Engineering of alanine dehydrogenase from Bacillus subtilis for novel cofactor specificity

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Abstract

The L-alanine dehydrogenase of Bacillus subtilis (BasAlaDH), which is strictly dependent on NADH as redox cofactor, efficiently catalyzes the reductive amination of pyruvate to L-alanine using ammonia as amino group donor. To enable application of BasAlaDH as regenerating enzyme in coupled reactions with NADPH-dependent alcohol dehydrogenases, we altered its cofactor specificity from NADH to NADPH via protein engineering. By introducing two amino acid exchanges, D196A and L197R, high catalytic efficiency for NADPH was achieved, with $k_{cat}$/K_M $= 54.1 \mu$M$^{-1}$ Min$^{-1}$ ($K_M = 32 \pm 3 \mu$M; $k_{cat} = 1,730 \pm 39$ Min$^{-1}$), almost the same as the wild-type enzyme for NADH ($k_{cat}$/K_M $= 59.9 \mu$M$^{-1}$ Min$^{-1}$; $K_M = 14 \pm 2 \mu$M; $k_{cat} = 838 \pm 21$ Min$^{-1}$). Conversely, recognition of NADH was much diminished in the mutated enzyme ($k_{cat}$/K_M $= 3 \mu$M$^{-1}$ Min$^{-1}$). BasAlaDH(D196A/L197R) was applied in a coupled oxidation/transamination reaction of the chiral dicyclic dialcohol isosorbide to its diamines, catalyzed by Ralstonia sp. alcohol dehydrogenase and Paracoccus denitrificans $\omega$-aminotransferase, thus allowing recycling of the two cosubstrates NADP$^+$ and L-Ala. An excellent cofactor regeneration with recycling factors of 33 for NADP$^+$ and 13 for L-Ala was observed with the engineered BasAlaDH in a small-scale biocatalysis experiment. This opens a biocatalytic route to novel building blocks for industrial high-performance polymers. © 2015 International Union of Biochemistry and Molecular Biology, Inc. Volume 63, Number 5, Pages 616–624, 2016

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1. Introduction

The L-alanine dehydrogenase (AlaDH) of Bacillus subtilis (EC 1.4.1.1; BasAlaDH) belongs to the family of amino acid dehydrogenases (AADHs) within the oxidoreductase superfamily [1, 2].

These enzymes reversibly catalyze the oxidative deamination of amino acids to their corresponding keto acids while liberating ammonia and reducing either NAD$^+$ or NADP$^+$. The enzyme subclassification of AADHs is based on the predominant amino acid substrate, such as glycine, serine, tryptophan, aspartate, and alanine. Currently, 23 different AADH classes are listed in the databases ENZYME (http://enzyme.expasy.org) [3] and BRENDA (http://www.brenda-enzymes.org) [4].

AADHs are physiologically important enzymes that act at the interface of nitrogen and carbon metabolism. Apart from pyridoxal phosphate (PLP)-dependent aminotransferases and deaminases, AADHs likewise catalyze the removal of the amino group from an amino acid and, thus, allow subsequent catalORIZATION of the carbon skeleton by glycolytic reactions or in the citric acid cycle. From an applied perspective, AADHs have the advantage of liberating the amino moiety as free ammonia or, in reverse direction, of directly incorporating ammonia into a keto acid. Furthermore, AADH link deamination/amination to the reduction/oxidation of a nucleotide cofactor, which enables coupling to a variety of energy consuming/generating processes [5].

AADHs have been studied extensively because of their ubiquitous distribution and also with regard to industrial use...
[6]. Reductive amination of keto acids was shown to be a convenient route to the synthesis of natural as well as unnatural amino acids. For example, 2-keto-6-hydroxy hexanoic acid was quantitatively converted into l-6-hydroxynorleucine at the preparative scale by beef liver glutamate DH using NADH cofactor recycling with D-glucose dehydrogenase from *Bacillus megaterium* [7, 8]. l-6-Hydroxynorleucine is a chiral intermediate useful for the synthesis of a vasopeptidase inhibitor [7]. Also, the NADH-dependent leucine DH from *Bacillus sphaericus* was employed for the amination of trimethylpyruvate to l-tert-leucine [9]. Other amino acids synthesized by the same method, such as neopentylglycine and 5,5-dimethyl-butylic, are building blocks for drugs including anti-tumor agents and HIV protease inhibitors [10].

