Fusion of Formate Dehydrogenase and Alanine Dehydrogenase as an Amino Donor Regenerating System Coupled to Transaminases

Valentina Marchini,[a] Ana I. Benítez-Mateos,[a] Sofia L. Hutter,[a] and Francesca Paradisi*[a]

Fusion enzymes are attractive tools for facilitating the assembly of biocatalytic cascades for chemical synthesis. This approach can offer great advantages for cooperative redox cascades that need the constant supply of a donor molecule. In this work, we have developed a self-sufficient bifunctional enzyme that can be coupled to transaminase-catalyzed reactions for the efficient recycling of the amino donor (L-alanine). By genetic fusion of an alanine dehydrogenase (AlaDH) and a formate dehydrogenase (FDH), a redox-complementary system was applied to recycle the amino donor and the cofactor (NADH), respectively. AlaDH and FDH were assembled in both combinations (FDH-AlaDH and AlaDH-FDH), with a 2.5-fold higher enzymatic activity of the latter system. Then, AlaDH-FDH was coupled to two different *S*-selective transaminases for the synthesis of vanillyl amine (10 mM) reaching up to 99% conversion in 24 h in both cases. Finally, the multienzyme system was reused for at least 3 consecutive cycles when implemented in dialysis-assisted biotransformations.

Introduction

Amine transaminases (ATAs, EC 2.6.1.-) have attracted considerable interest in their use as biocatalysts for the enantioselective synthesis of valuable chiral amine compounds, which are widespread within the pharmaceutical, agrochemical, and fine chemical industry.[1–7] Nonetheless, the insufficient stability of ATAs under operating conditions still constitutes a major hurdle for industrial applications.[8] An excess of the amine donor is often required to shift the unfavorable reaction equilibrium towards the production of the desired amine, but this may be detrimental for the enzyme.[9] The high concentration of amino donor may cause monomer dissociation, unfolding, and irreversible protein aggregation.[8,10] Furthermore, the need for a constant supply of amino donor reduces the cost-efficiency and sustainability of the enzymatic process. The use of smart amine donors has been an excellent step forward but not all ATAs accept those substrates and the byproduct needs to be dealt with.[11]

When simple amine donors are used, the enzyme can suffer from inhibition of the generated aldehydes or keto acids such as pyruvate.[10] In these cases, several methods have been developed to displace the equilibrium and avoid byproduct inhibition such as the coupling of a second enzyme. Lactate dehydrogenase (LDH) had been combined with an ATA using alanine as amino donor to convert the pyruvate into lactic acid at the expense of NADH, removing the byproduct from the reaction environment.[1] To recycle the cofactor (NADH), a third enzyme (glucose dehydrogenase) was added. The formed byproduct, however, caused a pH shift that affected the transaminase stability.[4] Similar approaches employed a pyruvate decarboxylase to remove pyruvate by converting it to acetaldehyde, but this molecule competes with the substrate to be aminated, leading to ethylamine as side product.[1,4]

Alternatively, another strategy based on an alanine dehydrogenase (AlaDH) has been applied to remove the pyruvate from the reaction bulk by regenerating alanine in situ (with the addition of NADH and NH_3) so that the amine donor is continuously available for the transaminase reaction.[12–15] Therefore, both equilibrium displacement and byproduct inhibition were addressed. The cofactor was then recycled by adding a third enzyme, formate dehydrogenase (FDH).[12,13]

Despite the advantages offered by multi-enzyme systems, the production and optimization of each individual enzyme is costly and time-consuming. Fusion proteins can address these difficulties by combining enzymes that are catalytically compatible into one single multifunctional enzyme,[16,17] and the genetic fusion can result into improved catalytic efficiency due to the closer proximity of the active sites.[18,19] Additionally, gene expression, folding, and enzyme stability can be potentially enhanced, but the design of the fusion enzyme must be carefully rationalized.[16]

In this work, we develop an easy-to-produce and efficient enzymatic system that can be coupled to transaminase reactions as the amino donor regeneration system. A bifunctional enzyme has been rationally designed by combining an alanine dehydrogenase from *Halomonas elongata* (HeAlaDH) with a formate dehydrogenase from *Candida boidinii* (CbFDH).[12,20] Two different genetic constructs were developed

[a] *Dr. V. Marchini, Dr. A. I. Benítez-Mateos, S. L. Hutter, Prof. Dr. F. Paradisi
Department of Chemistry
Biochemistry and Pharmaceutical Sciences
University of Bern, Freiestrasse 3, 3012 Bern (Switzerland)
E-mail: francesca.paradisi@unibe.ch*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200428

