VIP Enzyme Catalysis

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# Stereospecific Alkyne Reduction: Novel Activity of Old Yellow Enzymes**

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The use of oxidoreductase enzymes extends the repertoire of methods for regio- and stereospecific synthesis remarkably.[1] In particular, the flavin-containing "Old Yellow Enzyme" (OYE) and a growing number of discovered homologues are valued as a toolbox for asymmetric reduction of the carbon–carbon double bond.[2] While this is one of the best studied enzyme families with ongoing investigations of substrate range, biotechnological application, active-site catalytic mechanism, and natural function, their action on a triple bond is reported here for the first time. As an example, we studied the enzymatic reduction of the phenyl-substituted ynone **1**. To our knowledge, enzymatic reductions of ynones to enones have not been reported to date.

The *trans*-selective reduction of ynones to enones can be achieved by treatment with diisobutylaluminum hydride in the presence of hexamethylphosphoric triamide.[3] However, the yield and selectivity are only satisfactory. Alternatively, the *trans* enone can be produced by treatment of the ynone with a water-soluble triaryl phosphine, but this phosphine has to be applied in excess amount as it is oxidized to the corresponding oxide.[4] For the *cis*-selective reduction of ynones, a solid-supported palladium catalyst has been used.[5] The enzymatic reduction outlined herein may be an environmentally friendly alternative for *trans*-selective reductions of the alkyne bond: The nontoxic catalyst and co-substrate glucose are cheaply produced from renewable resources and are biodegradable. Furthermore, the reaction has low energy requirements as it operates at room temperature and atmospheric pressure.

Recombinant *E. coli* strains that each produce one of four different enzymes of the OYE family were investigated as whole-cell biocatalysts for the reduction of the ynone **1**. The product concentrations shown in Figure 1 illustrate that all tested enzymes, OYE 1, 2, and 3 from yeasts and NAD(P)H-dependent 2-cyclohexen-1-one reductase (NCR) from the bacterium *Zymomonas mobilis*, were able to catalyze the reactions shown in Scheme 1. The enzyme glucose dehydrogenase concurrently oxidized glucose and thus recycled $NADP^+$ to NADPH. Additional experiments showed that NADH can also serve as the reducing agent. It is apparent from Figure 1 that OYE 1, 2, and 3 reduced **1** to **2** at different rates (left), and $^1H$ NMR spectroscopy revealed that only the *trans* isomer of **2** was produced. However, when **2** was the substrate (right) all three enzymes reduced **2** to **3** at similar rates. As an exception, NCR can reduce **2** to **3** faster than the other enzymes, but **1** was reduced to **3** very slowly and without detectable intermediate. Knowledge of these catalytic differences may be useful for synthetic applications as well as for further studies of the structure–function relationship in this popular enzyme family. The observed progressive increase in ynone reduction from OYE 1 to OYE 3, for example, correlates with an amino acid sequence identity of 91% between OYE 1 and 2 and 80% between OYE 1 and 3.

The reverse reactions, the oxidation of **3** to **2** or **2** to **1**, were not observed in control experiments at pH 6.8 with $NAD^+$ added as an electron acceptor in the absence of

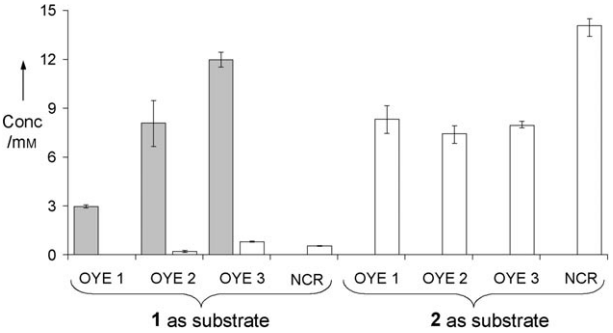
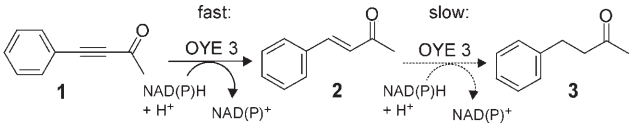



***Figure 1.*** Enzymatic reduction of ynones and enones. 20 mM ynone **1** (left) or enone **2** (right) was converted by 10 $g\,L^{-1}$ *E. coli* cells (containing the enzymes OYE 1–3 or NCR) at pH 6.8 and 30 °C over 1 h (shaded: product **2**; unshaded: product **3**). The average values of three biotransformations are given; error bars indicate the lowest and highest results.