For preparative purposes, the reduced cofactor NADH that is consumed during the reductive amination of keto acids can be regenerated via oxidation of ammonium formate to CO₂ using formate DH from *Candida boidinii* [6]. In this context, the reductive amination of 5-(1,3-dioxolan-2-yl)-2-oxopentanoic acid using phenylalanine DH from *Thermoactinomyces intermedius* with just catalytic amounts of NADH represents another example for the industrial application of AADHs [11]. The product (S)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid provides one of three chemical building blocks for the synthesis of the antihypertensive drug omapatrilat [8].

BasAlaDH, first described by Wiame and Pierard [12, 13], catalyzes the oxidative deamination of l-alanine to pyruvate and ammonia using NAD⁺ as exclusive hydride acceptor. The kinetic properties of this enzyme [11], including its reaction mechanism [14] and the limiting steps of catalysis [15], have been elucidated, whereas a crystal structure has not been reported to date. AlaDH follows an ordered catalytic mechanism in which NAD⁺ occupies the active site before the amino acid substrate is bound. Subsequently, ammonia and pyruvate are released, followed by liberation of NADH. The hydride transfer to NAD⁺ has been shown to occur at the prol(R) position of the nicotinamide ring, classifying BasAlaDH as a member of the A-stereospecific dehydrogenases [16].

Because of its pronounced substrate specificity, AlaDH can be applied only for the synthesis of l-alanine. An early example is the transformation of racemic lactate into l-alanine via the intermediate pyruvate, employing a combination of D- and l-lactate DHs with l-AlaDH as biocatalysts [17]. Another reaction cascade involving BasAlaDH was reported for the production of (S)-3-fluoroalanine, a potent antibiotic agent [18], via kinetic resolution of racemic 3-fluoroalanine by stereospecific oxidative deamination [19].

Recently, the concept of redox-neutral linear biocatalytic cascades has been extended beyond the use of nicotinamide-dependent enzymes. The ADH-catalyzed oxidation of primary alcohols was combined with the ωAT-catalyzed reductive amination of the intermediate aldehyde to form a primary amine [20]. Since the former reaction generates NADH from NAD⁺, whereas the latter converts the cosubstrate l-alanine into pyruvate, a third biocatalyst, BasAlaDH, was introduced [21] to recycle this cosubstrate and connect both transformations, also enabling the use of ammonia as nitrogen source. This strategy has been further applied to the amination of secondary alcohols, in particular the renewable platform chemical isosorbide (1,4:3,6-dianhydro-d-glucitol) [22, 23].

In this context, we found that the strictly NADP⁺-specific ADH from *Ralstonia* sp. (RasADH) is particularly suitable for the oxidation of the dicyclic chiral isosorbide substrate, and we determined the kcat and Km values for the forward and backward reactions [22, 24]. However, the NADP⁺ dependence of RasADH prevented efficient cofactor regeneration because of the NADH specificity of BasAlaDH. Therefore, we describe here an endeavor to alter the cofactor specificity of BasAlaDH from NADH to NADPH using bioinformatic analysis and rational protein engineering. The engineered enzyme is applied in a coupled reaction with two other biocatalysts to convert isosorbide into dicyclic diamines which constitute promising intermediates for high-performance polymers [22].

2. Materials and Methods

2.1. Bioinformatic analysis of BasAlaDH

Structural homologues of BasAlaDH were searched using the HHpred server (http://toolkit.tuebingen.mpg.de/hhpred) [25]. In this process, a global multiple sequence alignment was generated using HHblits [26] using three iterations with activated “secondary structure score”. The AlaDH from *Mycobacterium tuberculosis* (MtAlaDH) [27] appeared as the most similar homolog with known crystal structure (PDB ID: 2VHW) having 67% amino acid similarity and 51% identity. For comparison, BasAlaDH revealed 67% similarity (54% identity) to the functionally well-characterized AlaDH from *Shewanella* sp. (SheAlaDH) [28]. The crystal structure of holo-MtAlaDH was used as template for generating homology models of both BasAlaDH and SheAlaDH using the HHpred server. The resulting homology models were superimposed onto the crystal structure of MtAlaDH utilizing the function MatchMaker [29] of the Chimera modeling program (version 1.5.3) [30]. A structure-based sequence alignment between MtAlaDH, BasAlaDH, and SheAlaDH was generated with the MatchMaker function of Chimera and visualized with ALiNE [31].

