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Photochemical regeneration of flavoenzymes – An Old Yellow Enzyme case-study

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A B S T R A C T

Direct, NAD(P)H-independent regeneration of Old Yellow Enzymes represents an interesting approach for simplified reaction schemes for the stereoselective reduction of conjugated C=C-double bonds. Simply by illuminating the reaction mixtures with blue light in the presence of sacrificial electron donors enables to circumvent the costly and unstable nicotinamide cofactors and a corresponding regeneration system.

In the present study, we characterise the parameters determining the efficiency of this approach and outline the current limitations. Particularly, the photolability of the flavin photocatalyst and the (flavin-containing) biocatalyst represent the major limitation en route to preparative application.

1. Introduction

Ene-reductases from the Old Yellow Enzymes (OYEs) family have been known for close to a century [1]. They catalyse the stereospecific *trans*-hydrogenation of conjugated C=C-double bonds creating up to two new chiral centres (Scheme 1).

Their potential as catalysts for organic synthesis, however, has only been recognised by Faber, Hauer and coworkers a decade ago [2,3]. Since then, preparative applications of OYEs have increased exponentially [4,5] with the first examples of industrial application [6,7].

OYEs contain a flavin prosthetic group – mostly a flavin mononucleotide (FMN) - which in the first reduction half-reaction of the catalytic mechanism is reduced by the nicotinamide adenine dinucleotide cofactor (NAD(P)H); in the second oxidation half-reaction of the mechanism, the reduced flavin transfers a hydride ion in a Michael-type addition to the β-carbon atom of the enzyme-bound unsaturated substrate followed by protonation of the resulting enolate anion in a *trans* fashion.

In the past years, a range of alternative reductants have been reported replacing the costly and unstable nicotinamide cofactors [8]. Amongst them, chemical reductants such as synthetic nicotinamides [9–12] or transition metal catalysts [13,14] have been proposed. Also photochemical approaches are gaining attention [15–26]. The latter approaches mostly utilise FMN (the prosthetic group of OYEs) as photocatalyst to channel electrons from a sacrificial electron donor (e.g. EDTA) to the OYE's active site (Scheme 2). The photochemistry of the (yellow) flavin photocatalyst has been established decades ago [27–31], but a critical evaluation of their practical usefulness is so far missing. We therefore set out to investigate the scope and limitations of direct, photochemical regeneration of FMN to promote OYE-catalysed reduction reactions.

As a model reaction for our investigations, we chose the stereospecific reduction of 2-methylcyclohexenone to (*R*)-2-methylcyclohexanone catalysed by the OYE homologue from *Bacillus subtilis* (YqjM) (Scheme 2) [32–34].

2. Results and discussion

Recombinant YqjM was produced in *Escherichia coli* using an auto-induction medium [35,36]. His-Tag containing YqjM was purified by affinity chromatography using Ni-NTA chromatography.

For the photochemical reaction outlined in Scheme 2, we used commercial LEDs (λ = 450 nm) as light source wrapped around the reaction vessel (Figs. S1 and S2). With this setup, full conversion of 10 mM 2-methylcyclohexenone could be achieved within < 5 h (Fig. 1). No further side product was observed using gas chromatography for analysis.

The enantioselectivity of the reaction was superb (*ee* > 99.5%) as throughout the experiment only traces of the (*S*)-enantiomer were observed. This finding also confirms our assumption of the enzymatic reduction of the conjugated C=C-double bond as the direct reduction by the reduced mediator would have yielded racemic product. It is worth mentioning that anaerobic reaction conditions were essential for the reaction as, in the presence of molecular oxygen, lower reaction

