ChemPubSoc Europe

DOI: 10.1002/cbic.201500604

CHEMBIOCHEM Communications

Loop-Grafted Old Yellow Enzymes in the Bienzymatic Cascade Reduction of Allylic Alcohols

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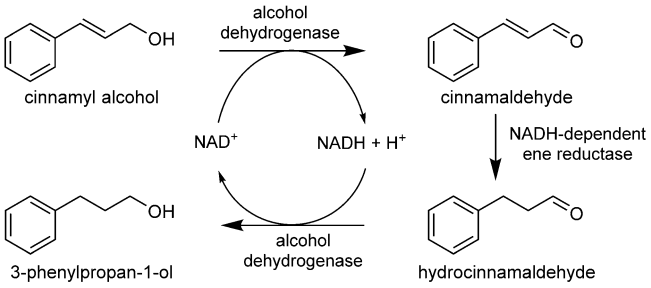
The enzymatic reduction of C=C bonds in allylic alcohols with Old Yellow Enzymes represents a challenging task, due to insufficient activation through the hydroxy group. In our work, we coupled an alcohol dehydrogenase with three wild-type ene reductases—namely nicotinamide-dependent cyclohex-2-en-1-one reductase (NCR) from *Zymomonas mobilis*, OYE1 from *Saccharomyces pastorianus* and morphinone reductase (MR) from *Pseudomonas putida* M10—and four rationally designed β/α loop variants of NCR in the bienzymatic cascade hydrogenation of allylic alcohols. Remarkably, the wild type of NCR was not able to catalyse the cascade reaction whereas MR and OYE1 demonstrated high to excellent activities. Through the rational loop grafting of two intrinsic β/α surface loop regions near the entrance of the active site of NCR with the corresponding loops from OYE1 or MR we successfully transferred the cascade reduction activity from one family member to another. Further we observed that loop grafting revealed certain influences on the interaction with the nicotinamide cofactor.

Asymmetric hydrogenation is an important tool in synthetic organic chemistry because it can be performed with a large variety of substrates, as well as with experimental simplicity and high levels of enantiocontrol. Asymmetric hydrogenation has been applied to Rh- and Ru-catalysed enantioselective reductions of ketones/aldehydes, imines, activated olefins, aromatic heterocycles and allylic alcohols.[1] Allylic alcohols are abundant in natural sources, including essential oils, and are widely used as starting materials and/or major components in the food, fragrance and pharmaceutical industries. Recently, several multienzymatic cascade reactions using ene reductases, alcohol dehydrogenases and Baeyer–Villiger monooxygenases, employed either as cell-free or whole-cell catalytic systems, have been described.[2–4] Further, biocatalytic approaches for the asymmetric reduction of allylic alcohols have been reported. Bakers' yeast has been used for the selective reduction of cinnamyl alcohols and derivatives in a whole-cell system.[5]

Furthermore, the groups of Sacchetti and Hollmann presented a three-step in vitro enzymatic redox isomerisation cascade reaction based on alcohol dehydrogenase and ene reductase enzyme catalysts.[6,7] The ene reductases applied in these two studies include the two bakers' yeast isoenzyme reductases OYE2 and OYE3[7] and the thermophilic *Ts*ER from *Thermus scoductus* SA-01.[6] Here, the alcohol dehydrogenase (ADH) converts the allylic alcohol into the corresponding aldehyde, with the aid of NAD^+ as cofactor, and this is followed by the reduction of the formed enal intermediate with the NADH-dependent ene reductase and the further ADH-catalysed reduction of the formed aldehyde to generate the corresponding alcohol product (Scheme 1).



Scheme 1. Bienzymatic three-step cascade reaction for the reduction of cinnamyl alcohol through the coupling of an alcohol dehydrogenase (ADH) with ene reductase wild-type enzymes and loop-grafted variants.