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

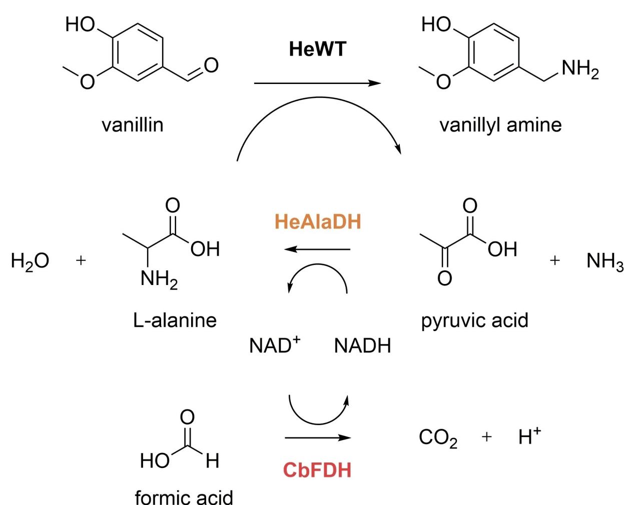
and studied. The efficiency of the bifunctional enzyme as amino donor regenerator was then tested in biotransformations for the amination of vanillin as a case of study. Two different ω-transaminases, one from *Halomonas elongata* (HeWT)[21] and one from *Chromobacterium violaceum* (CvTA)[22] were examined in combination with the bifunctional enzyme (Scheme 1).

Results and Discussion

Design and development of genetic constructions

The genetic construction of both recombinant proteins was developed using the original plasmid encoding the FDH (pET28b-CbFDH) as the backbone template (Figure S1).[19] The HeAlaDH gene was firstly inserted downstream of the FDH gene sequence (His-FDH-AlaDH) (Figure 1 and Table S1) with the inclusion of a flexible GSGGGGSAS linker between the two domains to facilitate folding. Indeed, the presence of glycine and serine as small polar amino acids has been shown to provide good flexibility and optimal stability in water.[23] A (6x)His-tag was also fused to the FDH domain for purification purposes as in a previous fusion protein including the CbFDH.[19]

As both enzymes are multimeric proteins (CbFDH is dimeric and HeAlaDH is hexameric), the assembly of the monomers can vary within the fusion protein, and this may affect the final enzymatic activity. Hence, a second genetic construct where the gene of HeAlaDH was inserted upstream of the CbFDH gene (AlaDH-FDH-His) was then developed (Figure 1). Indeed, past works on fusion proteins showed that the orientation of the domains can drastically change the activity as well as stability of the final protein.[24–27] For instance, the fusion of an alcohol dehydrogenase (ADH) with a cyclohexanone monooxygenase (CHMO) was studied and one orientation showed the expected catalytic efficiency, while the other one showed low to no activity at all.[24] In a another recent example, different genetic constructs of CbFDH fused to an azoreductase were analyzed, finding one conformation with a 20-fold lower activity.[25]



Scheme 1. One-pot transaminase reaction (HeWT) for the conversion of vanillin into vanillyl amine, coupled with HeAlaDH for the regeneration of the amino donor (L-alanine), while removing the inhibiting product (pyruvic acid). The CbFDH is used to continuously recycle the cofactor NADH.

Figure 1. Schematic diagram of the two fusion protein constructs. GSGGGGGSAS corresponds to the linker sequence. See more details about the genetic construction in Figure S1.

Production and purification of the fusion proteins

While the His-FDH-AlaDH construct yielded an active and soluble system after a first expression screening (Table S2 and Figure S2), AlaDH-FDH-His protein required an extensive optimization of the expression conditions to obtain the enzyme in a soluble form (Figure S3).

In a previous work, the fusion of a tetrameric levodione reductase (LR) with a dimeric ATA linked by a PAS spacer produced insoluble aggregates with a 4-amino acid linker, only 0.2 mg/L with a spacer of 20 residues, and 0.6 mg/L with 40 or 60 amino acids.[28] Hence, the proportion of soluble fusion protein was significantly increased with the linker length, due to the sufficient steric flexibility for efficient oligomerization of each enzyme moiety with its characteristic quaternary structure. Therefore, a higher spacer length could prevent protein aggregation into inclusion bodies.[28] In the present case, the distance between the different terminals might be shorter for the second fusion protein (AlaDH-FDH-His), preventing the correct folding of the two domains for insufficient space and flexibility in between.

Activity and assembly of the fusion proteins

The specific activity was determined for both catalytic domains (AlaDH and FDH), which was compared to the activity of the WT enzymes (Table 1). For the His-FDH-AlaDH construct, only 11–12% of the activity was retained with respect to the WT AlaDH. On the other hand, 23% of the activity of the FDH domain was retained compared to the WT protein, 2-fold higher than the activity of the AlaDH domain. This behavior may be due to a different ratio of correct folding between the protein domains. Indeed, FDH is active as a dimer, while AlaDH requires six assembled monomers to be active. Therefore, it can be expected that due to the higher structural complexity of AlaDH, the activity compared to the parental enzymes is lower than for the FDH. Yet, the AlaDH domain retained activity in the chimeric construct while many previous attempts to develop fusion proteins of, in principle, less complex protein structures,

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

Table 1. Specific activity of the purified fusion proteins compared to the normalized specific activity of the wild-type enzymes. Reaction conditions: FDH: 100 mM sodium formate and 3 mM of NAD^+ in potassium phosphate 25 mM pH 7.5. AlaDH amination: 0.3 mM of NADH, 2.5 mM sodium pyruvate and 250 mM ammonium chloride in 100 mM potassium phosphate buffer pH 8.0. AlaDH deamination: 40 mM L-alanine and 1 mM of NAD^+ in 100 mM $NaHCO_3$ pH 10. All reactions were performed at 25 °C.