***Scheme 1.*** Reductions catalyzed by Old Yellow Enzymes (OYEs). **1**: 4-phenyl-3-butyne-2-one; **2**: (*E*)-4-phenyl-3-butene-2-one; **3**: 4-phenyl-2-butanone; $NAD(P)^+$: nicotinamide adenine (phosphate) dinucleotide.

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glucose dehydrogenase and glucose. Under the experimental conditions as described in Figure 1, the *E. coli* host strain was not able to catalyze the reduction of the carbon triple or double bond, but the host had a very low background activity for the reduction of the keto group, with less than 2.4% of the substrate being converted into the corresponding alcohol.

As OYE 3 showed a much higher rate for the reduction of **1** (Figure 1, left) than for the reduction of **2** (Figure 1, right), this enzyme was selected for a biotransformation. The enzymes OYE 3 and glucose dehydrogenase were both produced by recombinant *E. coli* strains, which were added as whole cells because enzyme purification would otherwise add to production costs. The biotransformation profile in Figure 2 illustrates that **1** was rapidly reduced to **2**, which

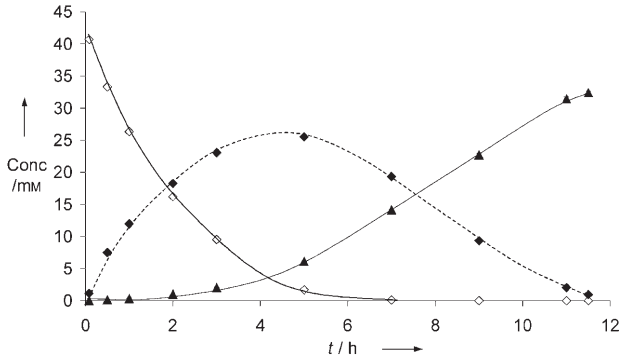


***Figure 2.*** Biotransformation of **1** by recombinant *E. coli* cells. *E. coli* (OYE 3) and *E. coli* (GDH) were used at 5 g L$^{-1}$ each (pH 6.8, 30 °C). The average values of two analyses from one reactor are given (**1**: ◇; **2**: ◆; **3**: ▲). Values were not corrected for dilution by addition of KOH. Considering the dilution, the mass balance at the end of the reaction was 5 mM lower than expected.

accumulated over the first 5 h at an overall rate of 0.76 g L$^{-1}$ h$^{-1}$ and with a theoretical molar yield of 65%. The velocity of the further reduction of **2** to **3** increased with decreasing concentrations of **1** and the conversion was complete in 11.5 h. Only 2.4% of **1** was reduced to the corresponding alcohol. The enzyme was very robust, as activity assays revealed that OYE 3 did not lose any activity over the 11.5 h.

The apparent $k_{cat}$ values in Table 1 illustrate that one active site of purified OYE 3 (one subunit of the dimer) was able to turn over 1.63 molecules of **1** per second, but the enzyme was about four times slower with **2**. The apparent half-maximal velocities were reached at low substrate concentrations (Michaelis–Menten constant, $K_m$), which indicates a high affinity of the enzyme to the substrates. The catalytic efficiency ($k_{cat}/K_m$) was 6.7-fold higher for **1** than for **2**. For an optimized enzyme, an increase of the $k_{cat}$ value for the ynone could be the target of improvement, for example, through evolutionary approaches. Alternatively, the desired product may be protected from further conversion through in situ removal as the established "ping-pong" mechanism with a shared site for substrate and NADPH binding[7] would require **2** to leave the enzyme before further reduction of **2** to **3** can occur. In situ product removal has been successfully established for other biotransformations.[8] A search for other enzymes that reduce a carbon triple bond only revealed nitrogenase, which reduces acetylene to ethylene in an ATP-dependent reaction.[9] However, the reduction of ynones to enones has not been reported.

