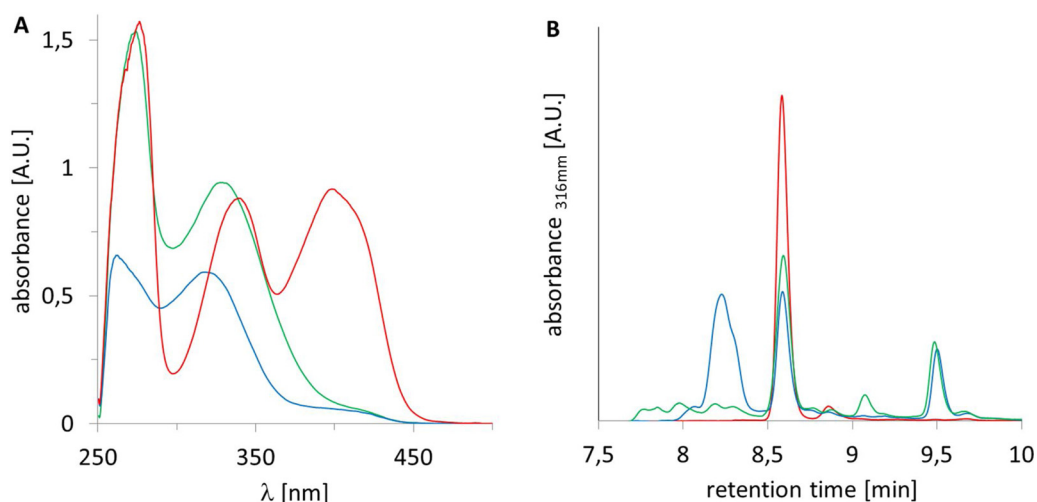


Fig. 1. Photochemical reduction of deazariboflavin (dRf) using NADH or EDTA as sacrificial electron donor. A: UV/Vis spectra of the various dRf species observed during the photoreduction of dRf^{ox} (red) with EDTA (blue) and NADH (green). General conditions: 75 μ M of dRf, 10 mM EDTA or 75 μ M NADH, 100 mM KPi buffer pH 6.0, Blue LED light setup, max light intensity, anaerobic conditions, RT. B: HPLC chromatograms recorded of photochemical reductions of dRf (red) using NADH (green) or EDTA (blue) as stoichiometric reductant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