2.2. Cloning of BasAlaDH

A synthetic codon-optimized gene encoding BasAlaDH was subcloned from pUC18-AlaDH (kindly provided by Evonik Industries, Marl, Germany) via PCR using the primers 5’-GCC ATC ATA GGG GTT CCT AAA GA-3’ and 5’-ACT GAT GAT GGT ACC TTA AGC ACC CGC CAC AGA-3’ onto pASK-IBA35(+) [32] using *Ehe* und *Kpn* restriction sites. The composition of the resulting expression plasmid was verified by analytical restriction digest and automated DNA sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Weiterstadt, Germany). The amino acid exchanges D196A, D196A/L197R, D196A/L197R/R201A, and D196A/L197R/N198S/R201A were introduced into BasAlaDH in a successive manner using the QuikChange
2.3. *Escherichia coli* expression and purification of BasAlaDH

The recombinant BasAlaDH equipped with an N-terminal His$_6$-tag [33] was produced as soluble protein in the cytoplasm of *E. coli* BL21 [34]. Shake flask cultures were grown at 30°C in 2 L LB medium [35] supplemented with 100 mg/L ampicillin. Gene expression was induced at OD$_{550}$ = 0.5 by adding 0.2 mg/L anhydrotetracycline [32]. After further shaking for 3 h, the cells were harvested by centrifugation, resuspended in 40 mM Hepes/NaOH pH 7.5, 0.5 M NaCl and disrupted using a French pressure cell (SLM Aminco, Urbana, IL, USA). Cell debris was removed by centrifugation, and the clear supernatant was applied to an IDA Sepharose column (GE Health-care, Munich, Germany) charged with ZnSO$_4$ [33]. The recombinant protein was eluted with a linear imidazole/HCl concentration gradient from 0 to 300 mM in the buffer from above.

Elution fractions were concentrated by ultrafiltration (Amicon Ultra-15 centrifugal filter units 30 kDa cut-off; Merck Millipore, Darmstadt, Germany) and subjected to size-exclusion chromatography (SEC) in the presence of 25 mM Hepes/NaOH pH 8.3 on a HiLoad 16/60 Superdex 200 column using an Akta purifier system (GE Healthcare). BasAlaDH eluted in a homogeneous peak with an apparent molecular size of approximately 90 kDa, corresponding to the homo-dimeric protein. The purified enzyme was analyzed by SDS-PAGE with Coomassie brilliant blue R-250 staining. Enzyme concentration was measured via absorption at 280 nm using an extinction coefficient of 21,890 M$^{-1}$ cm$^{-1}$ as calculated with the ExPASy ProtParam Tool [36].

2.4. Kinetic analysis of BasAlaDH and its variants

AlaDH enzyme activities were determined spectrophotometrically by measuring the substrate-dependent decrease in NADH or NADPH concentration using the following assay conditions: 5 mM pyruvate, NADH disodium salt or NADPH tetrasodium salt (Applichem, Darmstadt, Germany) at concentrations ranging from 10 to 500 µM, 200 mM NH$_4$Ac, 25 mM Hepes/NaOH pH 8.3, and 0.5 nM of the purified enzyme. Absorption at 340 nm was continuously measured at 30°C for 60 Min using a Synergy 2 microplate reader (BioTek Instruments, Bad Friedrichshall, Germany). $K_M$ and $k_{cat}$ values were determined from curve
2.5. Coupled reaction of BasAlaDH(D196A/L197R), RasADH, and PDωAT(L417M)

The triple biocatalytic reaction of isosorbide to its diamine diastereomers was performed with BasAlaDH(D196A/L197R) from this study together with RasADH and PDωAT(L417M), which were expressed and purified according to previously described procedures [22, 24]. The 250 µL assay solution contained 25 mM Hepes/NaOH pH 8.3 with 300 mM isosorbide (Evonik Industries) as substrate, 2 mM NADP⁺ sodium salt (Applichem) as hydride acceptor for the oxidation catalyzed by RasADH, 5 mM l-Ala and 0.3 mM PLP (Sigma, Munich, Germany) as (co-)substrates for PDωAT(L417M)-catalyzed transamination, as well as 100–300 mM NH₄Ac as ammonium donor for the regeneration of the l-Ala cofactor via the BasAlaDH variant. Enzymes were applied at the following concentrations: 132 µM RasADH, 40 µM PDωAT(L417M), and 24 µM BasAlaDH(D196A/L197R). After incubation at 30 °C for 96 h, the reaction was stopped and products were quantified by FMOCA/HPLC analytics of the mono- and diamino isosorbide derivatives as previously described [22].