***Table 1:*** Kinetic properties of purified OYE 3 from *S. cerevisiae*.[a]

| Substrate | $k_{cat}$ [s$^{-1}$] | $K_m$ [mM] | $k_{cat}/K_m$ [s$^{-1}$ mM$^{-1}$] |
|---|---|---|---|
| **1** | 1.63 ± 0.13 | 0.184 ± 0.019 | 8.8 |
| **2** | 0.40 ± 0.01 | 0.304 ± 0.014 | 1.3 |

[a] Apparent values with 0.3 mM NADPH at 30 °C, pH 6.8. Niino et al. estimated a $K_m$ value of 0.0058 mM for NADPH when measured with cyclohexenone as oxidant at 25 °C, pH 7.[6]

In conclusion, OYE and homologous enzymes display a potential for environmentally sustainable regio- and stereoselective hydrogenation of the carbon–carbon triple bond conjugated with a ketone or possibly with other polar groups such as aldehydes, esters, or nitro groups. Furthermore, the discovery of this novel activity of an "old enzyme" is also relevant for ongoing investigations of the enzyme structure–function relationship.

## Experimental Section

Unless otherwise specified, chemicals and enzymes were purchased from Sigma-Aldrich (Castle Hill, Australia). Recombinant *E. coli* strains that express the genes for the enzymes OYE 1 (from *Saccharomyces pasteurianus* formerly named *S. carlsbergensis*, sequence accession number Q02899, *E. coli* Lu13667), OYE 2 (from *S. cerevisiae*, Q03558, *E. coli* Lu13668), OYE 3 (from *S. cerevisiae*, P41816, *E. coli* Lu13669), NCR (from *Zymomonas mobilis*, Q5NLA1, *E. coli* Lu14079), and glucose dehydrogenase (*Bacillus subtilis*, M12276, *E. coli* Lu11598) were obtained from BASF (Ludwigshafen, Germany). The strains were grown in Luria Broth with the respective antibiotics and inducers for enzyme synthesis at 37 °C for 22 h. Cells were washed, and the wet biomass was stored at −20 °C until further usage. Biomass concentrations are given based on dry mass, which was determined after drying an aliquot of cells at 100 °C. (MES: β-morpholinoethanesulfonic acid; EDTA: ethylenediaminetetraacetic acid.)

Initial productivities (Figure 1) were determined in 4-mL glass vials containing a magnetic stirrer bar and 1 mL of the reaction mixture at 30 °C with a composition of 20 mM **1** or **2**, 10% propan-2-ol (v/v), 5 mM EDTA, 2 mM NADP$^+$, 50 mM D-glucose, 1 U mL$^{-1}$ cell-free glucose dehydrogenase from *Thermoplasma acidophilum* (Sigma-Aldrich, Castle Hill, Australia), *E. coli* equivalent to 10 g L$^{-1}$ dry biomass, and 50 mM MES/KOH pH 6.8. The batch biotransformation (Figure 2) was performed in a 50-mL glass reactor fitted with stirrer, pH controller, and water jacket for temperature control (30 °C). The biotransformation mixture contained 42 mM (6.1 g L$^{-1}$) **1**, 10% propan-2-ol (v/v), 300 mM D-glucose, 10 mM EDTA, 2 mM NADP$^+$, biomass equivalent to the dry mass of 5 g L$^{-1}$ *E. coli* (OYE 3) and 5 g L$^{-1}$ *E. coli* (glucose dehydrogenase), 300 mM MES/KOH at pH 6.8, controlled by the addition of 2.5 M KOH. For the determination of the activity of OYE 3, samples were treated with 5% (v/v) toluene for 15 min at room temperature to further permeabilize the cells. After phase separation by centrifugation at 3000 × g, Micro Bio-Spin 6 chromatography columns from Bio-Rad (Hercules, CA, USA) were used to remove reactants and any compounds smaller than 6 kDa from the supernatant. The activity of OYE 3 in this eluate was determined for the oxidation of NADPH in the presence of **1** using a photometer at a wavelength of 365 nm.