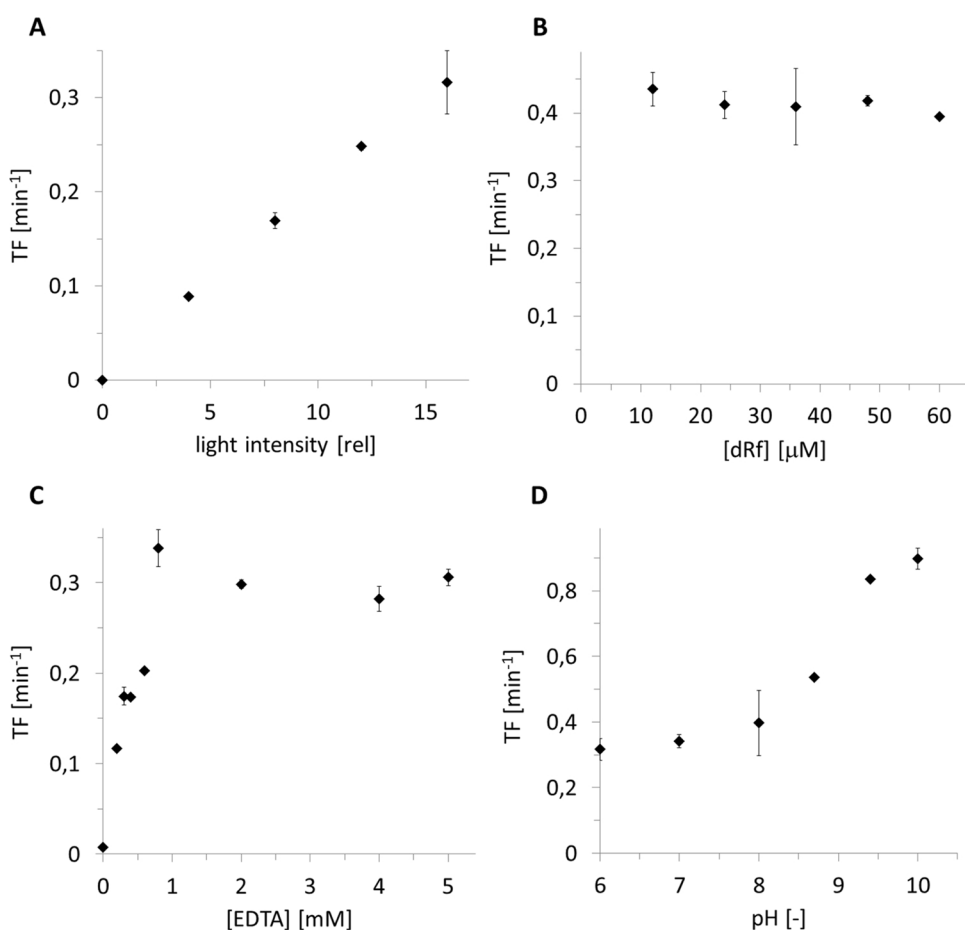


Fig. 2. Dependency of the dRf reduction rate (expressed as turnover frequency of dRf, TF) on A: intensity of the blue LED light source, B: dRf^{ox} concentration, C: Concentration of the sacrificial electron donor and D: pH. General conditions: 60 μ M of dRf^{ox}, 10 mM EDTA, 100 mM KPi buffer pH 6.0, blue LED light setup, max light intensity, anaerobic conditions, RT. $\text{TF} = (\text{initial dRf reduction rate } [\mu\text{M min}^{-1}]) \times (c(\text{dRf}) [\mu\text{M}])^{-1}$.

a two-step synthesis following a recently published procedure (Scheme 3) [61]. Overall, 457 mg of pure dRf (28% overall yield) were produced.

Having dRf at hand, we next investigated its photoreduction using either sodium ethylenediaminetetraacetate (EDTA, a single electron donor) or NADH (a hydride donor) (Fig. 1). In addition to EDTA also 3-(*N*-morpholino)propanesulfonic acid (MOPS) and other amino alcohols (such as TRIS) also served as single electron donors (Fig. S4.4). Also NADH can be replaced by the nicotinamide mimic benzyl dihydronicotinamide (BNAH). It is worth mentioning here that in all cases illumination was necessary to induce dRf reduction. The only exception

being BNAH, with which also in the dark, significant formation of H₂dRf was observed. One possible explanation for this may be the more negative redox potential of BNAH compared to NADH (−0.36 V and −0.315 V SHE, respectively) [63].

The spectra of single electron donor- and hydride-donor reduced dRfs differed significantly in shape suggesting different reduction products. This assumption was corroborated by chromatographic separation of the reaction products (Fig. 1B). While NADH-reduced dRf gave only one product (H₂dRf, as confirmed by its characteristic UV spectrum, Fig. S4.1) [56] EDTA-reduction of dRf yielded H₂dRf and another product (most likely assigned to the dimeric, half-reduced semiquinone

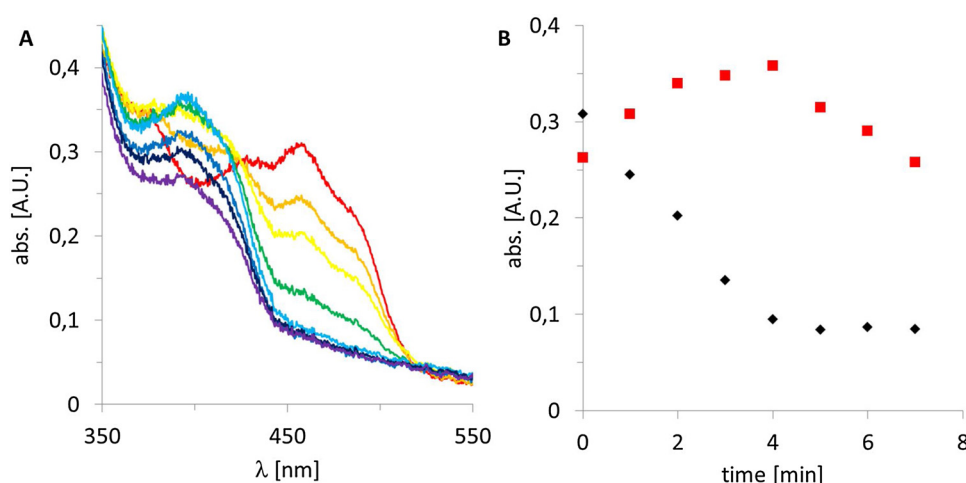


Fig. 3. Time course of the $(dRf)_2^{\text{red}}$ mediated reduction of YqjM. A: spectra recorded over time (1 min intervals) and B: time courses of the characteristic absorption maxima for YqjM-FMN^{ox} (290 nm, \blacklozenge) and dRf^{ox} (355 nm, \blacksquare). Conditions: A solution of 60 μM dRf^{ox} in 100 mM KPi buffer containing 0.5 mM EDTA was illuminated (blue LED) until full reduction was achieved, afterwards, this reaction mixture was supplemented with the same volume of 40 μM YqjM (in the same buffer) and subsequently illuminated using a blue LED [71]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

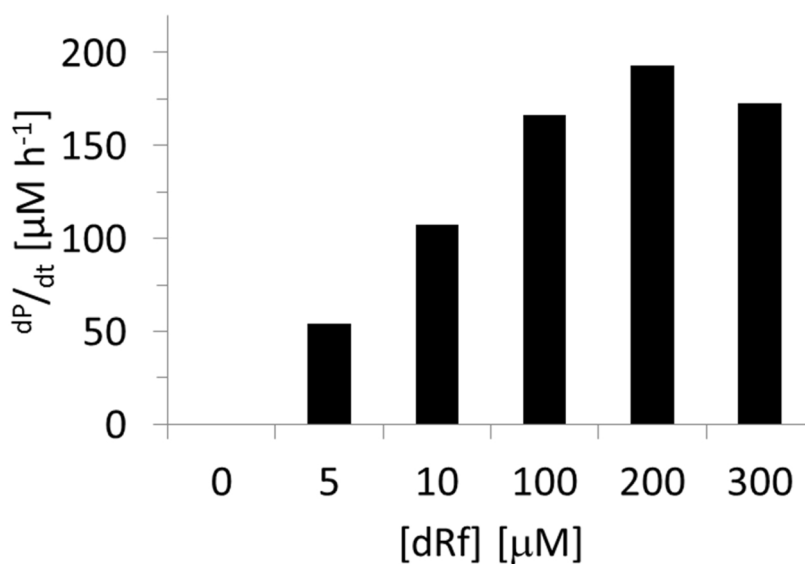
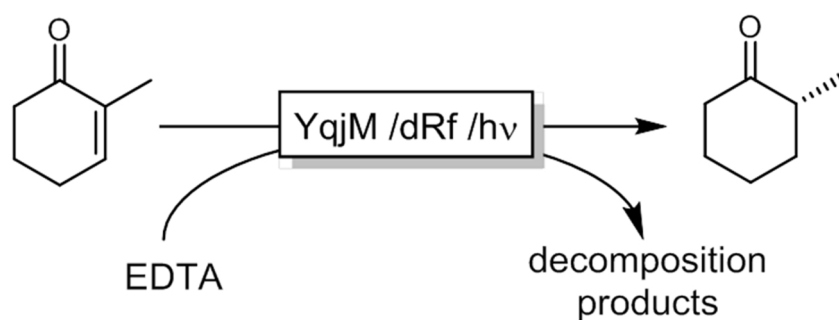


Fig. 4. Photoenzymatic reduction of 2-methyl cyclohexenone. General conditions: 5 μM YqjM, 1 mM 2-methyl cyclohexenone, 10 mM EDTA, 100 mM KPi pH 6.0, blue LED light setup, blue LED at maximal light intensity, anaerobic conditions, RT. It should be mentioned, that the solubility limit of dRf was around 250 μM , therefore, the sample at 300 μM dRf was turbid, which possibly influenced the regeneration reaction.

product) appearing simultaneously over time (Fig. S4.2). These findings are in line with previous reports using EDTA or NaBH_4 as reductants [58,64].

Next, we characterised the light-driven reduction of dRf^{ox} using EDTA as a sacrificial electron donor in some more detail. It is worth mentioning here that the photochemical reduction of dRf proceeded approximately 14 times slower than the reduction of ‘normal’ riboflavin. This is most likely attributed to the better overlap of the riboflavin absorption spectrum with the emission spectrum of the LED used

in this study (Fig. S4.5) [65]. However, also differences in the redox potentials of Rf (-0.146 V vs. SHE) and dRf (-0.237 V vs. SHE) may contribute [66]. Using the Nernst equation, the redox potential of dRf was estimated to be -0.332 V vs. SHE at pH 6.0 (see SI for the calculations).

The reduction rate of dRf was linearly dependent on the light intensity applied (Figs. 2 A, S4.6). Likewise the overall rate of dRf reduction linearly depended on the dRf concentration itself (Fig. 2B). Hence, we conclude that the *in situ* concentration of photoexcited dRf is

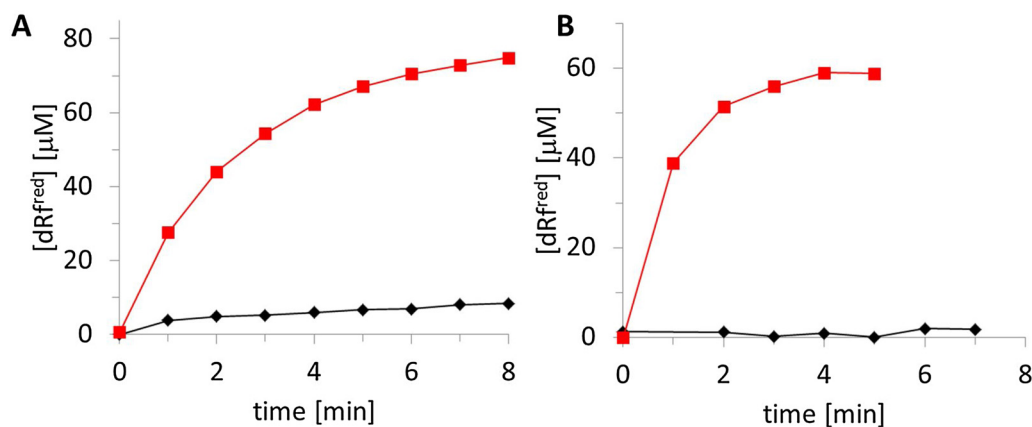


Fig. 5. Photochemical reduction of deazariboflavin using NADH (A) or EDTA (B) as sacrificial electron donor under aerobic (♦) and anaerobic (■) conditions. Conditions: 10 mM EDTA or 1 mM NADH, 100 mM KPi buffer pH 6.0, blue LED light setup, max light intensity, RT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Aerobic reoxidation rates observed for deazariboflavin reduced by EDTA or NADH.

	Reoxidation rate [$\mu\text{M h}^{-1}$]	
	Dark	Light
(dRf) ₂	1.7	96
H ₂ dRf	0.78	30

Conditions: 10 mM EDTA or 1 mM NADH, 100 mM KPi buffer pH 6.0, blue LED light setup, max light intensity, RT.

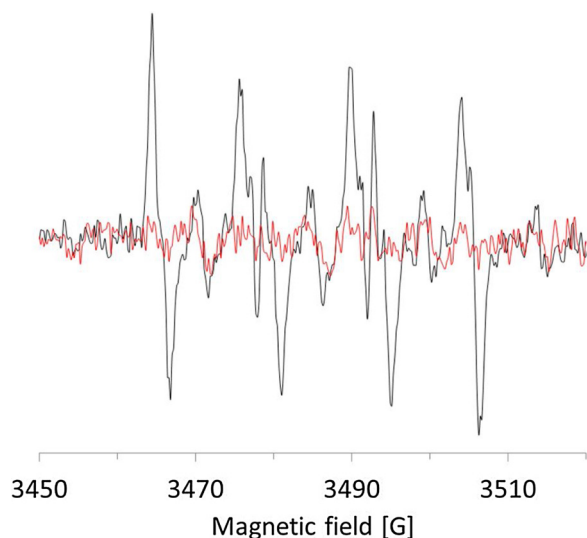


Fig. 6. EPR spectra recorded using the spin trap technique during the aerobic reduction of dRf with BNAH under illumination (black) and in dark (red). Conditions: 60 μM dRf, 1 mM BNAH, 1% v/v DMSO, 100 mM KPi pH 6.0, Kaiser Fibre Optic Lighting system 15 V, 150 W, on half intensity, aerobic conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overall rate limiting. Varying the concentration of the sacrificial electron donor (EDTA) revealed a saturation-type dependency of the dRf reduction rate on the EDTA concentration applied (Fig. 2C). Above approximately 1.5–2 mM EDTA (pH 6), no further increase of the dRf reduction rate was observed. Finally, there was a sigmoidal pH-dependency of the dRf^{ox} reduction rate with a turning point at approximately pH 9 (Fig. 2D). Interestingly, using MOPS instead of EDTA shifted this turning point to pH 7 (Fig. S4.7). Furthermore, the substitution pattern of the N-atom in the sacrificial electron donor had an influence on the reduction rate (Fig. S4.4). Overall, it appears that

higher electron densities around the N-atom of the donors facilitate the electron transfer to dRf. These observations are in line with the oxidation mechanism suggested by Kramer and coworkers [67]. These authors suggested a methylene radical intermediate being formed after single electron transfer of the amino acid donor and subsequent decarboxylation. The extent of hyperconjugative stabilisation of this intermediate radical should increase with the N-substitution pattern as well as with occurrence of a non-protonated N-substituent.

To further examine the applicability of photoreduced dRf^{red} to regenerate oxidoreductases, we used it to reduce the FMN-dependent old yellow enzyme homologue from *Bacillus subtilis* (YqjM) [36,68,69]. The different spectral properties of dRf^{ox} ($\lambda_{\text{max}} = 390 \text{ nm}$) and YqjM-bound FMN^{ox} ($\lambda_{\text{max}} = 455 \text{ nm}$) allow for the simultaneous determination and quantification of the electron transfer between photoregenerated dRf^{red} and YqjM-bound FMN^{ox} (Fig. 3). In accordance to previous findings by Massey and Hemmerich [70] we found that photoregeneration of YqjM was possible. Using e.g. 1.5 eq of (prior reduced) dRf full reduction of YqjM was observed within 4 minutes. This reaction was observed under blue light illumination only. Incubation of YqjM with prereduced dRf in the dark or upon illumination with other wavelengths yielded no significant reduction of the YqjM-bound FMN (Fig. S4.8). Currently, we are lacking a satisfactory explanation for this observation. Possibly the interaction of (dRf)₂^{red} with the enzyme-bound FMN is sterically hindered and photoexcitation of the latter may accelerated the long-distance electron transfer.

To test if catalytic turnover of both, dRf and YqjM is feasible, we used the enantioselective reduction of 2-methyl cyclohexanone to (*R*)-2-methyl cyclohexanone as model reaction (Fig. 4). In the absence of dRf no conversion of the starting material was observed indicating that direct photochemical reduction of YqjM-bound FMN by EDTA was not efficient. However, already in the presence of 5 μM dRf (equimolar to YqjM) a product formation rate of approximately 50 $\mu\text{M h}^{-1}$ was observed. Hence, a turnover frequency of approximately 10 h^{-1} was calculated for YqjM and dRf. This corresponds well to the YqjM-reduction rate observed before (Fig. 3) indicating that the reduction of the biocatalyst was overall rate-limiting. The overall rate of the photoenzymatic reduction reaction increased steadily with increasing photocatalyst concentration (up to 200 μM , representing the solubility limit for dRf). With it, the catalytic efficiency of YqjM increased to 40 h^{-1} . It is worth mentioning that in all experiments (*R*)-2-methyl cyclohexanone was formed almost exclusively (Fig. S4.9).