	FDH	Amination AlaDH	Deamination AlaDH
WT enzymes[a]	1.75 U/mg	58 U/mg	12 U/mg
His-FDH-AlaDH	0.4 ± 0.08 U/mg	6.5 ± 1 U/mg	1.4 ± 0.1 U/mg
Retained activity	23 ± 5 %	11 ± 2 %	12 ± 1 %
AlaDH-FDH-His	1.0 ± 0.2 U/mg	16.0 ± 2.3 U/mg	2.4 ± 0.5 U/mg
Retained activity	57 ± 11 %	28 ± 4 %	20 ± 4 %

[a] Normalized activity of the WT enzymes is shown as a reference value compared to the corresponding protein domain of the fusion protein. See more details on the calculation of the normalized activity in section 4 of the Supporting Information.

Table 2. Kinetic constants of the AlaDH-FDH-His fusion protein compared to the WT alanine- and the WT formate dehydrogenases.

	Fusion protein – AlaDH K_M [mM]	K_{cat} [s^{-1}][a]	WT – AlaDH K_M [mM][b]	K_{cat} [s^{-1}][a]
L-alanine	11.4 ± 1.5	1.7	10.3 ± 2.4	8.4
NAD^+	0.26 ± 0.02		0.20 ± 0.04	
Pyruvate	0.44 ± 0.06	11.2	0.60 ± 0.11	40.6
Ammonia	93.3 ± 16.2		77.8 ± 12.6	
NADH	0.18 ± 0.02		0.05 ± 0.01	
	Fusion Protein – FDH K_M [mM]	K_{cat} [s^{-1}]	WT – FDH K_M [mM][a]	K_{cat} [s^{-1}]
Formate	7.8 ± 1.4	0.7	5.0 ± 1.0	1.3
NAD^+	0.57 ± 0.03		0.17 ± 0.03	

[a] K_{cat} depicted corresponds to the deamination direction (L-alanine and NAD^+) and to the amination direction (pyruvate, ammonia, NADH). [b] K_M values for WT AlaDH were extracted from Ref. [12].

failed in retaining the structure, and thus the activity, of the WT enzymes.[29,30]

In order to analyze the assembly of the monomers, size exclusion chromatography (SEC) of the fusion protein was performed. The results of the SEC confirmed that AlaDH and FDH domains were not fully assembled in hexamers and dimers, respectively (Figures S6 and S8). Instead, the two main conformations were assembled in 8 and 12 subunits for the His-FDH-AlaDH. This may have led to the low retained specific activity compared to the WT enzymes.

The second bifunctional enzyme (AlaDH-FDH-His) showed higher retained activity than the first one (His-FDH-AlaDH) (Table 1). Around 60 % of FDH activity was retained after the fusion to the AlaDH, while the AlaDH domain retained 20–30 % of the original activity, considering both directions of reaction (amination and deamination). Overall, this fusion protein presented 2/2.5-fold higher activity than the His-FDH-AlaDH fusion protein for both protein domains. Notably, the percentage of activity of the FDH domain was twice that of the AlaDH domain also in this case. This could be beneficial to ensure a fast recycling of NADH without limiting the overall conversion.

Due to the higher enzymatic activity, AlaDH-FDH-His enzyme was chosen for further characterization. In this case, SEC revealed that AlaDH-FDH-His was mainly assembled in 12, 6, 2 and 1 subunits (Figures S7 and S9). This construct provided at least part of the hexameric structure that is required for the full activity. However, the presence of the other three quaternary structures reduced the overall retained activity.

Characterization of the AlaDH-FDH-His fusion protein

The affinity of the enzyme domains for the natural substrates was assessed as shown in Table 2. The resulting K_M values were comparable between fusion protein and WT domains with most of the substrates (while K_{cat} was more heavily impacted). Only NADH for the AlaDH domain and NAD^+ for the FDH domain showed a 3.5-fold higher K_M than the WT enzymes. A similar behavior was found in other fusion proteins. The GluDH domain in the GluDH-FDH protein presented a K_M 2-fold higher than the parental enzymes.[19] Similarly, the PheDH-FDH-His fusion protein had 2-fold higher K_M values for the substrates phenylpyruvate and formate than the WT enzymes.[31] In another examples, the K_M values of the FDH domain in the His-FDH-AzoRo fusion protein increased by at least 10-fold for formate and almost 2-fold for NAD^+.[25]

The stability of the fusion protein over different pH, temperature, and cosolvent (DMSO) concentration was then investigated. For the AlaDH domain, the profile of pH stability matches the WT enzyme (Figure S10).[12] The FDH domain did not seem to lose activity after incubation at very low pH, even if the AlaDH stability was considerably affected in the same conditions. Concerning the temperature stability, the FDH domain showed reduced stability at higher temperatures over the 24 and 48 hours compared to the WT FDH, while no difference was noticed between 25 °C and 34 °C (Figure S11). In contrast, the AlaDH domain exhibited a profile comparable to the WT AlaDH, without any significant loss of stability. As DMSO was previously used to solubilize the substrate in the further transamination reactions due to the good stability of HeWT,[21] the stability of AlaDH, FDH as well as AlaDH-FDH-His in the presence of this solvent was assessed. At 10–20 % DMSO, the AlaDH domain completely preserved the activity (Figure S12). In the case of the FDH domain, most of the activity was retained at 10 % DMSO, but 30 % activity loss was noted after long incubation times (72 h) in presence of 20 % DMSO.

