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Catalysis

Characterization of the Old Yellow Enzyme Homolog from *Bacillus subtilis* (YqjM)

Milja Pesic, Elena Fernández-Fueyo, and Frank Hollmann*[a]

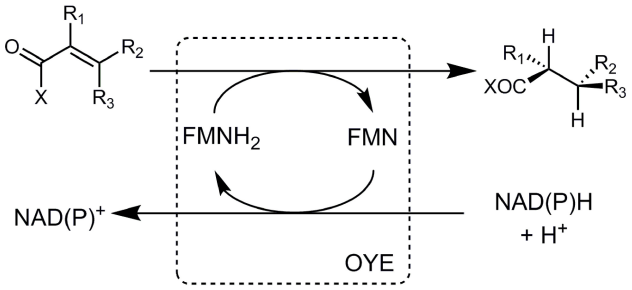
Old Yellow Enzymes (OYEs) have become practical catalysts for the stereoselective *trans*-hydrogenation of conjugated C=C-double bonds. Despite their importance, in-depth characterization of their kinetic parameters and factors influencing their activity and stability have largely been neglected in the past years. In this study, we close this gap for one of the most prominent OYEs, i.e. the OYE from *Bacillus subtilis* (YqjM).

Introduction

Since more than a decade, ene reductases from the Old Yellow Enzyme family are receiving considerable attention as catalysts for the preparative conversion of conjugated C=C-double bonds.[1] First reported in the early 20th century,[2] this family of enzymes has been subject to intensive mechanistic studies in the 1990s especially by Massey and coworkers.[3] Later, especially thanks to the pioneering work by Faber and Hauer, the scope of OYE-like biocatalysts has expanded tremendously and with it the scope of useful transformations.[1b,d,e]

The catalytic mechanism of OYE-homolog ene reductases is fairly well understood.[1c–e] In the natural cycle, an enzyme-bound flavin mononucleotide cofactor (FMN) first is reduced by a reduced nicotinamide cofactor (reductive half-reaction, Scheme 1 lower). In the second step of the catalytic mechanism (oxidative half reaction, Scheme 1 upper) the reduced $FMNH_2$ itself reduces the substrate in a Michael-type hydride transfer to the β-C-atom. Protonation of the resulting anion occurs from the opposite face of the C=C-double bond through a tyrosine moiety; overall resulting in a *trans*-hydrogenation. This catalytic mechanism also enables regeneration pathways independent from the natural nicotinamide cofactor such as substrate-coupled regeneration,[4] use of synthetic reductants[5] and electrochemical[6] or photochemical regeneration.[7]

Furthermore, OYE are currently experiencing an increasing interest from the catalysis community for the preparation of enantiomerically pure compounds from simple (prochiral) conjugated C=C-double bonds. For example, preparation of



Scheme 1. OYE-catalyzed *trans*-hydrogenation of conjugated C=C-double bonds. The catalytic mechanism is generally divided into an 'oxidative' and a 'reductive' half-reaction. In the latter, the enzyme-bound FMN cofactor is reduced by NAD(P)H resulting in $FMNH_2$ (or its partially deprotonated form) which then, in the oxidative half reaction donates a hydride to the enzyme-bound conjugated C=C-double bond in a Michael-type reaction.

chiral dicarboxylic acid(esters),[8] aldehydes and ketones,[9] nitriles,[10] nitro compounds[11] protected acyloins[12] or halogen-substituted acrylates[13] have been reported. Also, preparative scale applications are within reach.[14] Furthermore, a growing number of protein engineering studies is constantly filling the gaps of suitable enzyme variants for 'difficult' substrates.[15]

Another trend in this area is to combine OYE other redox enzymes to obtain redox-neutral redox cascades.[16]

Overall, OYEs are on the step to become truly practical catalysts for organic synthesis.[17]

Quite interestingly, however, while the scope of OYEs is rapidly increasing the number of studies dealing with an in-depth characterization of these enzymes is rather small. For example, for one of the most prominent members of the enzyme class, the OYE homolog from *Bacillus subtilis* (YqjM).[18] only a handful of kinetic studies (reporting e.g. K_M and k_{cat} values) have been reported.[18]

Therefore, the aim of this study was to fill this gap by providing some in-depth kinetic studies for YqjM to put a basis for process development and modelling.