3. Results and Discussion

3.1. Rationale for the alteration of BasAlaDH cofactor specificity

The availability of an NADPH-dependent AADH seems crucial for the efficient conversion of the dicyclic dialcohol isosorbide into the corresponding diamine(s) if employing the NADP⁺-dependent ADH from RasADH (RasADH) [24] in combination with the previously optimized ωAT from *Paracoccus denitrificans*, PDωAT(L417M)—which necessitates l-alanine as a recyclable cosubstrate [22]. In this multistep reaction (Scheme 1A), RasADH first oxidizes one hydroxyl group of isosorbide to a keto function by transferring a hydride ion (Scheme 1A), then the PLP-dependent transaminase catalyzes reductive amination to the aminoalcohol. After another cycle of oxidation and transamination, the final diamine product is obtained as a mixture of diastereomers (Scheme 1B).

Unfortunately, efforts to identify an NAD⁺-specific ADH that accepts the dicyclic secondary alcohol substrate with sufficient activity for industrial application have failed, whereas the NADP⁺-specific RasADH offers a suitable alcohol substrate spectrum [22]. Although an attempt to engineer RasADH for altered NADH versus NADPH specificity appeared in principle successful, this was accompanied by a significant loss in gross catalytic efficiency. Consequently, an NADPH-dependent AlaDH would enable recycling of both cosubstrates required by the optimal ADH and ωAT enzymes for this reaction. In such a setting, there is no net usage of redox equivalents and the overall reaction is only driven by ammonia (Scheme 1).

In this context, BasAlaDH offers a most promising starting point because it catalyzes the reductiveamination of pyruvate with an extraordinary turnover rate (*k*₅ₗ = 5.542 ± 237 Min⁻¹) and also possesses high affinity for this substrate (*K*ₐ₅ = 0.27 ± 0.04 mM), as previously described (albeit under reaction conditions slightly different from those applied in the present study) [22]. Furthermore, the recombinant enzyme can be produced in *E. coli* shake flask culture with a high yield of 25 mg/L as functional homo-dimeric protein.

To alter the cofactor specificity of BasAlaDH from NADH to NADPH, we first identified homologous enzymes with known three-dimensional structure using the HHpred server [25]. The closest relative, with 51 % amino acid sequence identity, was AlaDH from *M. tuberculosis* (MtAlaDH) [38, 39]. The crystal structures of both the apo-form and the NADH complex of this enzyme (PDB IDs: 2VHY and 2VHW) were determined to 2.3 and 2.0 Å resolution, respectively [27]. The quaternary structure of MtAlaDH in the crystal appears as a trimer of dimers: the core of the hexamer is formed by the six NADH-binding domains, whereas the substrate-binding domains are located at the apical positions.

We used the crystal structure of holo-MtAlaDH as template to generate a homology model of BasAlaDH and also of the related AlaDH from the antarctic bacterium *Shewanella* (SheAlaDH) [28], which is known to accept both NADH and NADPH. In fact, a change in NADPH/NADH cofactor specificity from 0.0004 to 13.8 was already described for this enzyme [40]. In this instance, better acceptance of NADPH was observed upon replacing residue Asp198 of SheAlaDH, which is conserved among various NADH-specific AADHs, by Ala.
Conversely, cofactor specificity was improved toward NADH by exchanging the neighboring residue Arg199 with Ile.

Based on both a structural superposition of the homology models of BasAlaDH and SheAlaDH on the crystal structure of MtAlaDH (Fig. 1), the corresponding multiple sequence alignment (Fig. 2) and in the light of the results from the previous mutagenesis studies on SheAlaDH, the following amino acid substitutions were successively introduced into the active site of BasAlaDH: D196A, D196A/L197R, D196A/L197R/R201A, and D196A/L197R/N198S/R201A.

