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Old Yellow Enzyme-Catalyzed Dehydrogenation of Saturated Ketones

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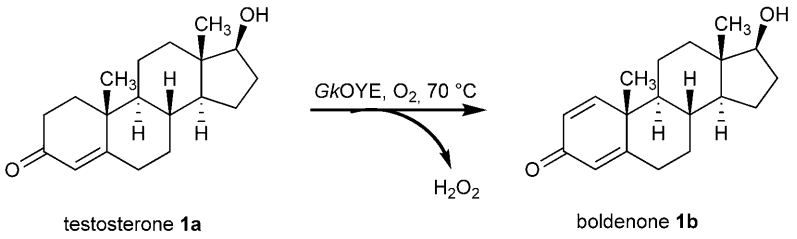
Abstract: Enzymes from extremophiles have always been of great interest for biotechnology because of their ruggedness against various stress factors. We have isolated, cloned, heterologously expressed and characterized a thermostable old yellow enzyme (OYE) from *Geobacillus kaustophilus*. In addition to the expected 'enone' reduction, *Gk*OYE also catalyzes the reverse reaction, i.e., the desaturation of C–C bonds adjacent to a carbonyl to give the corresponding α,β-unsaturated ketone. The reaction proceeds at the expense of molecular oxygen without the need for a nicotinamide cofactor and represents an environmentally benign alternative to known chemical dehydrogenation methods.

Keywords: dehydrogenation; desaturation; enzymatic oxidation; old yellow enzyme; oxidoreductases; thermophiles

α,β-Unsaturated ketones are versatile intermediates in organic synthesis and a number of strategies for their production from the saturated keto analogues has been described. Traditional protocols often employ toxic phenylselenium derivatives[1] or strong oxidants such as SeO_2, DDQ, or periodic acid.[2] More recently, *o*-iodoxybenzoic acid (IBX) has been described as a useful stoichiometric oxidant for the desaturation of carbonyl compounds.[3] Pd-catalyzed dehydrogenation of saturated ketones[4] or the corresponding silyl enol ethers[5] has also been explored, but often the yields and turnover numbers are very limited. In general, the applicability of the existing chemical methods is limited by chemo- and regioselectivity as well as their low environmental compatibility.

Enzymatic dehydrogenation of ketones would represent a valuable "green" alternative, but there are only a few examples described so far, the first being the enoate reductase-catalyzed dismutation reaction of cyclohex-2-enone moieties leading to the corresponding phenols and cyclohexanones.[6] Similarly, the aromatization to phenols was reported as catalytic promiscuity or side reaction.[7] Massey et al. created artificial old yellow enzyme (OYE) with increased redox-potential (−50 mV) compared to the natural enzyme (−207 mV). The apo-enzyme (lacking the natural flavin-cofactor in the active site) was reconstituted with a chemically modified (synthetic) flavin cofactor. With this artificial desaturase the dehydrogenation of several saturated ketones was demonstrated.[8] Herein, we report the first enzymatic desaturation that is catalyzed by a readily available, natural thermophilic old yellow enzyme from *Geobacillus kaustophilus*.

While screening microorganisms for active pharmaceutical ingredient-metabolizing activities, we found that testosterone **1a** was readily converted to its 1,2-didehydro analogue boldenone **1b** (Scheme 1) by the thermophilic microorganism *Geobacillus kaustophilus* DSM 7263. Boldenone was purified by preparative HPLC and identified by 2D-^{1}H NMR (see Supporting

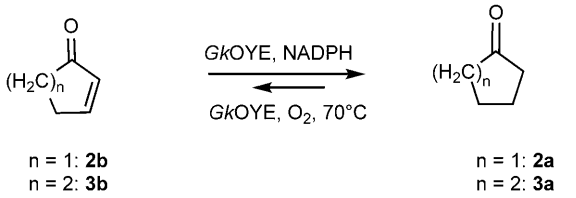


Scheme 1. Old yellow enzyme (*Gk*OYE)-mediated testosterone 1,2-desaturation reaction.