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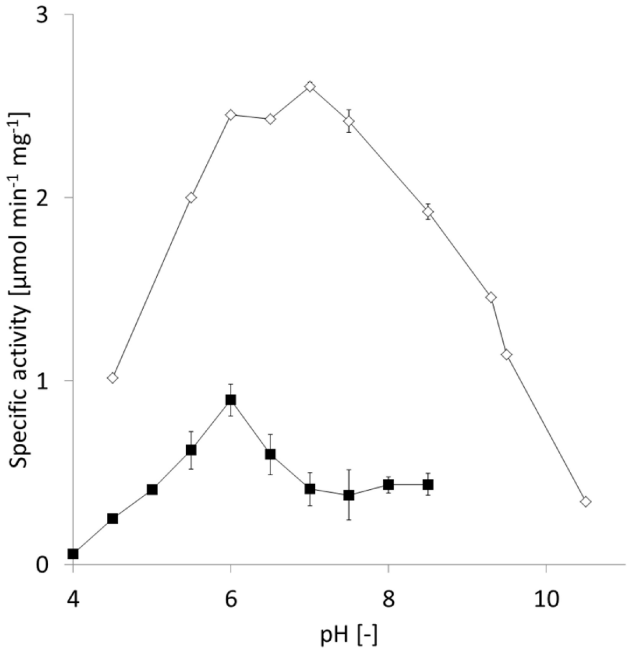
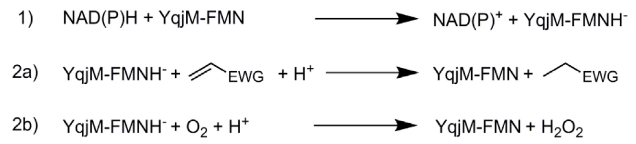


Figure 1. pH profile for the YqjM-catalyzed reduction of 2-cyclohexen-1-one (1 mM) (◇) and ketoisophorone (5 mM) (■). Conditions: 25 °C; 50 mM sodium citrate, potassium phosphate and sodium bicarbonate buffers for pH ranges 4.0-5.5, 6.0-9.3 and 9.5-10.5, respectively; 0.15 mM NADPH; 1 µM and 2 µM YqjM was used for the reduction of 2-cyclohexen-1-one and ketoisophorone, respectively. Specific activities were calculated by subtracting the activity measured in the presence of the substrate and the activity measured with the molecular oxygen as only substrate.



Scheme 2. Possible reoxidation pathways for the YqjM-bound reduced flavin (obtained from NAD(P)H-mediated reduction (reductive half-reaction), eq. 1). The reduced FMN cofactor can either transfer a hydride to the ene-substrate (eq. 2a) or to molecular oxygen (eq. 2b) (oxidative half-reaction).

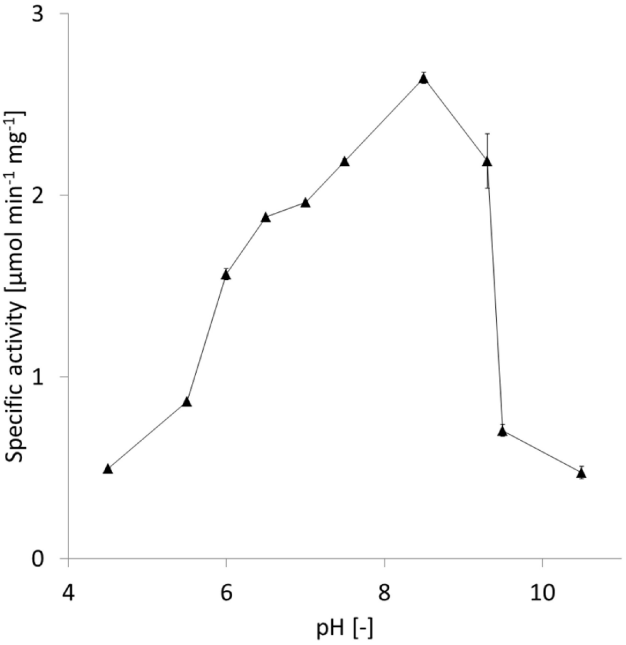


Figure 2. pH dependency of the background reaction. Conditions: 25 °C; 50 mM sodium citrate, potassium phosphate and sodium bicarbonate buffers for pH ranges 4.0-5.5, 6.0-9.3 and 9.5-10.5, respectively; 0.15 mM NADPH; 2 µM YqjM.