Old Yellow Enzymes are a large and widespread enzyme family known to catalyse the reduction of a wide range of substrates with use of a nicotinamide cofactor.[8,9] The natural asymmetric bioreduction proceeds by a ping-pong bi–bi mechanism, divided into reductive and oxidative half reactions (Figure S1 in the Supporting Information).[10] The substrate scope of ene reductases, however, is limited to compounds containing an electron-withdrawing group that activates the C=C bond, thus allowing the *trans*-specific enzymatic reduction. Substrates with non-activating functional groups, such as allylic alcohols, cannot directly be reduced by these enzyme catalysts.

Ene reductase family members are based on a (β/α) triosephosphate isomerase (TIM) barrel structure. The active site containing the prosthetic flavin mononucleotide (FMN) is located at the carboxy-terminal end of the barrel and surrounded by diverse and variable β/α surface loop regions.[11] Recently, we succeeded in demonstrating that loop-engineered variants of the nicotinamide-dependent cyclohex-2-en-1-one reductase (NCR) from *Zymomonas mobilis*[11,12] with different loop lengths (and thus amino acid compositions) alter the activity and the overall stability of this enzyme.[13,14] Given the broad substrate range of natural ene reductases, we set out to explore whether this diversity could be leveraged by rational loop engineering of NCR for the reduction of challenging allylic alcohols. Therefore, two β/α surface loop regions near the entrance of the

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201500604.

active site were selected for loop grafting experiments on the basis of structure and sequence alignments of the three well-known and described ene reductase family members: NCR, OYE1 from *Saccharomyces pastorianus*[11,15] and morphinone reductase (MR) from *Pseudomonas putida* M10[16,17] (Figures 1 and S2). The three selected ene reductases possess overall sequence identities between 29.4% (NCR/OYE1) and 42.4% (NCR/MR). The monomeric reductase NCR served as enzyme scaffold for the generation of a total of four single loop-grafted variants, named loop A_OYE1, loop A_MR, loop B_OYE1 and loop B_MR.

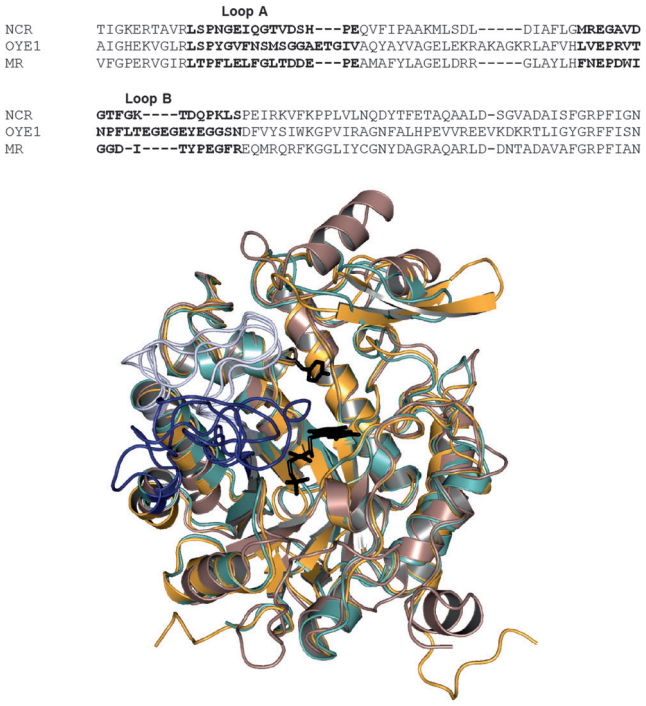


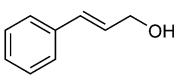
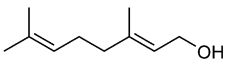
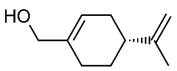
Figure 1. Sequence and structure alignment of selected ene reductases NCR, OYE1 and MR. Top: Excerpt of a multiple sequence alignment. Two loops, A and B (highlighted in bold), near the active site were selected for loop grafting. Bottom: Structural superposition of loop A (light blue) and loop B (dark blue) of the three ene reductases NCR (PDB ID: 4A3U, cyan), MR (PDB ID: 1GWJ, orange) and OYE1 (PDB ID: 1OYA, purple). Catalytically active tyrosine and prosthetic FMN are shown as black sticks. Pictures are generated with PyMol.