The substitution D196A, which corresponds to position 198 in SheAlaDH, was sought to prevent hydrogen bonding with the 2'-hydroxyl and 3'-hydroxyl groups of the adenosine ribose of NADH and to create space for the phosphate group linked to the 2'-hydroxyl group in NADPH (Fig. 1). Furthermore, Leu197, equivalent to position 199 in SheAlaDH, was replaced by Arg to allow formation of a salt bridge to the phosphate group of NADPH. Apart from that, the residues Asn198 (Ser200 in SheAlaDH) and Arg201 (Ala203 in SheAlaDH) were expected to prevent optimal binding of NADPH by BasAlaDH due to hydrogen bonding with the 2'-hydroxyl group of the adenosine ribose of NADH; consequently, they were mutated to Ser and Ala, respectively, to match the corresponding amino acids present in SheAlaDH (Figs. 1 and 2).
TABLE 1  
K<sub>M</sub> and k<sub>cat</sub> values for BasAlaDH and its engineered variants toward NADH and NADPH

<table>
<thead>
<tr>
<th>Substrate investigated and enzyme variant</th>
<th>K&lt;sub&gt;M&lt;/sub&gt; (µM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (Min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;M&lt;/sub&gt; (µM&lt;sup&gt;-1&lt;/sup&gt; Min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wtBasAlaDH</td>
<td>14 ± 2</td>
<td>838 ± 21</td>
<td>59.9</td>
</tr>
<tr>
<td>D196A</td>
<td>429 ± 101</td>
<td>2,164 ± 285</td>
<td>5.0</td>
</tr>
<tr>
<td>D196A/L197R</td>
<td>887 ± 360</td>
<td>2,669 ± 719</td>
<td>3.0</td>
</tr>
<tr>
<td>D196A/L197R/R201A</td>
<td>n.s.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>D196A/L197R/N198S/R201A</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>NADPH:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wtBasAlaDH</td>
<td>448 ± 135</td>
<td>666 ± 114</td>
<td>1.5</td>
</tr>
<tr>
<td>D196A</td>
<td>390 ± 88</td>
<td>2,435 ± 296</td>
<td>6.2</td>
</tr>
<tr>
<td>D196A/L197R</td>
<td>32 ± 3</td>
<td>1,730 ± 39</td>
<td>54.1</td>
</tr>
<tr>
<td>D196A/L197R/R201A</td>
<td>219 ± 51</td>
<td>759 ± 77</td>
<td>3.5</td>
</tr>
<tr>
<td>D196A/L197R/N198S/R201A</td>
<td>119 ± 35</td>
<td>994 ± 100</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Assay conditions: 5 mM pyruvate, 200 mM NH₄Ac, 10–500 µM NAD(P)H, 0.5 nM enzyme in 25 mM Hepes/NaOH pH 8.3 at 30°C.

<sup>a</sup>Not saturated.

3.2. Preparation of the engineered BasAlaDH enzymes

BasAlaDH and its four variants described above were produced in E. coli at the shake flask scale, followed by two-step purification to homogeneity from the soluble whole cell extract via immobilized metal ion affinity chromatography (IMAC) and SEC. The enzyme parameters k<sub>cat</sub> and K<sub>M</sub> for the nicotinamide cosubstrates NADH and NADPH were determined by photometrically following their specific UV absorption at 340 nm upon conversion of pyruvate and NH₄Ac to l-alanine (Table 1).

As expected, the wild-type BasAlaDH showed pronounced specificity for NADH as cofactor. However, this NADH propensity was dramatically reduced as result of the single amino acid exchange D196A while the recognition of NADPH was improved, leading to similar k<sub>cat</sub>/K<sub>M</sub> values for both cosubstrates—albeit tenfold lower than for wtBasAlaDH. Thus, elimination of the Asp side chain that is highly conserved among NADH-specific AADHs [41–43] led to a 30-fold increase in the K<sub>M</sub> value for NADH while essentially maintaining the (still low) NADPH affinity. The measured kinetic values for the BasAlaDH variant D196A with NADPH as cofactor (K<sub>M</sub> = 390 µM, k<sub>cat</sub> = 2,435 Min<sup>-1</sup>) are almost identical to those published for the corresponding variant D198A of SheAlaDH (K<sub>M</sub> = 350 µM, k<sub>cat</sub> = 2,628 Min<sup>-1</sup>; [40]).