Results and Discussion

pH optimum of YqjM

We started the characterization of YqjM by investigating the influence of the pH value on the enzyme activity. In particular we investigated the influence of pH on the reduction of cyclohexenone and ketoisophorone as substrates (Figure 1). It is worth noting that the specific activities have been determined in a spectrophotometric assay following the depletion of NADPH (at 340 nm) after the substraction of the background activity (i.e. the non-substrate-related reoxidation of the enzyme-bound, reduced flavin, *vide infra*). YqjM accepts both, NADH and NADPH with a slight preference for the phosporylated cofactor.[18b]

For both substrates, the optimal pH value was in the slightly acidic to neutral range. The pH optimum in the neutral range compares well with those determined for other ene reductases for example the ones from *Meyerozyma guilliermondii*,[19] *Clavispora lusitaniae*,[20] *Yersinia bercovieri*[21] *Chryseobacterium*[22] and others.[22–23]

Interestingly, the background activity (Scheme 2) increased steadily with pH until approx. pH 8.5 and then rapidly decreased. Also, it is noteworthy that the rate of this background reaction was in the same order of magnitude as the (desired) C=C-bond reduction activity (Figure 2).

This rather significant contribution of the background reation to the overall NADPH consumption was rather unexpected as this 'uncoupling reaction' has been reported for flavo enzymes but generally only contributes to 5–10% of the overall NAD(P)H consumption.[24] It is worth mentioning here that under anaerobic conditions (either using the glucose oxidase / glucose system or performing the experiments in the glove box) no background reaction (oxidation of NAD(P)H in the absence of substrate) was observed.

The stability of YqjM was rather pH-independent. Incubation of the enzyme at different pH values for 24 h at 25°C yielded almost 90% activity recovery at pH 4.5 and a slightly lower residual activity of 65% at pH 9.5 (Figure 3).

Temperature optimum of YqjM

Aiming at determining the optimal reaction temperature of YqjM we investigated both, activity of YqjM at varying temper-

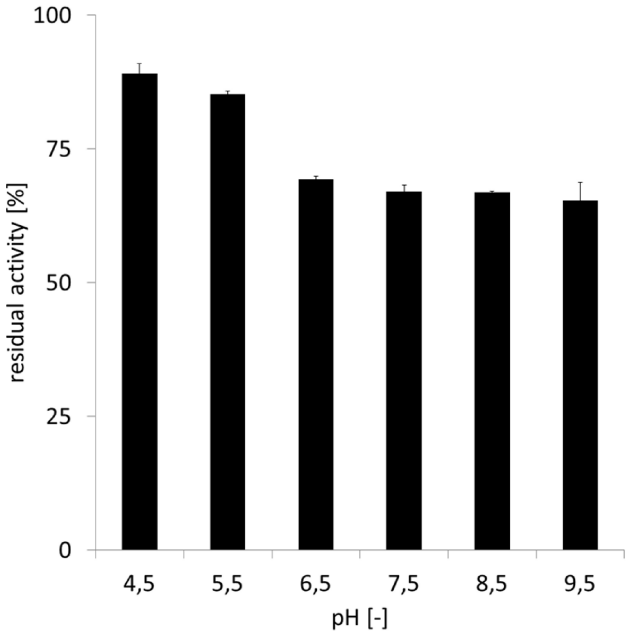


Figure 3. pH stability of YqjM at different pH values. Conditions: YqjM was incubated for 24 h in 50 mM sodium citrate, potassium phosphate or sodium bicarbonate buffers for pH ranges 4.5-5.5, 6.5-8.5 and 9.5, respectively. Residual activity was measured by standard UV assay at 340 nm and 25°C in 50 mM potassium phosphate buffer pH 6.5: 1 mM 2-cyclohex-1-one; 0.15 mM NADPH; 20 mM glucose; 10 U/mL glucose oxidase; 1 μM YqjM.