In order to facilitate the asymmetric reduction of allylic alcohols in a bienzymatic cascade, we tested several commercially available alcohol dehydrogenases for their ability to catalyse the oxidation and reduction of a small set of substrates by using NADH/NAD^+ (data not shown). The commercially available ADH equine from Sigma–Aldrich turned out to be the most active alcohol dehydrogenase (Table S1). Wild-type enzymes and the generated loop variants were purified and applied in in vitro cascade reactions with the ADH equine together with cinnamyl alcohol, geraniol or (−)-perillyl alcohol as substrates.

Of the three wild-type enzymes expressed, purified and tested, only OYE1 and MR showed good to excellent activities towards the reduction of the three allylic substrates. Surprisingly, the activity of NCR wild type was fairly low (Table 1). When the bienzymatic one-pot reduction reactions of the three allylic alcohol substrates were performed with the generated NCR loop variants, however, good to excellent product formation could be observed.

The grafting of the corresponding loop A and loop B regions from OYE1 and MR, respectively, into the NCR scaffold had therefore resulted in NCR variants with new activities. It is also interesting to note that the reduction of (*S*)-(−)-perillyl alcohol resulted in the formation of two different diastereomers of the resulting shisool alcohol (Figure S3). The two wild-type enzymes OYE1 and MR demonstrated opposite stereochemical behaviour in the reduction of (*S*)-(−)-perillyl alcohol. From literature data on the reduction of perillyl aldehyde and car-

Table 1. Product formation in in vitro one-pot bienzymatic reductions of cinnamyl alcohol, geraniol and (*S*)-(−)-perillyl alcohol with the selected ADH equine and seven purified (purity > 95%) ene reductase wild-type enzymes and variants.

		Product formation [%]			
Substrates		cinnamyl alcohol 	geraniol 	(*S*)-(−)-perillyl alcohol[a] 	
Products		3-phenylpropan-1-ol	citronellol	*trans*-shisool	*cis*-shisool
wild type	NCR	4.3 ± 0.4	3.3 ± 0.6	1.3 ± 0.1	0.1 ± 0.1
	OYE1	97.5 ± 0.6	99.1 ± 0.1	95.4 ± 0.1	0.5 ± 0.1
	MR	85.9 ± 0.4	25.9 ± 1.0	12.0 ± 5.3	71.4 ± 2.4
loop variants of NCR	A_OYE1	57.6 ± 8.5	10.3 ± 1.0	94.2 ± 0.2	3.4 ± 0.1
	A_MR	97.6 ± 0.7	20.9 ± 4.1	93.4 ± 0.4	3.4 ± 0.7
	B_OYE1	94.4 ± 1.1	42.0 ± 4.8	82.9 ± 1.4	3.8 ± 0.7
	B_MR	30.7 ± 1.3	45.4 ± 1.2	6.2 ± 1.0	84.2 ± 0.9

Reactions were performed in triplicate in 24 h reaction time. [a] The reduction of (*S*)-(−)-perillyl alcohol resulted in the formation of two possible different diastereomers (Figure S3).

vone,[18,19] we assume that OYE1 produced nearly exclusively *trans*-shisool (95.4%) and MR *cis*-shisool (71.4%, see Table 1). Additional experiments should clarify these observations in detail. By looking at the stereoselectivity of the generated loop-grafted variants, we also observed that the ability to produce *cis*-shisool was transferred from MR to NCR during the loop grafting of the loop B region (84.2% *cis*-shisool was formed with loop B_MR). In contrast, the grafting of the loop A region had no impact on the stereoselective output of the enzyme. These results go in hand with results related to the influence of β/α surface loop regions on enzyme selectivity (unpublished data) previously obtained in our lab.