Information). The responsible enzyme (Genbank YP 148185) was identified by MALDI-TOF after partial purification (see Supporting Information). It is a homologue of the well characterized YqjM from *Bacillus subtilis*[9] (66% amino acid sequence identity/80% similarity) and therefore belongs to the "old yellow enzyme" (OYE) family of flavoproteins. The corresponding gene was then cloned[10] and expressed in *E. coli* at expression levels of up to 8 g L^{-1}.

For further characterization, *Geobacillus kaustophilus* OYE (*Gk*OYE) was purified from *E. coli* lysates by standard chromatographic purification procedures. Alternatively, highly pure enzyme was obtained by simple heat treatment of the lysate. Thereby, most *E. coli* proteins were precipitated while the thermostable *Gk*OYE remained in the supernatant. As common among OYEs,[11] the active site prosthetic flavin is non-covalently bound, as shown by trichloroacetic acid precipitation. MS analysis of the supernatant revealed the active site flavin to be flavin mononucleotide (FMN, see Supporting Information), a finding subsequently corroborated by the elucidation of the X-ray crystal structure of *Gk*OYE (see also Figure 3).

As expected for an enoate reductase, *Gk*OYE exhibited high reductase activity on typical substrates such as cyclopentenone **2b** and cyclohexenone **3b** (Scheme 2) in the presence of NADPH. The reverse reaction, i.e., the dehydrogenation of **3a** to yield **3b**, is known to be strongly endothermic (+109 kJ mol^{-1}).[12] Since the dehydrogenation at the expense of molecular oxygen is not spontaneously happening and may require high activation energy, we expected that the reaction may be favoured at elevated temperatures only. Hence, the thermostability of the enzyme for this biocatalytic dehydrogenation reaction was ex-



Scheme 2. Enone reduction and reverse reaction catalyzed by *Gk*OYE.

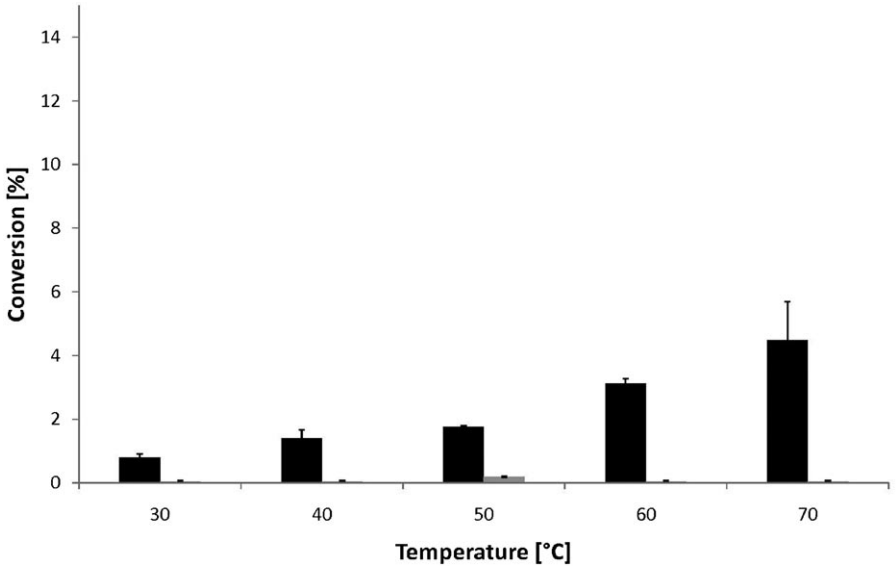


Figure 1. Temperature dependence of desaturation of **3a** under screening conditions (crude *E. coli* lysates, <1 mg total protein mL^{-1}, 24 h). Comparison of *Gk*OYE (black) and YqjM (grey). For normalization of expression effects, enzyme amounts which resulted in the same saturation activity (substrate **3b**, 37 °C) were used.

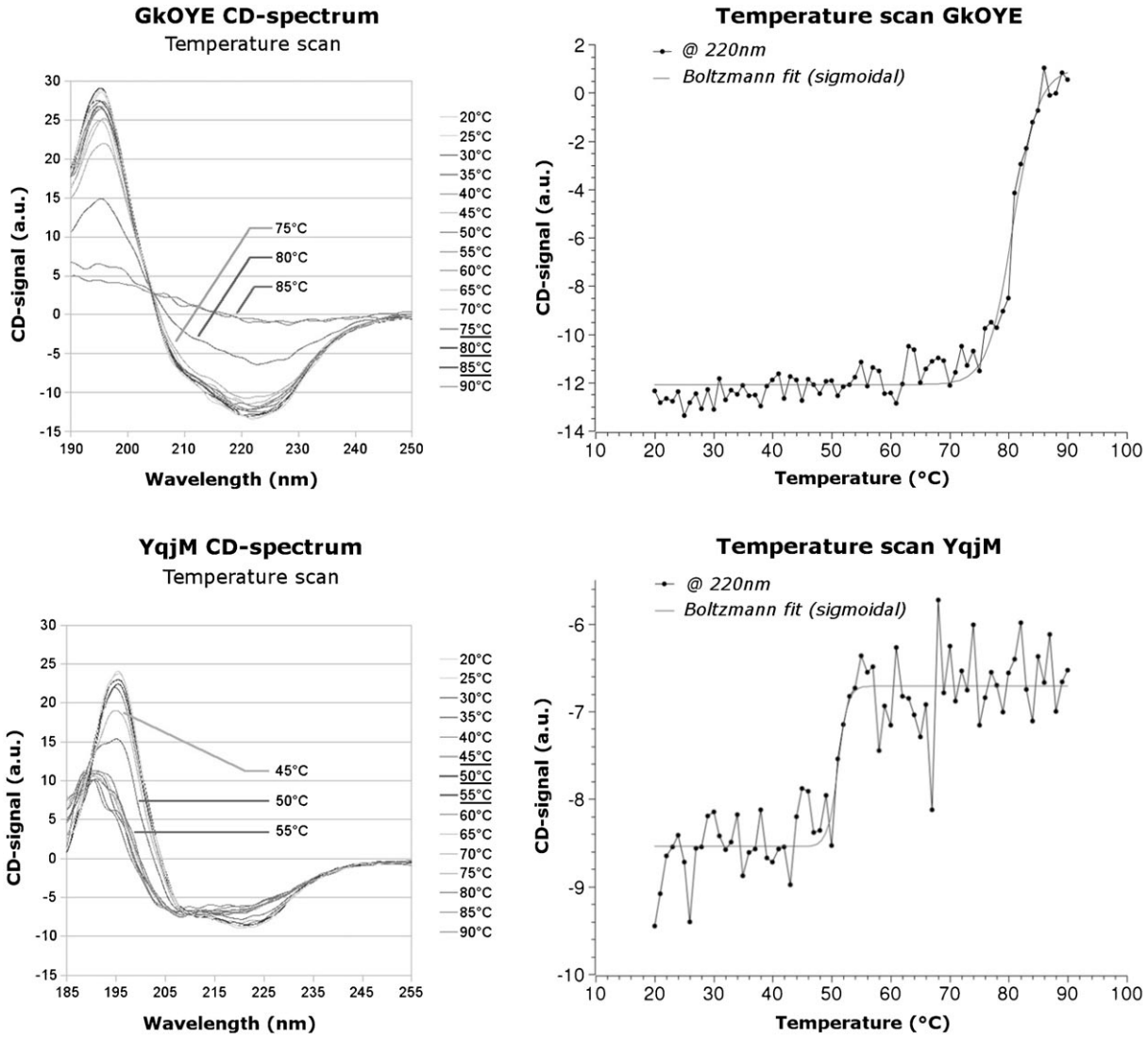


Figure 2. Far UV CD-spectroscopic analysis of *Gk*OYE (*top*) and YqjM (*bottom*). The original CD signals at 220 nm measured at 1 °C intervals together with a fitted sigmoidal curve are shown on the *left*. CD spectra measured at temperatures between 20 and 90 °C with an interval of 5 °C are shown on the *right*.

ploited.[13] Using **3a** as the model substrate we indeed found an increase in conversion with rising temperature, reaching an optimum at 70 °C (Figure 1). Temperatures above 75 °C led to unfolding of the protein with concomitant release of the flavin cofactor (Figure 2). While dynamic light scattering experiments and FMN fluorescence measurements (see Supporting Information) demonstrated aggregation of YqjM and only partial FMN release at elevated temperature, *Gk*OYE remained in tetrameric structure and denatured at 76-82 °C with total release of FMN.