The results confirmed that both AlaDH and FDH domains retained their overall properties when combined into a fusion protein. Besides, it should be noted that both fusion partners show biochemical compatibility at the reaction conditions that would be required for the biotransformations of vanillin (pH 8.0, 37 °C, and 10 % DMSO).

Batch biotransformations for the amination of vanillin

Transaminases are employed as biocatalysts for the synthesis of flavors and fragrances.[32,33] Herein, we have evaluated the

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

transaminase HeWT for the model amination of vanillin to yield vanillyl amine in batch biotransformations. The equilibrium of this reaction is indeed unfavorable, requiring an excess of the amino donor equivalents to shifts it towards the product.[12,21] Hence, this aromatic aldehyde is a very suitable substrate to test our system. The fusion protein AlaDH-FDH-His was coupled as a bifunctional biocatalyst to recycle the amino donor. To this end, no excess of amino donor was employed, only 1 equivalent of L-alanine. In parallel, the same reaction was performed with the WT enzymes, adding a protein concentration comparable to the corresponding protein domain of the fusion protein.

After 1 hour of reaction, HeWT coupled with the WT enzymes (AlaDH and FDH) achieved 94% of conversion, while the coupling with the fusion protein reached only 66% conversion (Figure S13). Both enzymatic systems reached the maximum conversion after 8 h. Despite the lower catalytic rate of the fusion protein, these results confirmed the feasibility of this system to recycle the amino donor (L-alanine) by removing the by-product (pyruvate). Considering the lower activity of the fusion protein domains compared to the WT enzymes and to make a more comparable evaluation, biotransformations containing the same activity (units) of fusion protein and WT enzymes were carried out. In this case, both enzyme preparations performed similarly (Figure 2). In comparison, the reaction using only HeWT, with no recycling system, stopped at 20% conversion after 24 h.

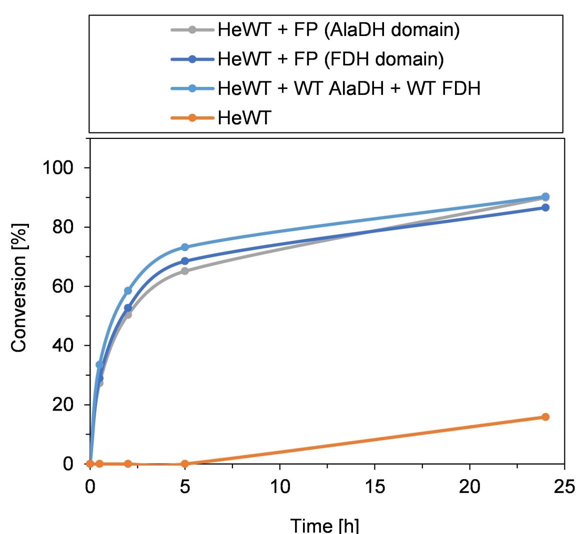


Figure 2. Batch biotransformations for the synthesis of vanillin amine comparing the fusion protein (AlaDH-FDH-His) and the WT enzymes. Reaction components: 0.36 mg of HeWT, 10 mM vanillin, 10 mM L-alanine, 100 mM ammonium formate, 1 mM NAD+, 0.1 mM PLP, 100 mM phosphate buffer pH 8.0, 10% DMSO. The reaction of "FP (AlaDH domain)" contained 0.15 units of AlaDH-FDH-His, the reaction "FP (FDH domain)" contained 0.12 units of AlaDH-FDH-His, and the reaction "WT AlaDH + WT FDH" contained 0.15 units of WT AlaDH and 0.12 units of WT FDH. Reactions were performed in 1 mL at 37 °C and 250 rpm. The consumption of vanillin was monitored by HPLC.

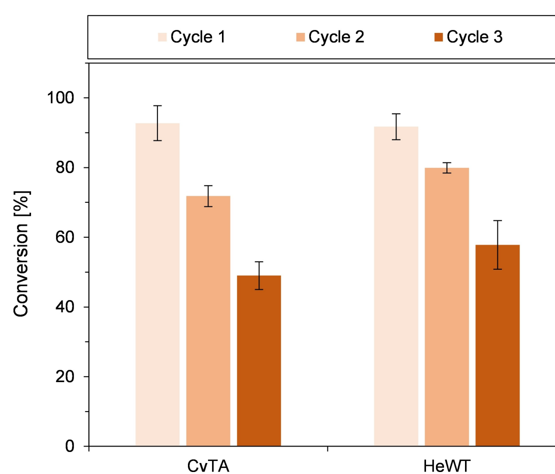


Figure 3. Dialysis assisted biotransformations for the synthesis of vanillin amine. 1 mg of transaminase (either HeWT or CvTA) and 1.5 mg of AlaDH-FDH-His were mixed inside a dialysis bag (cellulose membrane, cut-off 12 KDa). The reactions were performed in a glass vial incubated in a water bath at 37 °C with stirring (250 rpm). The 5 mL volume contained 1 mM NAD^+, 100 mM ammonium formate, 10 mM vanillin, 10 mM L-alanine, 0.1 mM PLP in 100 mM potassium phosphate buffer pH 8.0. Each reaction cycle corresponds to 24 h. The consumption of vanillin was monitored by HPLC.