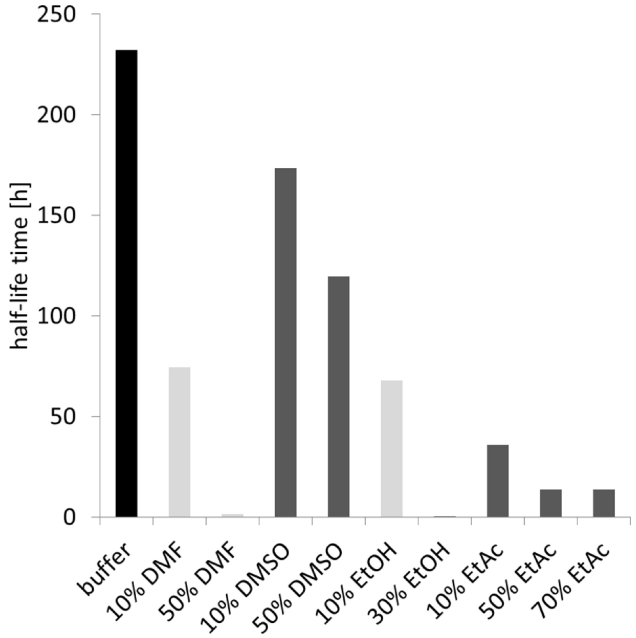


Figure 5. Robustness of YqjM against some selected co-solvents. Conditions: 25°C, 50 mM potassium phosphate buffer pH 6.5 containing the corresponding concentration (*v/v*) of the co-solvent. Activity was measured by standard UV assay at 340 nm and 25°C in 50 mM potassium phosphate buffer pH 6.5: 1 mM 2-cyclohex-1-one; 0.15 mM NADPH; 20 mM glucose; 10 U/mL glucose oxidase; 1 μM YqjM.

atures and the inactivation rate of YqjM at these temperatures (as half-life times) (Figure 4).

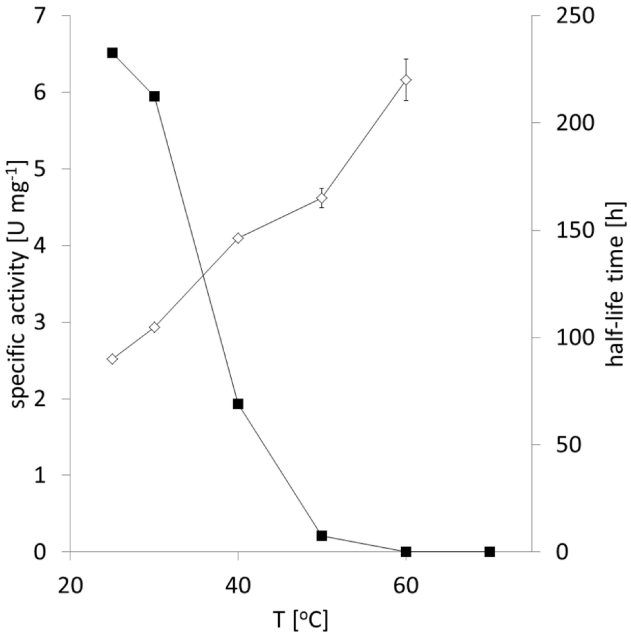


Figure 4. Temperature-dependency of the specific activity (◇) and the half-life time (■) of YqjM. Conditions: 50 mM potassium phosphate buffer pH 6.5; 1 mM 2-cyclohex-1-one; 0.15 mM NADPH; 20 mM glucose; 10 U/mL glucose oxidase; 0.5-1 μM YqjM.

Interestingly, the specific activity of YqjM increased only linearly with temperature up to 60°C. Beyond this temperature, even in the short term spectrophotometric assay (duration approx. 1 minute) the activity decreased. This behavior is also reflected by the decreasing stability of YqjM with increasing temperature. For example, at 25°C, the half-life time was approx. 9 days whereas at 60°C this value dropped to less than 2 minutes. From this, we conclude that the optimal temperature of YqjM (i.e. the temperature at which the highest total turnover number of the catalyst can be achieved) is 30°C (or possibly even lower).