By introducing the additional mutation L197R into BasAlaDH, the ability to convert NADH was further decreased, with a twofold elevated K<sub>M</sub>, whereas the binding of NADPH was more than 10-fold improved. Notably, the wtSheAlaDH (as well as its D198A variant) carries already an Arg residue at the corresponding position 199 [40]. With k<sub>cat</sub>/K<sub>M</sub> = 54.1 µM<sup>-1</sup> Min<sup>-1</sup>, the catalytic efficiency of the BasAlaDH variant D196A/L197R for NADPH was almost identical to the one of the wild-type enzyme for NADH (59.9 µM<sup>-1</sup> Min<sup>-1</sup>).

Thus, we successfully altered the cofactor specificity of BasAlaDH from NADH to NADPH while fully maintaining the high catalytic efficiency of this enzyme. The effects of the two amino acid mutations were similar to those previously described for SheAlaDH. However, the latter has features typical of cold-adapted enzymes, and both the optimal temperature for catalytic activity and its thermostability are lower [28]. Notably, further improvement of NADPH acceptance by introducing the additional substitutions N198S and/or R201A into the BasAlaDH variant D196A/L197R could not be observed as the resulting substrate affinities and turnover numbers were clearly diminished (Table 1).

3.3. Isosorbide amination by coupled triple reaction with RasADH, PD<sub>ω</sub>AT(L417M), and the engineered BasAlaDH

The highly active NADPH-dependent BasAlaDH variant D196A/L197R was applied as regenerating enzyme in the context of the coupled oxidation/transamination reaction from isosorbide to its mono- and diamines to allow recycling of both cosubstrates, that is, NADP<sup>+</sup> and l-Ala (cf. Scheme 1).

Compared with the previously described reaction utilizing L. aquatica levodione reductase in combination with the engineered PD<sub>ω</sub>AT(L417M) [22], this new setup permitted the use of
The NADPH-dependent RasADH, which is much more active on the isosorbide dialcohol substrate, as explained further above. RasADH and PDωAT(L417M) were individually produced in *E. coli* at the shake flask scale and purified to homogeneity via IMAC and SEC as published before [22, 24].

All three purified enzymes were directly combined in the coupled reaction mixture with 300 mM isosorbide substrate and varied concentration of an ammonium donor, 100–300 mM NH_4Ac, as well as catalytic concentrations of NADP⁺ and L-Ala. The formation of intermediate monoamines as well as the diamine end products (four and three possible diastereomers, respectively, cf. Scheme 1B) after a reaction time of 96 H was analyzed via FMOC derivatization and quantitative HPLC separation on a Zorbax SB-C8 column (Fig. 3). Product peaks were observed at retention times of 14.472, 15.113, 15.720, and 16.580 Min, which could be assigned to the four possible stereoisomeric forms of the intermediate monoamines (aminoalcohols) on the basis of corresponding standards (cf. Figs. 3A and 3B). These peaks did not appear in the negative control reaction which had the analogous setup but lacking RasADH (cf. Fig. 3C). Smaller product peaks were also detected at later retention times of 37.479 and 38.135 Min. Using appropriate standards (see Fig. 3A), these signals could be unambiguously assigned to the isosorbide diamines 2,5-diamino-1,4:3,6-dianhydro-1-idit(ol) (DAI) and 2,5-diamino-1,4:3,6-dianhydro-2,5-didesoxy-D-sorbit(ol) (DAS).

Notably, the best NH_4Ac concentration in this setup was 100 mM, whereas higher concentrations led to lower conversion yields (Fig. 4). A similar observation was already described for the asymmetric amination of sec-alcohols to the corresponding α-chiral primary amines with BasAlaDH using ammonium.
FIG. 4  HPLC analysis after FMOC derivatization of monoamines (left) and diamines (right) formed during the amination of isosorbide via concerted biocatalysis by RasADH, PDω-AT(L417M), and BasAlaDH(D196A/L197R) at different concentrations of the ammonium donor.

chloride or ammonium formate as ammonium donor [23]. Possibly, high ammonia salt concentrations inhibit enzymatic activity as also reported for the AlaDH from soybean nodule bacteroids [44].

Formation of the aminoalcohol IV (cf. Scheme 1B) as main intermediate and of DAI as predominant diamine end product was detectable in concentrations of up to 52 mM and 25 µM, respectively (Fig. 4). These yields demonstrate that the regeneration of NADP⁺ and l-Ala cofactors with the engineered BasAlaDH(D196A/L197R) enzyme was quite effective, indicating recycling factors of 33 for NADP⁺ and of 13 for l-Ala. Otherwise, the combined yield of intermediate and end products would have been limited to around 2 mM by the available NADP⁺ in this reaction setup. Thus, in principle, the successful coupling of all three enzymatic reactions, that is, oxidation, transamination, and cofactor recycling (cf. Scheme 1A), was proven, resulting in respectable yields of the aminoalcohol intermediates (~22% yield in relation to the isosorbide substrate) and small, but clearly detectable amounts of the diamines DAI and DAS (LC–MS verified by Aqura, Marl, Germany) as desired end products.

With regard to the small yield of the diamine products, one should consider that, in order to convert isosorbide into a diamine, both types of enzymes in the applied coupled reaction, that is, dehydrogenase and transaminase, must convert at least two different substrates: isosorbide/aminoalcohol and hydroxyketone/aminoketone, respectively. However, it has to be assumed that the enzymes do not accept both substrates equally well. In the setup described here, the dehydrogenase and transaminase were chosen such that they optimally act on isosorbide as well as the corresponding hydroxyketone, respectively. Notably, for the following step in the reaction cascade (cf. Scheme 1A) a second set of enzymes would be desirable that are more specific for the aminoalcohol (possibly even accounting for diastereomers) and the aminoketone as substrates to eventually achieve higher overall efficiency. This problem was already extensively discussed in a preceding study [22]. The fact that we are able to produce detectable amounts of diamine, at all, means that we have now good starting chances to achieve this goal.

Interestingly, the predominant production of the intermediate aminoalcohol IV reveals that the hydroxyl group of the isosorbide substrate in R-configuration is the better substrate for RasADH compared with the other one in S-configuration. Whereas RasADH hardly shows stereochemical preference for alcohols with less-restricted configuration, for example, (R/S)-1-phenylethanol [24], this might be explained by the higher nucleophilic character of this hydroxyl substituent in isosorbide because of its intramolecular hydrogen bond to the ether O atom of the opposite tetrahydrofuran ring, which is in close spatial proximity [45, 46]. On the other hand, the formation of DAI as main end product (Fig. 4) is attributed to the stereoselectivity of PDω-AT(L417M) [22].

Taken together, with the help of the engineered BasAlaDH, we have provided the first proof of concept for the dual amination of the dicyclic, chiral dialcohol isosorbide in a triple coupled biocatalytic reaction including efficient cofactor recycling. Nevertheless, a couple of issues need to be addressed in order to improve the yield of the diamine end products and to make the enzymatic reaction competitive with chemical synthesis routes [47].

4. Conclusions

Our bioinformatic analysis of the biotechnologically relevant enzyme BasAlaDH has provided an explanation of its strict NADH dependence. Based on this insight, we successfully engineered a BasAlaDH variant showing catalytic efficiency for NADPH comparable to the one of the wild-type enzyme for NADH. This accomplishment proved to be crucial for the biocatalytic conversion of isosorbide to its diamines utilizing the NADPH-dependent RasADH [24] in combination with the engineered PDω-AT(L417M) [22]. The observed increase in overall yield in the present redox-neutral enzyme cascade is mainly attributable to the better affinity of RasADH (K_M = 72 mM) for the isosorbide substrate (as well as a higher turnover number by a factor 2) compared with levodione reductase (K_M = 4,266 mM), which had been employed for this reaction before [22].
The mutated BasAlaDH with its NADPH-adapted catalytic activity also shows potential for applications in other established amination reactions of primary or secondary alcohols, for example, 1-hexanol, benzyl alcohol, and 1-phenyl-propan-1-ol [23,48]. Generally, the availability of an NADPH-dependent version of BasAlaDH opens a greater choice in the coupling with suitable ADHs (and ATs) regarding substrate specificity for the initial alcohol oxidation step.

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