These results served as the basis for examinations of NCR, OYE1 and MR wild-type enzymes as well as the four loop-grafted variants in the reduction of the formed activated aldehyde intermediates. We thus tested the wild-type and loop-grafted variants in the reduction of the two substrates cinnamaldehyde and geranial, corresponding intermediates in the cascade reaction. Interestingly, NCR wild type clearly demonstrated the highest product formation with both intermediate aldehydes tested. Decreased reduction rates were obtained for OYE1 and MR wild types and the generated NCR loop variants (Figure 2). Therefore, we conclude that the grafting of β/α loop regions from the less active biocatalysts OYE1 and MR into the NCR scaffold resulted in reduced activities for these substrates.

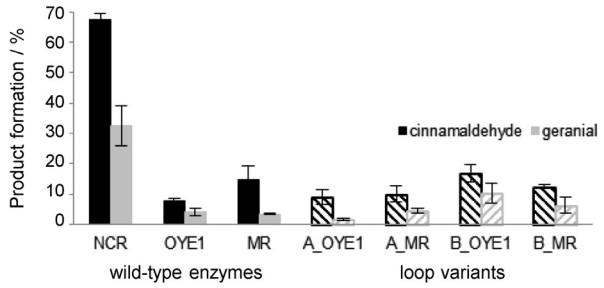


Figure 2. Initial activities for the reduction of the formed intermediates cinnamaldehyde (black) and geranial (grey) with wild-type enzymes NCR, OYE1 and MR (solid) and the four designed loop-grafted variants loop A_OYE1, loop A_MR, loop B_OYE1 and loop B_MR (dashed lines). Reactions were performed in triplicate in 2.5 h reaction time (Table S2).

Despite the fact that NCR wild type was able to catalyse the reduction of the aldehyde compounds, a reduced activity coupled to ADH was observed. Thus, we assume that the nicotinamide cofactor might directly influence the activity of ene reductases.[20] During the natural bioreduction, as well as in the bienzymatic cascade setup, the required reduced nicotinamide cofactor (NADH) is consumed both by the ene reductase and by the alcohol dehydrogenase. In parallel, the oxidised form (NAD^+) accumulates in the reaction broth and might modulate the redox balance of this cascade reaction. In order to elucidate the impact of the oxidised cofactor NAD^+ on the reduction activity of ene reductase enzymes, biotransformation reactions with different reduced/oxidised nicotinamide cofactor ratios were performed. In all biotransformations an excess of the reduced NADH cofactor was used to enable complete conversion of the substrate. As model substrate cinnamaldehyde was employed. This substrate demonstrated the highest conversion rates in the reduction reaction with all enzymes tested (Figure 2).

As expected, the highest level of product formation was obtained with the NCR wild type in a reaction setup without any oxidised cofactor present at the beginning of the biotransformation. Interestingly, NCR was found to be quite sensitive towards the presence of oxidised NAD^+ in the reaction mixture. An increase in NAD^+ led to a considerably decreased activity of NCR (−80.7% with a ratio of 1:1, Table 2). MR and OYE1 wild-type enzymes and loop-grafted variants loop A_OYE1, loop A_MR, loop B_OYE1 and loop B_MR demonstrated more flexible behaviour than NCR towards the presence of oxidised NAD^+ (minor alterations in yield of reduced products, Table 2). Because NADH represents an opportunistic substrate in the ping-pong bi–bi reaction mechanism, we assume that the NCR wild type should be inhibited by the oxidised NAD^+. The higher flexibility of OYE1 and MR towards the oxidised cofactor could be transferred to the NCR backbone through the grafting of the two β/α surface loops. These results indicate that the loops of the ene reductases are possibly involved in the interaction both with the reduced and with the oxidised nicotinamide cofactor. The exact type of interaction between the β/α loop regions and the nicotinamide cofactor is currently under investigation in our laboratory.

Table 2. Product formation in the conversion of cinnamaldehyde with NADH present in the reaction mixture, together with the percentage alterations in product yields with different amounts of NAD^+ present at the beginning of the reaction (NADH/NAD^+ ratio 4:1 and 1:1).