Other samples were analyzed by GC after extraction with chloroform using *n*-decane as internal standard. Concentrations

  

were determined by using external standards that were extracted by the same method. An RTX5-MS column (30 m × 0.25 mm, 0.25-μm phase thickness) from RESTEK (Bellefonte, USA) was used with nitrogen as carrier gas (0.6 mL $min^{-1}$) and split injection (1:60). The temperature program was 80 °C (1 min), then 16 °C $min^{-1}$ to 280 °C (1 min). The injector was set at 250 °C and the flame ionization detector was set at 310 °C. The identity of compounds was confirmed by co-elution with standards and by GC/MS (Agilent Technologies 5971GC/MSD, Santa Clara, CA, USA). The retention times were as follows: *n*-decane 5.51 min, **3** 8.30 min, **1** 8.82 min, and **2** 9.48 min. GC/MS was performed as described for GC with a flow rate of 0.7 mL $min^{-1}$ and splitless injection. $^1$H NMR spectra were recorded in $CDCl_3$ on a 300 MHz (Bruker DPX 300) instrument. The *trans* configuration of **2** was concluded from the coupling constant of the protons of the double bond, which was identical (3 H, $J = 16.2$ Hz) for sample and standard.

To determine the kinetic properties of purified OYE 3 (kindly provided by BASF), the oxidation of NADPH was monitored at 365 nm and 30 °C ($\varepsilon_{365\,nm} = 3.515 \times 10^3\,M^{-1}cm^{-1}$) by using a temperature-controlled CaryBio100 spectrophotometer (Varian Inc., Palo Alto, CA, USA). Control experiments showed that activities were not limited by the NADPH concentration. The assay contained 2.5–13 μg $mL^{-1}$ OYE 3, 2% propan-2-ol (v/v), 5 mM EDTA, 0.3 mM NADPH, 50 mM MES/KOH pH 6.8, and 26.5 μM–1.95 mM of **1** or 202 μM–2.99 mM of **2**. To minimize oxidation of reduced OYE 3 by $O_2$, the buffer was degassed and an $O_2$-consuming system was included (10 U $mL^{-1}$ glucose oxidase from *Aspergillus niger* and 10 mM D-glucose).[10] Kinetic parameters were calculated from double-reciprocal plots using CaryWinUV software 02.00 (Varian Inc.). For the calculation of $k_{cat}$ the protein concentrations of OYE 3 were determined by the method of Bradford.[11]

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[1] A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258.
[2] A. S. Bommarius, K. M. Polizzi, *Chem. Eng. Sci.* **2006**, *61*, 1004; A. Müller, B. Hauer, B. Rosche, *J. Mol. Catal. B* **2006**, *38*, 126; M. Wada, A. Yoshizumi, Y. Noda, M. Kataoka, S. Shimizu, H. Takagi, S. Nakamori, *Appl. Environ. Microbiol.* **2003**, *69*, 933; R. E. Williams, N. C. Bruce, *Microbiology* **2002**, *148*, 1607.
[3] T. Tsuda, T. Yoshida, T. Kawamoto, T. Saegusa, *J. Org. Chem.* **1987**, *52*, 1624.
[4] C. Larpent, G. Meignan, *Tetrahedron Lett.* **1993**, *34*, 4331.
[5] M. Gruttadauria, L. F. Liotta, R. Noto, G. Deganello, *Tetrahedron Lett.* **2001**, *42*, 2015.
[6] Y. S. Niino, S. Chakraborty, B. J. Brown, V. Massey, *J. Biol. Chem.* **1995**, *270*, 1983.
[7] K. M. Fox, P. A. Karplus, *Structure* **1994**, *2*, 1089.
[8] B. Buhler, A. Schmid, *J. Biotechnol.* **2004**, *113*, 183; D. Meyer, B. Buehler, A. Schmid, *Adv. Appl. Microbiol.* **2006**, *59*, 3.
[9] B. K. Burgess, D. J. Lowe, *Chem. Rev.* **1996**, *96*, 2983.
[10] T. B. Fitzpatrick, S. Auweter, K. Kitzing, T. Clausen, N. Amrhein, P. Macheroux, *Protein Expression Purif.* **2004**, *36*, 280.
[11] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.

  