AlaDH-FDH-His fusion protein in dialysis assisted biotransformation

A dialysis-assisted set up has been employed to test the scalability and the reusability of the fusion protein coupled to transamination reactions.[12] The soluble AlaDH-FDH-His and HeWT were contained in a dialysis membrane bag which was then submerged in the reaction mixture (Figure S14A). Under these conditions, full conversion of vanillin (10 mM, 5 mL) was achieved in 24 hours (Figure S14B). To test the versatility of AlaDH-FDH-His as amino donor recycling system, the fusion protein was also combined with another *S*-selective transaminase, CvTA, for the amination of vanillin. Full conversion was also achieved in this case in 24 h (Figure 3).

Then, the reusability of the fusion protein was tested with both HeWT and CvTA. In both cases, the enzymatic preparation could be reused for at least 3 cycles in batch maintaining at least 50% of molar conversion after the third cycle (Figure 3). Although the reaction conditions differ, the fusion protein reported herein shows a similar reusability compared to our previous work in which twice the amount of enzymes were used for the amino donor regeneration (1.5 mg in this work, 3 mg in Ref. [12]).

Conclusion

After rational optimization of the expression conditions, two bifunctional fusion proteins of AlaDH and FDH were produced (His-FDH-AlaDH and AlaDH-FDH-His) to recycle alanine in combination with enzymatic transaminations. The two enzymes were active, although AlaDH-FDH-His fusion protein demon-

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

strated 2–2.5-fold higher activity compared to the His-FDH-AlaDH assembly. In this regard, SEC indicated that the lower activity was related to the different quaternary assembly of the subunits compared to the original enzymes. AlaDH-FDH-His was characterized, confirming similar stability and affinity compared to the WT HeAlaDH and CbFDH. Moreover, the efficiency of the fusion protein was proved in combination with two transaminases (HeWT and CvTA) for the amination of vanillin, showing similar conversion rates to the WT enzymes. Finally, the enzymatic preparations could be reused as a dialysis-assisted bioreactors. In summary, this work supports the use of fusion proteins as a simpler and more cost-efficient strategy to produce multi-functional biocatalysts although further optimizations could increase the enzyme activity.

Experimental Section

Materials

Chemicals, reagents, and medium component, unless stated otherwise, were obtained as analytical grade from Sigma-Aldrich and Fisher Scientific. All the materials and kits employed for the cloning were acquired from New England Biolabs (NEB). Primers were synthetized by Microsynth AG; NADH and NAD^+ were purchased from Apollo Scientific Ltd.

Genetic construction

The genes of CbFDH and HeAlaDH were previously cloned from the respective genomes.[12,34] Herein, both genes were inserted in the pET28b(+) vector. A linker of Gly-Ser-Gly-Gly-Gly-Gly-Ser-Ala-Ser was included between the two domains. Four restriction sites were added as reported for a previous fusion protein construction.[19] The plasmid of the CbFDH (UniProt ID: O13437) was used as template for PCR amplification (vector fragments). For the development of the AlaDH-FDH-His construct, the vector FDH-pET28b was firstly mutated in a single position (6242 bp) where the stop codon was located. To encode for the His-tag at the FDH C-terminal of AlaDH-FDH-His fusion protein, a one-point mutation was performed to remove the stop codon at the end of the FDH gene. The vector FDH-pET28b was mutated in a single position (6242 bp). The mutation resulted in the exchange of the base A contained in the TAA stop codon for a C base, developing a TCA triplet, which translates for a serine in the amino acidic structure. For the purpose, a Q5 site-directed mutagenesis kit was employed following the protocol given by the supplier.

The alanine dehydrogenase gene (UniProt ID: E1 V931) was amplified from pRSETb-HeAlaDH to obtain the insert fragments. All the designed primers are listed in Table S1. The genes were amplified by PCR using Q5 High-Fidelity DNA polymerase and the obtained fragments (insert and vector) were used for cloning the fusion construct with the Gibson Assembly Cloning kit. A previous step of *DpnI* digestion with further heat-inactivation as well as sample refinement with the PCR purification kit were done before the assembly of the HeAlaDH with the vector containing the CbFDH sequence. After completing the Gibson Assembly of His-FDH-AlaDH gene, the assembled product was transformed into DH5α *E. coli* comptetent cells, and the grown colonies were subjected to colony PCR. The amplification was done using the T7 promoter as forward primer and T7 terminator as reverse primer. All the amplified fragments were analyzed by electrophoresis and the colony related to the sample showing a 2.5 Kb size was chosen for plasmid propagation, sequencing, and further studies. Regarding the AlaDH-FDH-His, one colony was taken after transformation of the assembled product into *E. coli* DH5α (included in the kit, protocol given by the supplier), which was directly used for plasmid replication, isolation, and sequencing.