YqjM from *Bacillus subtilis* – although similar to *Gk*OYE in active site structure (Figure 3) and exhibiting highly similar redox potential (-207 ± 2 mV *vs.* -208 ± 3 mV) – proved less suitable for the ketone dehydrogenation. Traces of **3a** were oxidized to **3b** (Figure 1), but the limited thermal stability of YqjM (protein unfolding ~50 °C, see Figure 2) impedes its useful application for this transformation. However, due to the different inactivation behaviour, YqjM remained partially active at higher temperature, thereby allowing minimal conversions even at 50 °C.

Testing other substrates, we were pleased to find that ketones other than cyclohexanone were converted even more readily by *Gk*OYE at 70 °C (Table 1). The low conversion of the initially discovered oxidation of testosterone **1a** to boldenone **1b** for example, was increased to 71% (*Gk*OYE 20 mg mL^{-1}, 24 h, 70 °C, 10 mM substrate). Lower substrate concentration (5 mM) and prolonged incubation time (48 h) led to almost quantitative conversion for this substrate. Cyclopentanone **2a**, for example, led to 55% conversion.

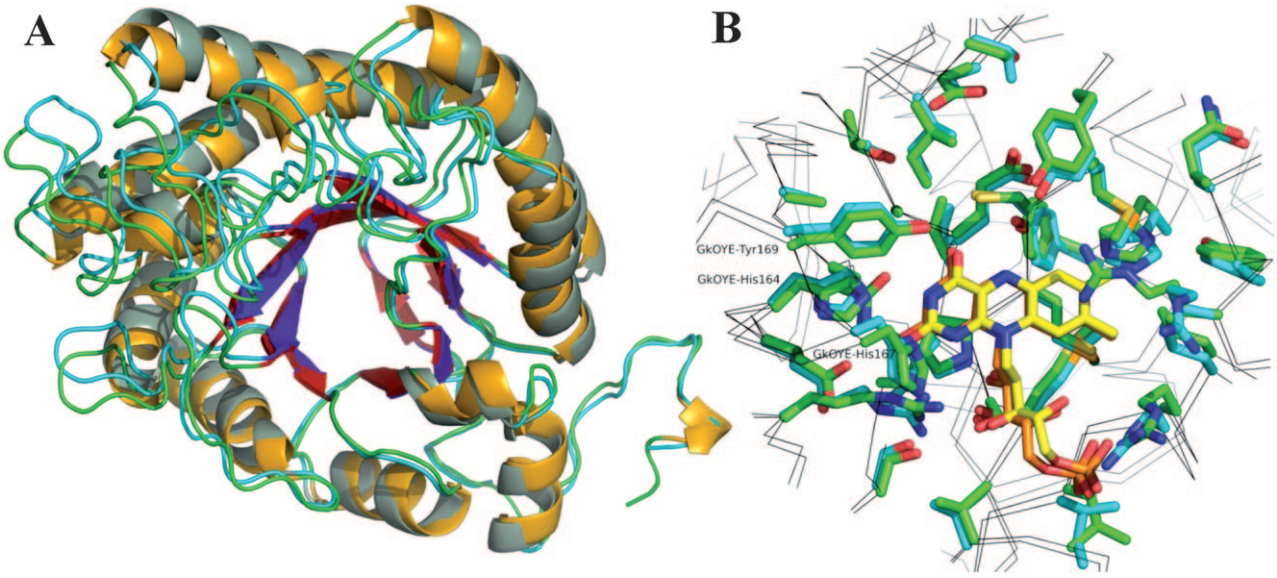


Figure 3. A: Structural superposition of *Gk*OYE and YqjM. Both monomer structures are shown in a cartoon representation and are coloured according to their secondary structures. *Gk*OYE: red (β-strands), gold (α-helices), green (loops); YqjM: blue (β), grey (α), cyan (loops). **B:** Close-up view of the active centers of the aligned enzymes *Gk*OYE (green; FMN cofactor yellow) and YqjM (cyan; FMN cofactor orange). Active site residues in 10 Å distance to the FMN-N5 are represented as stick model.

Table 1. Conversion of ketones (10 mM) to the corresponding α,β-unsaturated compounds by *Gk*OYE (20 mg mL^{-1}, 24 h, 70 °C).

Entry	Substrate	Product	Conversion [%]
1	**1a**	**1b**	71[a]
2	**2a**	**2b**	55[b]
3	**3a**	**3b**	5[b,c]

[a] Determined by HPLC-MS.
[b] Determined by GC-MS.
[c] **3b** is further oxidized to phenol.

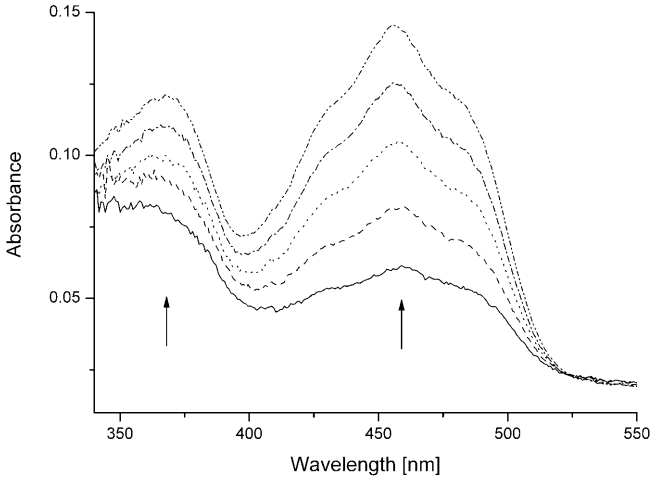
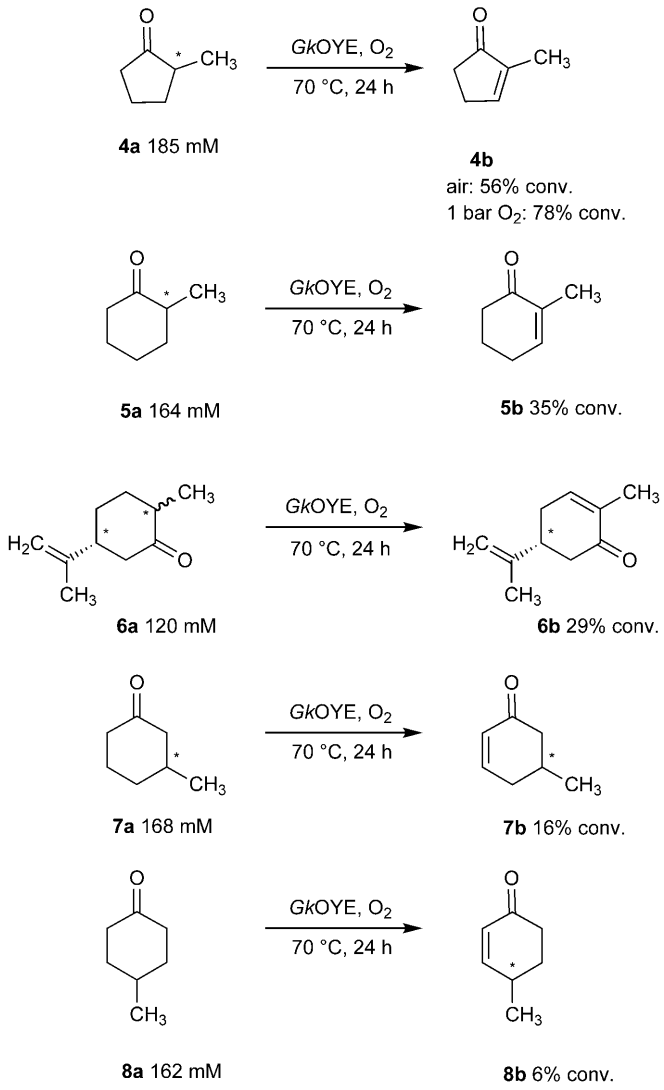


Figure 4. Selected spectra during the reoxidation of *Gk*OYE by molecular oxygen showing the reappearance of oxidized FMN over a time span of 20 s.

In contrast to the enone reduction, no nicotinamide cofactor was required for these oxidative transformations, suggesting that the catalytic cycle was closed *via* aerobic reoxidation of $FMNH_2$.[14] As a proof for oxygen consumption, initial experiments were carried out with oxygen limitation, which resulted in dramatically reduced yields. Subsequently, stopped-flow experiments revealed that molecular oxygen is indeed able to re-oxidize the reduced flavin mononucleotide (FMN) of *Gk*OYE (Figure 4).

To get a more detailed view of the perspectives of this enzyme, regio- and enantioselectivities were determined using various substituted cyclic ketones as substrates. Dehydrogenation of racemic 2-methylcyclopentanone **4a** occurred exclusively on the more sterically demanding side of the ketone, thus 2-methylcyclopent-2-enone **4b** was formed with 56% conversion (185 mM substrate, 70 °C, 24 h). In contrast to the reported observations with artificial OYE,[1] in our experiments, both enantiomers were transformed at comparable rates ($E = 1.2$), thus allowing theoretically full conversion (Scheme 3, Table 2). In a similar fashion, 2-methylcyclohexanone **5a** was converted to 2-methylcyclohex-2-enone **5b** and dihydrocarvone **6a** to carvone **6b**. In contrast to **4a**–**6a**, *Gk*OYE dehydrogenated 3-methylcyclohexanone **7a** on the less hindered side leading to 5-methylcyclohex-2-enone **7b** with 23% *ee*. The prochiral substrate 4-methylcyclohexanone **8a** was converted to 4-methylcyclohex-2-enone **8b** and also in this case the stereopreference was low (30% *ee*). The tendency of the reaction prod-



Scheme 3. Regioselective ketone dehydrogenation catalyzed by *Gk*OYE.

Table 2. Regioselective conversion of ketones to the corresponding α,β-unsaturated compounds by *Gk*OYE (5 mg mL^{-1}, 24 h, 70 °C).

No	Subst.	Prod.	Conv.[a] [%]	ee_S[b] [%]	ee_P[b] [%]	E[c]
1	**4a**	**4b**	56	8	n.a.	1.2
2	**5a**	**5b**	35[d]	20	n.a.	2.6
3	**6a**	**6b**	29	46[e]	>99	n.a.
4	**7a**	**7b**	16	3	23	1.4
5	**8a**	**8b**	6[f]	n.a.	30	n.a.

[a] Determined by GC-MS.
[b] Determined by GC.
[c] Enantioselectivity.
[d] 2% of *o*-cresol formed as by-product.
[e] Diastereomeric excess, commercial **5a** is a mixture of (2*R*,5*R*) and (2*S*,5*R*)-isomers with a *de* of 57%.
[f] 6% of *p*-cresol formed as by-product. n.a.: not applicable.

ucts to undergo double desaturation and thus aromatization proved to depend on the substitution pattern. While no cresol by-product was observed in the dehydrogenation of **7a**, the desaturated products **5b** and **8b** were converted further to *o*-cresol and *p*-cresol, respectively (Table 2 footnote). All reaction products were identified by comparison of both their retention times and mass spectra with those of authentic standards (see Supporting Information).

Since the enzyme requires molecular oxygen, and the solubility of molecular oxygen in water decreases with higher temperature, the dehydrogenation was also tested in the presence of 1 bar molecular oxygen instead of air. By using this set-up the conversion of 2-methylcyclopentanone was significantly increased from 56% to 78% within 24 h.

The enzymatic transformation described here represents a proof of principle for an environmentally benign biocatalytic option[15] for the preparation of α,β-unsaturated ketones starting from their corresponding saturated keto analogues.

Concluding, the enzymatic desaturation catalyzed by *Gk*OYE opens new synthetic routes to a number of α,β-unsaturated compounds. New ketosteroids might thus be accessible. In contrast to known ketosteroid dehydrogenases,[16] *Gk*OYE does not rely on expensive nicotinamide cofactors or artificial electron acceptors, because the reoxidation of FMN is solely O_2 dependent. Unlike ketosteroid dehydrogenases, *Gk*OYE's application is, however, not limited to steroids, allowing the synthesis of small α,β-unsaturated ketones, which are often valuable flavour and aroma defining compounds.[17] Elevated temperatures appear to be the key factor for the desaturase activity of old yellow enzymes; whereas enzyme structure, redox potential and several other physicochemical properties of YqjM and *Gk*OYE are highly similar, their thermal behavior differs. *Gk*OYE is available in high amounts and is, in contrast to OYEs with artificial cofactor,[8] the first natural desaturase.

Experimental Section

Typical Desaturation Reaction

Protein solution (500 μL, 5–20 mg mL^{-1}) was added to a 10 mM substrate solution in 500 μL of a 50 mM KP_i buffer, pH 7, and incubated at 70 °C for 24 h. All reactions were analyzed by HPLC-MS, GC-FID or GC-MS using authentic standards (where available) for comparison.

CD Measurements

Freshly purified samples of *Gk*OYE and YqjM were diluted to a concentration of 1 mg mL^{-1} in 50 mM potassium phosphate buffer pH 7.3. To clarify if FMN is set free before or concomitant with protein unfolding, two independent tem-

perature scans were performed on a Jasco J-715 spectropolarimeter at the absorption maxima of FMN (373 nm) and the protein (220 nm), respectively. These temperature scans ranged from 20 °C up to 90 °C with a step size of one degree. A 0.02 cm cuvette was used. In addition, a whole wavelength scan (185–255 nm) was collected every 5 °C. For the FMN measurements, the concentrations of the enzyme samples were in the range of 4.5 mg mL^{-1} and a 0.1 cm cuvette was used. This relatively high concentration was necessary to get a measurable CD signal.

Protein Crystallization

*Gk*OYE was crystallized using sitting drop vapour diffusion yielding two different crystal forms (hexagonal and orthorhombic). The structures were solved by molecular replacement using a homology model based on the structure of YqjM and were refined against 2.3 and 2.5 Å datasets collected on a rotating anode x-ray source as well as the EMBL/DESY in Hamburg. Coordinates and structure factors were deposited in the Protein Data Bank (PDB) under entry codes 3GR7 (hexagonal) and 3GR8 (orthorhombic).

Stopped Flow Experiments: Reoxidation of FMN

YqjM and *Gk*OYE were reduced with substoichiometric amounts of NADPH under anaerobic conditions and subsequently reoxidized with air-saturated buffer (KP_i 50 mM pH 7.0, ~240 μM O_2). Experiments were performed in a glove box with a nitrogen atmosphere (1 ppm O_2) and spectral changes were recorded with a stopped-flow device (SF-61DX2, TgK Scientific) using a KinetaScanT dioade array detector.

Determination of Regio- and Enantioselectivity

10 mg of *Gk*OYE lyophilisate (from heat precipitation, protein concentration: 25%) were dissolved in KP_i buffer (50 mM, pH 7.5) and substrate (10 μL) was added. The samples were incubated at 70 °C and 1400 rpm on a thermoshaker for 48 h. The solution was extracted with EtOAc (600 μL) and centrifuged. The organic phase was separated, diluted with EtOAc (600 μL) and dried over Na_2SO_4. Conversion was determined by GC-MS analysis and substrate *ee* by GC analysis on a chiral stationary phase.

Reactions under O_2 Atmosphere

In 4 mL glass vials with septum-equipped screw caps, 10 mg of *Gk*OYE lyophilisate (from heat precipitation, protein concentration: 25%) were dissolved in KP_i buffer (50 mM, pH 7.5) and substrate (10 μL) was added. The vials were evacuated and O_2 was added from a syringe. The samples were incubated at 70 °C and 1400 rpm on a thermoshaker for 24 h. The solution was extracted with EtOAc (600 μL) and centrifuged. The organic phase was separated, diluted with EtOAc (600 μL) and dried over Na_2SO_4. Conversion was determined by GC-MS analysis and substrate *ee* by GC analysis on a chiral stationary phase.

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