Stability of YqjM in the presence of solvents

Considering the poor water solubility of most substrates of interest for preparative applications also the robustness of OYEs against some water-miscible solvents is of interest. Therefore, we determined the half-life-time of YqjM in the presence of varying concentrations of common cosolvents such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol and ethyl acetate (Figure 5).

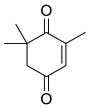
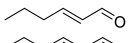
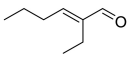
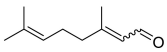
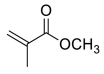
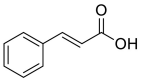
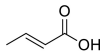
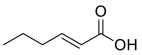
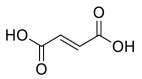
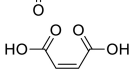
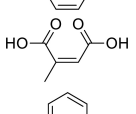
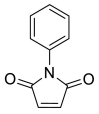
YqjM showed a very distinct robustness behaviour against the different solvents investigated: essentially showing decreased robustness in their presence. DMSO appeared to be the least harmful cosolvent leading to a reduction of half-life time of 'only' 25–50% if present at 10 or 50% (v/v). DMF, ethanol and ethyl acetate were much more potent inactivators of the enzyme.

Bommarius and coworkers observed something very similar with OYEs.[23b] Possibly, non-water soluble organic solvents will be more efficient to achieve overall high substrate loading.

Substrate scope of YqjM

Finally, we set out to determine the kinetic parameters of some known substrates of YqjM. In previous studies most of these substrates have already been established for YqjM[8a,9a] but kinetic parameters are missing. Since the determination of substrate-related activities is rather complex for YqjM (and possibly for all OYE-like ene reductases) due to the prominent O_2-related background reaction, we performed the following experiments under essentially anaerobic conditions. This was achieved by administering glucose together with glucose oxidase to reductively remove dissolved O_2 from the reaction mixture. Table 1 summarizes the kinetic parameters determined for some of the most common YqjM substrates. We also performed two experiments quantifying the reagents (substrates and products) using chiral GC analysis. Comparison with comparable UV/Vis results gave essentially identical specific activities (see supporting information Figs. S3 and S4 for details).

Table 1. Kinetic parameters for some representative substrates for YqjM.

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\cdot s^{-1}$)
	0.6 ± 0.1	3.6	6.4
	1.8 ± 0.1	1.8	1.0
	1.2 ± 0.2	2.2	1.9
	6.9 ± 1.3	1.5	0.2
	4.0 ± 0.5	2.2	0.6
	2.5 ± 0.9	1.3	0.5
	3.4 ± 0.7	0.06	0.02
	16.0 ± 1.0	0.7	0.04
	-	-	-
	-	-	-
	-	-	-
	-	-	-
	12.5 ± 1.4	0.41	0.03
	25.0 ± 3.6	0.30	0.01
	1.2 ± 0.7	3.93	3.42
	5.9 ± 1.2	3.64	0.61
	0.010 ± 0.004	4.18	415

Conditions: 25°C, 50 mM potassium phosphate buffer pH 6.5; 0.15 mM NADPH; 20 mM glucose; 10 U/mL glucose oxidase; 0.5-2 μM YqjM.

2-Cyclohexen-1-one was reduced by YqjM with the catalytic efficiency of 6.43 $mM^{-1}s^{-1}$. Introduction of a methyl group in α-position reduced the catalytic efficiency more than 6-fold both due to a reduced k_{cat} and an increased K_M value. Cyclic ketones containing substituents in the β-position were not included in this study since it was previously reported that they cannot be transformed by YqjM.[9a] The ring size had a significant effect on the kinetic parameters as 2-cyclopenten-1-one was approximately 3.5-fold lower than the one for 2-cyclohexen-1-one.

Aliphatic enals were rather modest substrates for YqjM with k_{cat} values reaching 2.2 s^{-1} for *trans*-hex-2-enal. Introduction of an α-ethyl substituent reduced the k_{cat} by approx. 50%. However, increasing the chain length as e.g. in case of citral dramatically reduced k_{cat}.

The highest affinity and activity was observed with *N*-phenylmaleimide, the substrate bearing two activated carbonyl bonds next to C=C bond, resulting in catalytic efficiency of 415.31 $mM^{-1}s^{-1}$.

Furthermore, three structurally related α,β-unsaturated dicarboxylic acids were tested. The (*E/Z*)-configuration of the substrate had a very strong impact on the kinetics of YqjM catalysed reduction. *E*-configured fumaric acid was found to be a poor substrate for YqjM, with the K_M value of 25 mM and the catalytic efficiency value of only 0.012 $mM^{-1}s^{-1}$. On the contrary, *Z*-configured maleic acid and its derivative, citraconic acid, were easily accepted by YqjM, although the high substrate inhibition was observed in both cases, with K_i values of 2.57 and 2.08 mM, respectively. Acrylic acid and acrylamide were converted rather sluggishly and any further modification (either as ester or as substituents in α- or β-position) essentially lead to complete inactivation of the enzyme.

These experimental findings to some extent were also confirmed by *in silico* docking studies. The crystal structure of YqjM in complex with *p*-nitrophenol (PDB: 1z44)[18a] was used after removing all co-crystallized ligands, except from FMN (details in SI). For example, while maleic acid smoothly binds to YqjM (i.e. orienting to the H-bonding histidines and having suitable distanced to the FMN–N5 (hydride donor) and tyrosine (proton donor)) the isomeric fumaric acid in its minimal energy conformation was too far away for efficient hydride transfer and reprotonation (Figure 6). Similar observations also explain the differences between *trans*-2-hexenal and *trans*-2-hexenoic acid as well as the non-acceptance of the other carboxylic acids shown in Table 1 (details in SI). In contrast, acrylic acid and acryl amide were embedded nicely in the H-bonding framework.

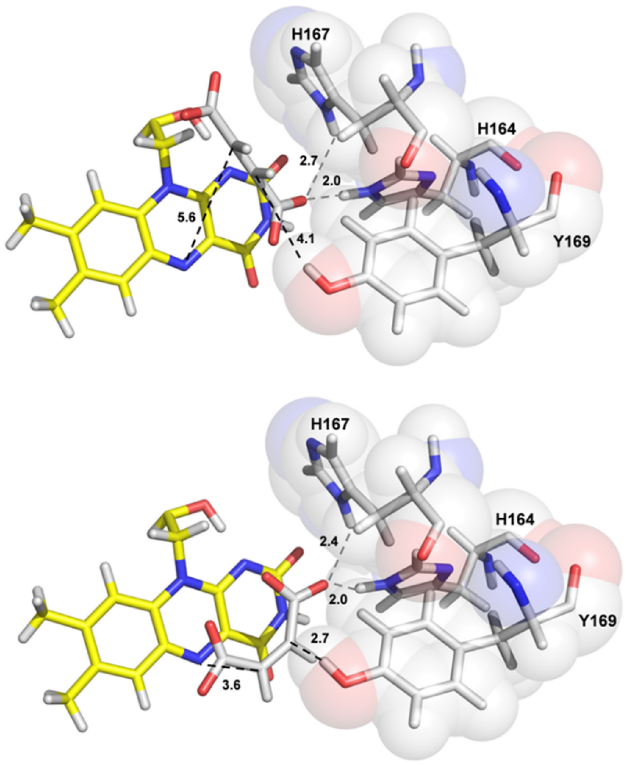


Figure 6. Docking results of fumaric acid (upper) and maleic acid (lower) to YqjM (PDB: 1z44).

Therefore, we conclude that the non-acceptance of conjugated carboxylic acid (esters) is more due to insufficient binding of the substrates rather than due to poor electronic activation of the C=C-bond.

Conclusions

In this contribution we report an in-depth characterization of the commonly used OYE homolog from *Bacillus subtilis* (YqjM). This will put the basis for the development and modelling of future processes using this enzyme.

Supporting Information Summary

A detailed description of the experimental procedures and additional experimental data can be found in the Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: catalytic reduction · kinetic characterization · old yellow enzymes · oxygen dilemma · YqjM

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