Gene expression and protein purification

HeWT, HeAlaDH and CbFDH were produced as previously described.[12] The plasmid pET28b harboring the gene of the fusion protein was transformed into *E. coli* BL21(DE3) competent cells by heat-shock at 42 °C for 45 s, followed by 2 min on ice. After adding 0.5 mL of LB media and incubation at 37 °C for 1 h, the cells were plated on LB agar with the 50 μg/mL of kanamycin. Then, 1 L flasks containing 300 mL of LB media supplemented with 50 μg/mL of kanamycin were inoculated with 3 mL of an overnight culture and incubated at 37 °C (150 rpm) until the optical density (OD_{600}) was 0.5–0.6. To induce the gene expression of His-FDH-AlaDH, 0.1 mM IPTG was added after 30 minutes of cold shock (incubation on ice for 30 min). Afterwards, flasks were left at 20 °C overnight. Instead, the overexpression to produce the recombinant fusion protein AlaDH-FDH-His was induced by the addition of 0.1 μM IPTG and the culture was grown at 16 °C for an overnight. Cells were harvested by centrifugation at 4,500 rpm (20 min, 4 °C) and resuspended in 50 mM potassium phosphate buffer, 100 mM NaCl and 30 mM imidazole at pH 7.5. The suspension was placed on ice and sonicated at 40 % amplitude for 8 min, with pulses of 5 s ON, 10 s OFF using the sonicator Fisherbrand™ Model 120 Sonic Dismembrator. After centrifugation at 14,500 rpm for 45 min, the supernatant was filtered (0.45 μm pore size) and the fusion proteins were purified from the supernatant by a Ni-NTA column (GE Healthcare) in the ÄKTA Pure system. The proteins were eluted in 50 mM potassium phosphate buffer, 100 mM NaCl and 300 mM imidazole at pH 7.5. The purified enzymes were dialyzed twice in 50 mM potassium phosphate buffer at pH 7.5. The protein concentration was estimated by measuring the absorbance at 280 nm in the EPOCH2 spectrophotometer (nanodrop Tek3 plate), using the predicted molar extinction coefficients (ε) and molecular weights: 24,500 $M^{-1}cm^{-1}$ and 42 KDa for HeAlaDH, 51,465 $M^{-1}cm^{-1}$ and 43 KDa for CbFDH, 74,260 $M^{-1}cm^{-1}$ and 82 KDa for the AlaDH and FDH fusion proteins. The ε values were obtained from https://web.expasy.org/protparam. The purity of the purified proteins was analyzed by SDS-PAGE.

Size exclusion chromatography

The previously reported protocol was followed.[19] Briefly, the purified fusion protein was applied to a gel filtration column (Superdex 200 10/300 GL, GE Healthcare), which was equilibrated with buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5) and mounted onto an ÄKTA Pure instrument (GE Healthcare). The flow rate was set at 0.5 mL/min. A calibration curve was made by plotting the elution volumes of the protein standards (Carbonic anhydrase from bovine erythrocytes (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase from yeast (150 kDa), β-amylase from sweet potato (200 kDa), apoferritin from horse spleen (443 kDa), bovine thyroglobulin (669 kDa), blue dextran (2,000 kDa)). The elution of the standard proteins and the sample was followed by absorbance readings at 280 nm. The molecular weight of the fusion protein was calculated through the elution volume fitted in the equation obtained from the calibration curve.

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

Activity assays

HeWT and CvTA: enzymatic activity was determined as described elsewhere.[21] Briefly, 2.5 mM pyruvate, 2.5 mM *S*-MBA (*S*-(−)-α-Methylbenzylamine), and 0.1 mM PLP in phosphate buffer at pH 8.0 was added to the enzyme solution. The formation of acetophenone was following at 245 nm (ε: 12,000 $M^{-1}cm^{-1}$) for 2 minutes. One unit of activity was defined as the amount of enzyme needed to produce 1 μmol of acetophenone per minute at 30 °C.

AlaDH and FDH: One unit of activity was determined as the amount of enzyme needed to produce or consume 1 μmol of NADH per minute at 25 °C. The activity assay was performed in a 96-well plate and detected by UV absorbance at 340 nm (ε: 6,220 $M^{-1}cm^{-1}$). Each protein domain was evaluated singularly (AlaDH oxidative deamination: 40 mM L-alanine in 100 mM $NaHCO_3$ pH 10; 2.5 mM sodium pyruvate and 250 mM ammonium chloride in 100 mM potassium phosphate buffer 50 mM pH 8.0 for AlaDH reductive amination; FDH: 100 mM sodium formate in potassium phosphate 25 mM pH 7.5). The appropriate cofactor was added in the reaction mixture (0.3 mM of NADH for AlaDH reductive amination, 1 mM of NAD^+ for AlaDH oxidative deamination, 3 mM of NAD^+ for FDH activity, as final concentrations). The specific activity was calculated in U/mg considering the overall concentration of the fusion protein. See more details on the calculation of the normalized activity (U/mg) in section 4 of the supporting information.