Cinnamaldehyde reduction reaction		Cofactor ratio NADH/NAD^+ 1:0 Yield of reduced products	4:1 Alteration in yield of reduced products relative to 1:0 [%]	1:1 Alteration in yield of reduced products relative to 1:0 [%]
wild type	NCR	98.4±1.6	−61.5	−80.7
	OYE1	59.4±0.8	−36.2	−63.0
	MR	34.9±0.7	−38.1	−47.3
loop variants	A_OYE1	92.6±4.6	−35.4	−55.2
	A_MR	89.0±8.8	+0.2	−56.4
	B_OYE1	56.6±0.2	−22.8	−42.9
	B_MR	39.1±2.1	−2.0	−67.0

For all performed reactions NADH was used in excess amount in order to achieve complete conversion of the substrate. However, further reduction of hydrocinnamaldehyde to the corresponding alcohol 3-phenylpropan-1-ol has been observed with excess amounts of NADH. For detailed information and deviations see Scheme S1 and Table S3.

In conclusion, the grafting of β/α surface loop regions between different ene reductases enables the alteration and the transfer of new reaction activities within these family members. Furthermore, differences in the interaction with the nicotinamide cofactor were noted. We expect that the proposed concept of loop grafting of flexible surface loop regions will stimu-

late the engineering of enzymes with modified and improved enzyme properties.

Experimental Section

General remarks: Restriction endonucleases, *Pfu* DNA polymerase and T4 DNA ligase were purchased from Fermentas (Thermo Scientific). Primers were obtained from Metabion (Matrinsried, Germany). Unless otherwise specified, chemicals were obtained from Fluka, Sigma–Aldrich, Carl Roth, SAFC (Hamburg, Germany) or BASF SE. GC-FID analyses were carried out with a Shimadzu GC-2010 instrument equipped with an AOC-20i auto injector and a HP-5 capillary column (Agilent technologies, 30 m × 0.25 mm × 0.25 μm) with H_2 as carrier gas (1.38 mL min^{-1}). Standard molecular biology techniques such as overlapping extension PCR, restriction digestion, ligation, heat shock transformation as well as protein concentration determination were used as previously described.[11,12]

Protein expression and purification: The two wild-type enzymes NCR and MR, as well as the four loop-grafted variants, were cloned in pET-28a(+) and expressed in TB medium with kanamycin (50 μg mL^{-1}) in *Escherichia coli* BL21(DE3). Enzyme expression was induced at an OD_{600} of 0.5–0.6 with IPTG (0.2 mM) at 30 °C for 20 h. After harvesting by centrifugation (9000 *g*, 30 min, 4 °C), the cells were disrupted with a French press (EmulsiFlex-C5, Avestin, Mannheim, Germany) and purified by immobilised metal affinity chromatography with an ÄKTA system. OYE1 wild-type enzyme was cloned into a pDHE vector and also expressed in *E. coli* BL21(DE3) under the following conditions: 37 °C, 180 rpm in TB medium (400 mL) containing ampicillin (100 mg). When the cultures reached an OD_{600} of 1.3–1.6, they were supplemented with L-rhamnnose (0.2%) for protein expression. After 14 h of incubation at 30 °C (160 rpm), cells were harvested by centrifugation (8000 *g*, 15 min, 4 °C) and resuspended in potassium phosphate buffer (50 mM, pH 7.4) containing phenylmethanesulfonyl fluoride (PMSF, 0.1 mM). Cell pellets were disrupted in two or three cycles with a French press (EmulsiFlex-C5, Avestin, Mannheim, Germany) at 4 °C. The resulting crude extracts were centrifuged (37000 *g*, 30 min, 4 °C), and the supernatants with the soluble proteins were recovered. Protein purification was performed in three steps: 1) ammonium sulfate precipitation, 2) fast protein liquid chromatography (FPLC), and 3) hydrophobic interaction chromatography (HIC). Protein precipitation of the lysate was performed with ammonium sulfate (24% final concentration). Subsequently, the lysate was centrifuged (7000 *g*) for 15 min at 4 °C, and the protein pellet was discarded. The ammonium sulfate concentration was then increased to an end concentration of 45%, followed by centrifugation (7000 *g*, 15 min, 4 °C), and the supernatant was discarded. Afterwards the proteins were resuspended in potassium phosphate buffer (50 mM, pH 7.8). FPLC was carried with Q-Sepharose FF columns (GE Healthcare) packed to a volume of 275 mL and a maximum flow of 20 mL min^{-1}. The column was washed (10 mL min^{-1} working flow) by a step gradient protocol with potassium phosphate buffer (50 mM, pH 7.8) containing ammonium sulfate (1.4 M). The elution of the OYE1 proteins was observed at a concentration of 400 mM ammonium sulfate. In addition to the characteristic total protein detection at 280 nm, OYE1 was identified by its absorbance at 455 nm. HIC was performed with a phenyl Sepharose HP column (GE Healthcare, Freiburg, Germany). The column was packed with a maximum flow of 18 mL min^{-1} to a volume of 240 mL. The elution buffer contained potassium phosphate (50 mM, pH 7.2) and ammonium sulfate (1.4 M). The elution of the OYE1 proteins was observed at a concentration of 850 mM ammonium sulfate. The purification was followed by a filtration and desalting step with Vivaspin ultrafiltration spin columns (Vivaspin 10 kDa, Sartorius, Göttingen, Germany) and concentrated in Tris·HCl reaction buffer (50 mM, pH 7.5). The purified enzyme was stored at −20 °C until further use.

Biotransformation reactions: All biotransformation reactions were carried out with purified enzyme (protein purity > 95%). Biotransformations were performed with NADH (5 mM), purified enzyme (100 μg mL^{-1}), ADH equine (0.2 U, Sigma–Aldrich) and Tris·HCl (1 mL, 50 mM, pH 7.5). The biotransformation was started by adding substrate [2 mM (cinnamyl alcohol, geraniol, (*S*)-(−)-perillyl alcohol]. After incubation (24 h, 30 °C, 180 rpm), the reaction was stopped by extraction with methyl-*tert*-butylether (MTBE; 2 × 500 μL). Activity tests towards cinnamaldehyde were performed with cinnamaldehyde (2 mM), NADH (2.5 mM) and purified protein (50 μg mL^{-1}) in Tris·HCl (1 mL, 50 mM, pH 7.5) for 2.5 h at 30 °C and 180 rpm, followed by extraction with MTBE (2 × 500 μL). Assays with varying cofactor concentrations contained cinnamaldehyde (2 mM), NADH/NAD$^+$ cofactor (1:0, 4:1, 1:1, 5 mM) and purified enzyme (100 μg mL^{-1}) in Tris·HCl (1 mL, 50 mM, pH 7.5) for 24 h at 30 °C and 180 rpm, and were stopped by extraction with MTBE (2 × 500 μL).

GC-FID analytics: Levels of conversion were determined from the percent area of the product. Samples were directly analysed from the MTBE phase without derivatisation by using a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm, Agilent) and one of two different temperature programs. The program for cyclic substrates (cinnamyl alcohol, (*S*)-(−)-perillyl alcohol and cinnamaldehyde) was 70 °C, 15 °C min^{-1} to 200 °C, 30 °C min^{-1} to 320 °C, hold 1 min. The program for the aliphatic substrate (geraniol) was 3 min at 60 °C, 10 °C min^{-1} to 150 °C, 50 °C min^{-1} to 300 °C, hold 1 min. The injector temperature was 250 °C. Compounds were detected by flame ionisation detection(FID) at 325 °C and identified by coelution with standards.

Acknowledgements

We gratefully acknowledge financial support from the Landesgraduiertenförderung (LSFG) Baden–Württemberg and the European Community's Seven Framework Program (FP7/2007–2013) for the Innovative Medicine Initiative under Grant Agreement no. 115360 (Chemical manufacturing methods for the 21st century pharmaceutical industries, Chem21).

Keywords: allylic alcohols · biocatalysis · cascade reactions · loop grafting · oxidoreductases · reduction

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Manuscript received: November 12, 2015
Accepted article published: January 14, 2016
Final article published: February 23, 2016