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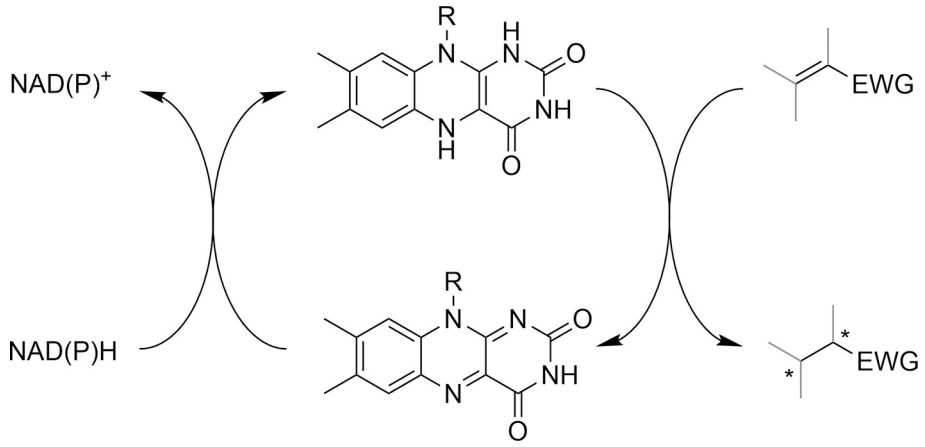
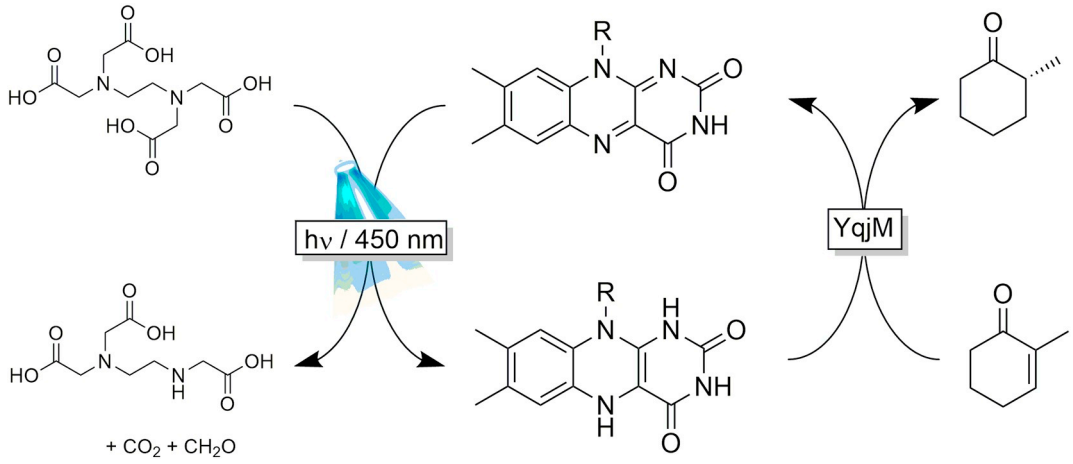


Scheme 1. Reduction of activated C=C-double bonds by Old Yellow Enzymes (OYEs). EWG = electron withdrawing group (typically a carbonyl group).



Scheme 2. Photoenzymatic reduction of 2-methylcyclohexenone to (*R*)-2-methylcyclohexanone using YqjM as biocatalyst. *In situ* regeneration of the reduced flavin prosthetic group is achieved using photoexcited FMN in the presence of EDTA as sacrificial electron donor.

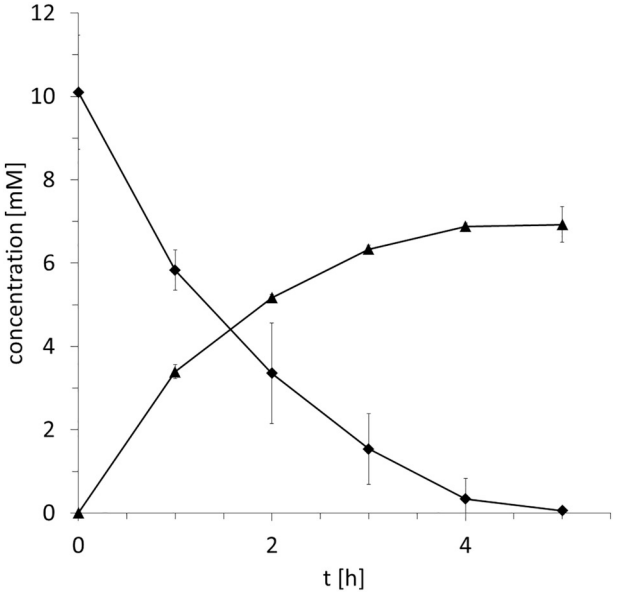


Fig. 1. Time-course of the photoenzymatic reduction of 2-methylcyclohexenone (♦) to (*R*)-2-methylcyclohexanone (▲). Conditions: 100 mM potassium phosphate buffer (pH 6.5), c(2-methylcyclohexanone) = 10 mM; c(FMN) = 0.2 mM, c(YqjM) = 6.9 μM, c(EDTA) = 50 mM, T = 30 °C, light source: blue LED (450 nm, 80 μW.cm^{-2}). During the reaction, anaerobic conditions were maintained leading a N_2 stream over the reaction.

rates were observed (2.04 mMh^{-1} instead of 3.39 mMh^{-1}, Fig. S8). This is in line with some previous findings about the high reactivity of reduced flavins in the presence of O_2 [37–42]. Therefore, we applied a gentle N_2 stream over the reaction mixture to maintain anaerobic reaction conditions. This, however, also lead to some increased evaporation of the reagents (as confirmed in appropriate control experiments), explaining the rather poor mass balance of approx. 70%. Improved reaction setups including condensers to minimise the reagent evaporation will circumvent this technical issue.

The catalytic performance of YqjM in terms of turnover frequency (TF = amount of product formed in time divided by the amount of YqjM used) was only in the range of 8 min^{-1} and thereby fell back significantly behind the activities reported using NADPH as reductant [32]. We therefore decided to investigate the parameters influencing the reaction rate in more detail. Particularly, we systematically varied the concentration of the photocatalyst (Fig. 2A), the biocatalysts (Fig. 2B), the light intensity (Fig. 2C) and the concentration of the sacrificial reductant (Fig. 2D).

Varying the photocatalyst concentration, we observed a saturation-type behaviour of the reaction rate with increasing FMN concentrations. Half-maximal reaction rates were observed at FMN concentration around 200 μM. This is almost 100-fold higher than the K_M value determined for the natural reductant (NADPH) [34] suggesting that the interaction of the (reduced) FMN to the active site of YqjM may be overall rate-limiting due to the sterically impaired interaction and suboptimal electron transfer from the photocatalytic FMN to the enzyme-bound FMN.

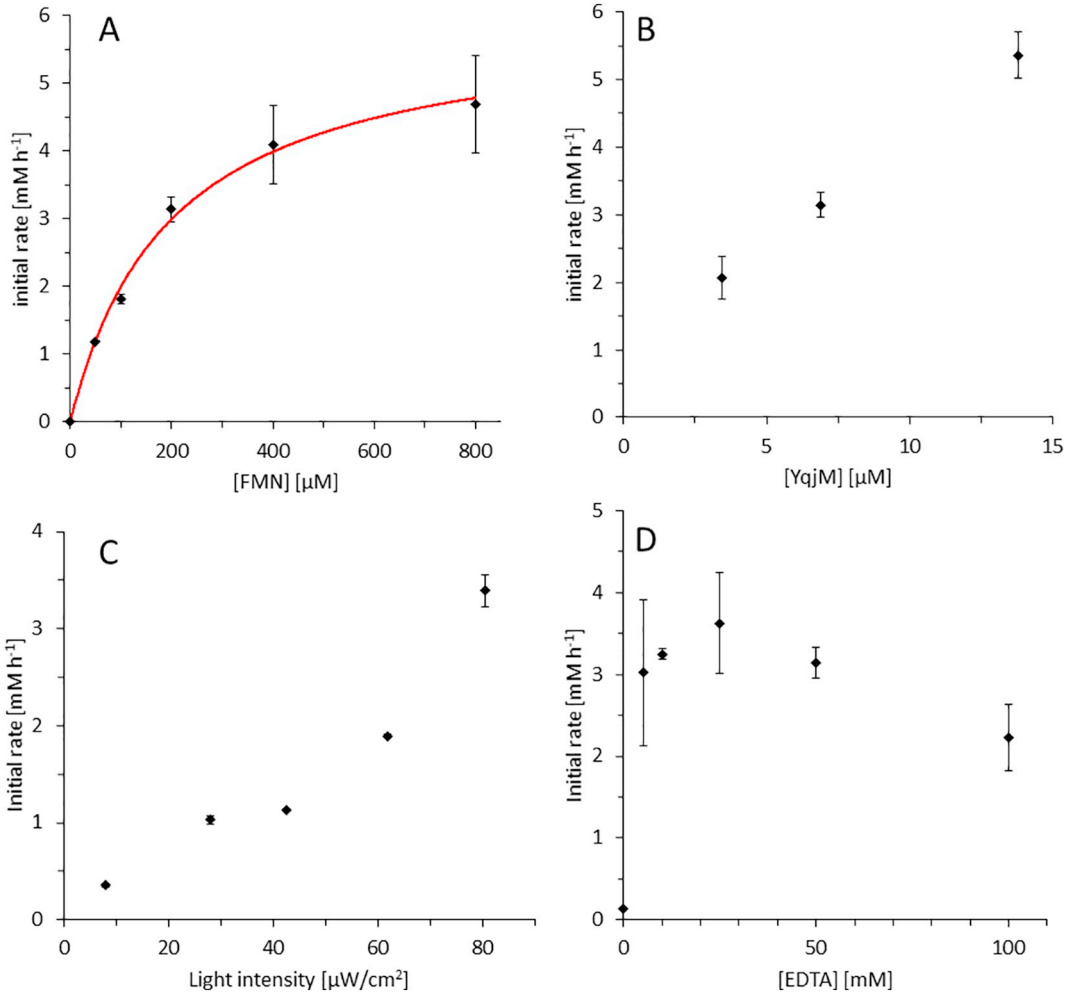


Fig. 2. Influence of the photocatalyst concentration (A), the enzyme concentration (B), the light intensity (C) and the sacrificial electron donor concentration (D) on the reduction rate of 2-methylcyclohexenone. Unless stated in the graph the following conditions were used: 100 mM potassium phosphate buffer (pH 6.5), c(2-methylcyclohexenone) = 10 mM; c(FMN) = 0.2 mM, c(YqjM) = 6.9 µM, c(EDTA) = 50 mM, T = 30 °C, light source: blue LED (450 nm, 80 µW.cm^{-2}). During the reaction, anaerobic conditions were maintained leading a N_2 stream over the reaction.

Increasing the biocatalyst concentration resulted in an almost linear increase of the overall reaction rate, which is in-line with the assumption of the reduction of YqjM being overall rate-limiting. The same is true for the observed influence of the light intensity. Higher photon fluxes are expected to yield higher concentrations of photoexcited flavins and therewith increase the concentration of reduced external flavins. The concentration of the sacrificial electron donor (EDTA) had only a minor influence on the overall rate. Only marginal conversion was observed in the absence of EDTA, which may be explained by the photochemical reduction of FMN by traces of 'contaminating' reductants (*vide infra*). Between an EDTA concentration of 5 to 50 mM, the overall rate remained largely unchanged and slightly decreased at higher EDTA concentrations. Activity assays of YqjM in the presence of elevated EDTA concentrations gave no indication for an inhibitory effect of EDTA (e.g. by removal of structurally relevant metal cations). Hence, at present stage we are lacking a plausible explanation for the decreased productivity of the photocatalytic system at higher EDTA concentrations.

Encouraged by these results we aimed at scaling up the reaction by increasing the substrate concentration to 50 mM. Unfortunately, product accumulation ceased after approximately 5 h (Fig. S6), which was also the time span of the experiment shown in Fig. 1. We ascribe this observation to a poor stability of YqjM under the reaction conditions. Indeed, product formation, resumed upon addition of fresh biocatalyst (Fig. S7) resulting in the accumulation of 36 mM of enantiopure product (72% yield and 100% conversion due to the above-mentioned evaporation issue). YqjM performed > 2500 catalytic cycles in this experiment.

Therefore, we investigated the stability of YqjM under different conditions (Fig. 3). If incubated in buffer in the presence of FMN but in the dark, YqjM was relatively stable with a half-life of approx. 33 h. This value, however, dramatically dropped upon illumination with blue light (both in the presence and absence of external FMN) and the apparent half-life of YqjM dropped to < 2 h. Under reducing conditions, i.e. in the presence of EDTA (or NADH), this decrease was somewhat less pronounced ($t_{½} \approx 5.5$ h). Furthermore, illumination with either red or green light had a far less detrimental effect on the stability of YqjM.

These results are most probably due to photodegradation of the enzyme-bound FMN [43–46]. Under dark conditions, virtually none of the YqjM-tightly bound FMN is photoexcited and therefore stable. The emission spectra of the green and red LEDs used in this experiment hardly overlap with the FMN absorption spectrum [40] and therefore only lead to minor photoexcitation and photodegradation on YqjM-tightly bound FMN (green light overlaps somewhat more and therefore also decreases the half-life of YqjM more pronouncedly). The effect of the reducing conditions may be explained by the significantly reduced absorption of reduced flavins at 450 nm. This assumption is also supported by the spectral changes recorded for both YqjM and free FMN

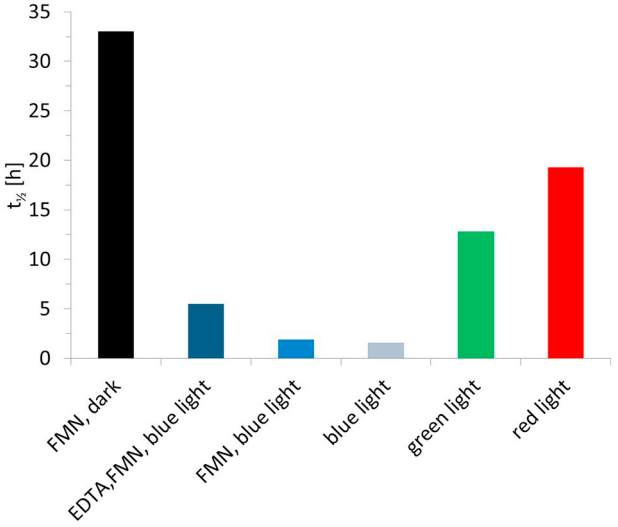


Fig. 3. Stability of YqjM under different incubation conditions. Incubation conditions: 6.88 μM of YqjM in KPi buffer 100 mM pH 6.5 in presence of either 200 μM of FMN and/or 50 mM of EDTA and illuminated by either blue, red or green LED or protected from any light source, T = 30 °C. Activity assay conditions: 20 mM of glucose, 150 μM of NADPH, 10 U/mL of glucose oxidase, 1 mM of 2-methylcyclohex-2-en-1-one and 0.85 μM of YqjM.

upon illumination with blue light (Fig. 4).

In the course of our experiments we also frequently observed a precipitate, which we believed to be denaturated YqjM. SDS-PAGE gel analysis revealed a loss of soluble protein under blue light illumination (Figs. S24–S25). Furthermore circular dichroism analysis (Figs. S22–S23) of the illuminated enzyme pointed towards some loss in the integrity of the tertiary structure of the enzyme. Size exclusion chromatography of a light treated YqjM sample showed an aggregation of enzymes over time (Fig. S21). We also performed a proteolytic digest of the illuminated and native YqjM using trypsin and analysed the resulting peptides by mass spectrometry for degradation products (Figs. S3–S4). This revealed that in the illuminated YqjM, two methionine residues (M14 and M134) were oxidised to the corresponding sulfoxides (Fig. S5). Although some oxidation of another methionine was observed already for the non-illuminated protein, the degree of sulfoxidation increased significantly after exposure to light. Illumination of the YqjM samples occurred under anaerobic conditions (using a stream of N_2 gas) which makes H_2O_2 a rather unlikely oxidant for this reaction. Possibly, photoexcited flavins can directly oxidise methionine via a so far unknown mechanism and electron sink. It is also worth mentioning that this analysis did not cover the entire YqjM polypeptide, therefore, at present time we cannot exclude further modifications of the protein to account for the decreasing stability under illumination. As these amino acids are at the surface of the protein, oxidation of these amino acids may well account for the protein aggregation and precipitation observed. Similar effects have been already described for oxidative stress related biological aging and degradation [57]. Future experiments with YqjM mutants devoid of these methionine residues will show if such mutants exhibit higher robustness under the photochemical reaction conditions [58].

3. Conclusions

Overall, we have confirmed that direct, NAD(P)H-independent regeneration of YqjM can productively be achieved using photoregenerated, reduced FMN photocatalysts/mediators.

The main limitation of the current setup lies with the poor photostability of the enzyme-bound, as well as the free FMN cofactor. As consequence, comparably low turnover numbers ($TON = mol_{Product} \times mol_{Catalyst}^{-1}$) in the range of 2000 and 30–40 for YqjM and FMN, respectively, were observed. While these numbers are in the range of state-of-the-art OYE reactions [3,59,60] they are orders of magnitude too low for economical synthesis of non-highly value-added products [61]. Therefore, further work will have to concentrate on increasing the robustness of the photoenzymatic reaction. Particularly we will focus on photocatalysts/mediators such as methylene blue and its derivatives [62] exhibiting an absorption maximum in the (infra)red range. This way, the photodegradation of OYEs can be minimised. Ideally, these photocatalysts/mediators exhibit more efficient interaction with the enzyme active site to accelerate the electron transfer from the reduced mediator to the oxidised, enzyme-bound flavin [63].

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

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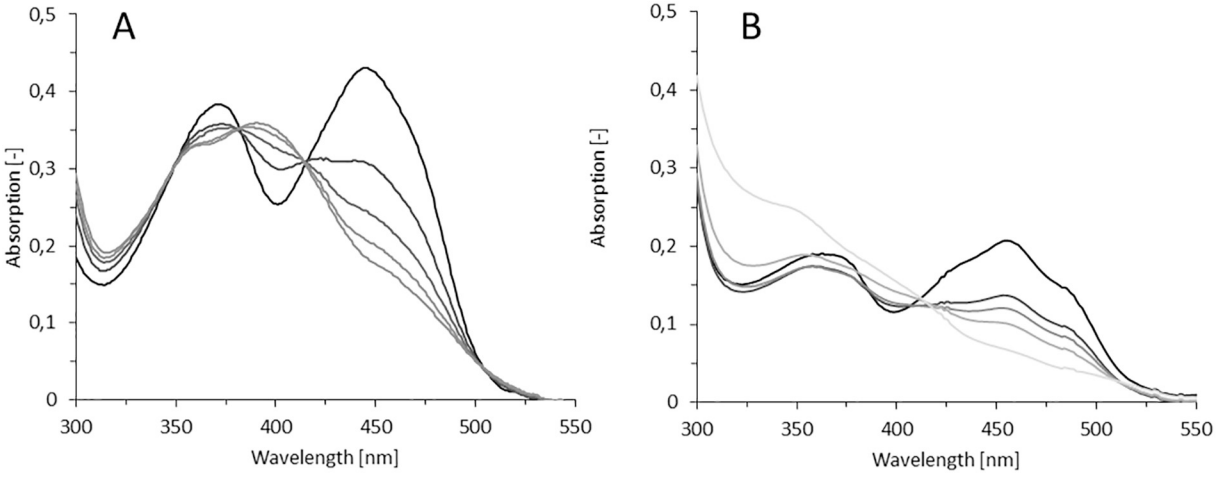

Fig. 4. Spectral changes observed for FMN (A) and YqjM (B) upon illumination with blue light. FMN: spectra were taken at 0, 1, 2, 4 and 8 min (from dark to light); YqjM: spectra were taken a 0, 1, 2, 5 and 23 h (from dark to light). General conditions: 20 μM of YqjM or FMN in KPi buffer 100 mM pH 6.5 illuminated by blue LED, 80 $\mu W.cm^{-2}$, T = 30 °C.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2019.140303.

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