Kinetic parameters

The kinetics of the AlaDH (WT and AlaDH-FDH-His) in the deamination direction were determined by measuring the activity of the enzyme when either the concentration of alanine or NAD^+ was varied. The concentration ranges used were as follows: 0.5–60 mM alanine and 0.1–5 mM NAD^+. In the aminating reacting, the kinetic parameters of pyruvate (0.05–5 mM), NADH (0.01–0.5 mM) and ammonia (10–50 mM) were determined. For the FDH (WT and AlaDH-FDH-His), the kinetic parameters of NAD^+ (0.1–3 mM) and formate (2–135 mM; with 3 mM NAD^+). The activity of the enzyme was measured as described in the previous section unless otherwise specified. All assays were carried out at least in duplicate. The data was fitted to Michaelis-Menten plots in GraphPad Prism 7 which was also used to calculate the kinetic parameters. For Kcat calculations, the following data were employed: V_{max} (U/mg) shown in Table 1, Mw (g/mol) and protein in reaction (mg) of only one domain (AlaDH or FDH) in case of the fusion protein.

Affinity assays

The kinetic constants were found in triplicate by measuring the dependence of initial rate on substrate concentration at 25 °C in the same buffers mentioned before. For oxidative deamination reaction of AlaDH, L-alanine concentration was varied from 0.1 to 60 mM in the presence of 1 mM NAD^+, while the cofactor concentration ranged from 0.1 to 3.0 mM (40 mM L-alanine). For the reverse reaction, pyruvate concentration was varied from 0.1 to 4 mM at a fixed concentration of 250 mM ammonium chloride; ammonium chloride was ranged between 10 to 500 mM at 2.5 mM sodium pyruvate. In both cases, the concentration of NADH was 0.5 mM. The kinetic constants for formic acid in the reaction of FDH were found by using a range between 2–135 mM sodium formate concentration at 3 mM NAD^+. The cofactor affinity was also evaluated by changing the concentration of NADH from 0.03 to 0.5 mM (2.5 mM pyruvate, 250 mM ammonium chloride) and 0.1–3.0 mM for NAD^+ (40 mM L-alanine for AlaDH and 100 mM formate for FDH). Reactions were made by adding an appropriate amount of enzyme in 10 μL solution, and the 20-fold concentrated cofactor solution (10 μL volume) in the 96 well-plates. The measurement started after mixing the substrates (180 μL) and following the change in absorbance at 340 nm at 25 °C.

Stability assays

The stability measurements at different temperatures, DMSO concentrations, and pH were executed in triplicate by incubating the biocatalyst solution at different temperatures (pH 7.5) or at pH values from 3 to 10 (temperature 4 °C) and withdrawing samples at regular times (2, 24, 48 hours) for proceeding with specific activity assays of the fusion protein in parallel with the WT enzymes.

Batch biotransformations

Reactions were performed with appropriate enzymes concentration, and the desired substrates. Dialysis assisted reactions were done as previously published.[12] Briefly, 10 mM vanillin, 10 mM L-alanine, 100 mM ammonium formate, 1 mM NAD^+, 0.1 mM PLP, 100 mM phosphate buffer pH 8.0, 10 % DMSO were added together with a proper amount of enzymes.

At different times, a volume of 50 μL was quenched with 225 μL HCl 0.2 % and 225 μL of acetonitrile. These samples were then analyzed by HPLC (Dionex UltiMate 3000 UHPLC Thermo Fisher Scientific), implemented with a C18 column (Waters X-Bridge, 3.5 μm, 2.1 × 100 mm). The flow rate was left at 0.8 mL/min and the oven was set at 45 °C. The samples were run using a gradient method from 5 : 95 to 95 : 5 (MilliQ water and acetonitrile with 0.1 % TFA) over 4 minutes. Conversions were calculated using A calibration curve of vanillin (5.2 min retention time) and vanillyl amine (4.05 min).

Dialysis assisted biotransformations

A mix of enzymes at the desired concentration were added inside a dialysis cellulose membrane tubing with a cut-off (MWCO) of 14 KDa (D9527-100FT from Sigma Aldrich, St. Louis, MO, USA). The membrane secured in both sides and submerged into a glass vial with total volume of 5 mL. The glass vials were covered with tape to avoid loss of solution by evaporation and hold in a water bath via a polystyrene sheet, ensuring that all the reaction solution is inside the water. Reactions were left stirring (250 rpm) at 37 °C. As a negative control, a vial containing all the substrates but the enzymes (replaced with buffer solution) was also employed. At the desired timepoints, samples were taken from the reaction bulk and analyzed by HPLC.

Author contributions

F. P. conceptualized the idea and supervised the project. V. M., A. I. B. M. and S. L. H. performed the experiments. V. M. and A. I. B. M wrote the initial draft. All the authors discussed the results and reviewed the final version of the work.