While these numbers are comparable to recently reported photoenzymatic systems,[46] the catalytic performance of YqjM falls back by orders of magnitude behind its potential (1.8 s^{-1} using NADPH as reductant) [36]. A plausible explanation for this is to assume an unfavourable interaction of the reduced dRf mediators with the enzyme-bound FMN resulting in poor electron transfer rates. Both, cofactor- and enzyme engineering may generate artificial binding sites and thereby accelerate the regeneration reaction [72,73].

Finally, we re-visited the O₂-stability of reduced dRfs. As mentioned above, significant reduction of YqjM-bound FMN and product formation in the photoenzymatic system was observed only under strictly anaerobic conditions. We therefore investigated the influence of O₂ on the photochemical reduction of dRf (Fig. 5). Independent from the sacrificial electron donor used (NADH or EDTA) reduction of dRf was observed under anaerobic conditions only while it was negligible in the presence of O₂. In the latter cases, H₂O₂ was detectable as well as NADH oxidation (in case of Fig. 5A) suggesting dRf^{red} reoxidation occurring simultaneously.

Therefore, we determined the reoxidation rates of photochemically reduced dRf in the dark and under illumination (Table 1). Both, the dimeric (dRf)₂ as well as the fully reduced H₂dRf were rather stable against O₂ in the dark whereas they swiftly reoxidized in the presence of (blue) light. Similar observations had previously been made by Hemmerich and coworkers [58].

The influence of light can be rationalised assuming that (trace amounts) of photoexcited, oxidised dRf synproportionate with H₂dRf or (dRf)₂ forming a reactive semiquinone radical reacting with O₂ via single electron transfer. This reaction should be autocatalytic, for which we have found indications in the reoxidation time-course (Figure S 4.10). Furthermore, EPR-measurements during BNAH-mediated reduction of dRf in the presence and absence of light support this assumption (Figs. 6 and S4.12). As dRf also is a photosensitizer capable of generating singlet oxygen (¹O₂) [74], an involvement of ¹O₂ cannot be ruled out completely. ¹O₂-oxidations, however, generally do not involve radical species, which is in contrast to the observations of radicals mentioned above.

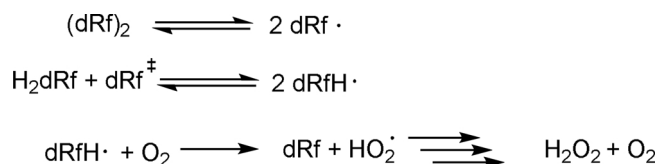
Conclusions

Direct reductive regeneration of flavoenzymes circumventing the expensive and unstable nicotinamide cofactors is an attractive alternative to established systems utilizing the nicotinamide cofactor together with an (enzymatic) regeneration system.

In case of O₂-dependent enzymes, the *Oxygen Dilemma*, however, severely impairs the utility of these approaches. While the high reactivity of 'normal flavins' with molecular oxygen is well-known [75], reduced deazaflavins are considered to be much more stable due to the spin-forbidden character of the reoxidation with triplet oxygen (³O₂) [52]. This motivated us to evaluate 5-deazariboflavin (dRf) as photocatalyst and mediator to regenerate flavin-dependent enzymes. Indeed, fully reduced H₂dRf is fairly stable in the presence of molecular oxygen. In the presence of oxidised dRf (especially if illuminated), however, radical species are formed via synproportionation, which react quickly with molecular oxygen (Scheme 4) [58].

Indeed, neither accumulation of reduced dRf or catalytic turnover was observed under aerobic conditions. Therefore, we conclude that a simple one-pot reaction cascade entailing photochemical reduction of dRf and biocatalytic reaction is not feasible due to the uncoupling reaction described above.

Possibly, technical solutions such as compartmentalised reaction



Scheme 4. Aerobic reoxidation of reduced deazaflavin species. The reactive semiquinone radical (dRf) reacts fast with O₂ eventually leading to H₂O₂ and O₂. dRf itself is formed either via the dimerization equilibrium of (dRf)₂ or through synproportionation of H₂dRf with (photoexcited) dRf. For reasons of simplicity, protonation equilibria have not been included in this scheme.

setups (anaerobic reduction of the flavin followed by aerobic biocatalytic reaction) as suggested by Schmid and coworkers [43] may be doable.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mcat.2018.04.015>.

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