Acknowledgements

This project was supported by the University of Bern "Seal of Excellence Fund" Postdoctoral Fellowship (SELF19-03 BIORPHAN-

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

DRUG, A. I. B. M.) and the SNSF (200021_192274, F. P.). Open Access funding provided by Universität Bern.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: alanine dehydrogenase · biocatalysis · formate dehydrogenase · fusion proteins · transaminases

[1] I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal.* **2017**, *7*, 8263–8284.
[2] F. Guo, P. Berglund, *Green Chem.* **2017**, *19*, 333–360.
[3] D. Boison, *xPharm Compr. Pharmacol. Ref., Elsevier* **2007**, 1–2.
[4] M. Fuchs, J. E. Farnberger, W. Kroutil, *Eur. J. Org. Chem.* **2015**, 6965–6982.
[5] S. Wu, R. Snajdrova, J. C. Moore, K. Baldenius, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2021**, *60*, 88–119; *Angew. Chem.* **2021**, *133*, 89–123.
[6] A. Gomm, E. O'Reilly, *Curr. Opin. Chem. Biol.* **2018**, *43*, 106–112.
[7] S. A. Kelly, S. Pohle, S. Wharry, S. Mix, C. C. R. Allen, T. S. Moody, B. F. Gilmore, *Chem. Rev.* **2018**, *118*, 349–367.
[8] T. Börner, S. Rämisch, S. Bartsch, A. Vogel, P. Adlercreutz, C. Grey, *ChemBioChem* **2017**, *18*, 1482–1486.
[9] T. Börner, S. Rämisch, E. R. Reddem, S. Bartsch, A. Vogel, A. M. W. H. Thunnissen, P. Adlercreutz, C. Grey, *ACS Catal.* **2017**, *7*, 1259–1269.
[10] J. S. Shin, B. G. Kim, *Biotechnol. Bioeng.* **2002**, *77*, 832–837.
[11] A. Gomm, W. Lewis, A. P. Green, E. O'Reilly, *Chem. Eur. J.* **2016**, *22*, 12692–12695.
[12] D. Roura Padrosa, Z. Nissar, F. Paradisi, D. M. Reactors, *Catalysts* **2021**, *11*, 520.
[13] D. Koszelewski, I. Lavandera, D. Clay, G. M. Guebitz, D. Rozzell, W. Kroutil, *Angew. Chem. Int. Ed.* **2008**, *47*, 9337–9340; *Angew. Chem.* **2008**, *120*, 9477–9480.
[14] N. Richter, J. E. Farnberger, D. Pressnitz, H. Lechner, F. Zepeck, W. Kroutil, *Green Chem.* **2015**, *17*, 2952–2958.
[15] S. Klatte, V. F. Wendisch, *Microb. Cell Fact.* **2015**, *14*, 1–10.
[16] D. T. Monterrey, I. Ayuso-Fernández, I. Oroz-Guinea, E. García-Junceda, *Biotechnol. Adv.* **2022**, *60*, 108016.
[17] Y. Ma, N. Zhang, G. Vernet, S. Kara, *Front. Bioeng. Biotechnol.* **2022**, *10*, 944226.
[18] I. Wheeldon, S. D. Minteer, S. Banta, S. C. Barton, P. Atanassov, M. Sigman, *Nat. Chem.* **2016**, *8*, 299–309.
[19] V. Marchini, A. I. Benítez-Mateos, D. Roura Padrosa, F. Paradisi, *Front. Catal.* **2021**, *1*, 1–13.
[20] J. M. Bolivar, L. Wilson, S. A. Ferrarotti, R. Fernandez-Lafuente, J. M. Guisan, C. Mateo, *Biomacromolecules* **2006**, *7*, 669–673.
[21] L. Cerioli, M. Planchestainer, J. Cassidy, D. Tessaro, F. Paradisi, *J. Mol. Catal. B* **2015**, *120*, 141–150.
[22] U. Kaulmann, K. Smithies, M. E. B. Smith, H. C. Hailes, J. M. Ward, *Enzyme Microb. Technol.* **2007**, *41*, 628–637.
[23] D. K. Patel, D. V. Menon, D. H. Patel, G. Dave, *Protein Expression Purif.* **2022**, *191*, 106012.
[24] F. S. Aalbers, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 7557–7565.
[25] A. C. R. Ngo, F. P. J. Schultes, A. Maier, S. N. H. Hadewig, D. Tischler, *ChemBioChem* **2022**, *23*, e202100643.
[26] S. Elleuche, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 1545–1556.
[27] K. Yu, C. Liu, B. G. Kim, D. Y. Lee, *Biotechnol. Adv.* **2015**, *33*, 155–164.
[28] A. Lerchner, M. Daake, A. Jarasch, A. Skerra, *Protein Eng. Des. Sel.* **2016**, *29*, 557–562.
[29] P. Ljungcrantz, H. Carlsson, M.-O. Mansson, P. Buckel, K. Mosbach, L. Bülow, *Biochemistry* **1989**, *28*, 8786–8792.
[30] K. Du, R. Li, D. Zhang, W. Feng, *ChemBioChem* **2019**, *20*, 701–709.
[31] W. Jiang, B. S. Fang, *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 577–584.
[32] M. L. Contente, F. Dall'Oglio, L. Tamborini, F. Molinari, F. Paradisi, *ChemCatChem* **2017**, *9*, 3843–3848.
[33] G. Smit, B. A. Smit, W. J. M. Engels, *FEMS Microbiol. Rev.* **2005**, *29*, 591–610.
[34] Y. Sakai, A. P. Murdanoto, T. Konishi, A. Iwamatsu, N. Kato, *J. Bacteriol.* **1997**, *179*, 4480–4485.

Manuscript received: July 27, 2022
Revised manuscript received: September 5, 2022
Accepted manuscript online: September 6, 2022
Version of record online: September 29, 2022

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH