

The triple variant K170D/N174L/D239A compensates the destabilizing effect of variant K170D/N174L in β -hydroxyacid dehydrogenase (β HAD) from *Arabidopsis thaliana*

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1. Abstract

Chiral amines are essential building blocks in biologically active compounds, fine chemicals, agrochemicals and pharmaceuticals. In the last ten years, various enzymes were identified as new biocatalysts for chiral amine synthesis. Promising enzymes for the synthesis of primary, secondary, and tertiary amines are NADPH-dependent imine reductases (IREDs).^[1–5] Bioinformatics analysis revealed that IREDs are closely related to β -hydroxyacid dehydrogenases (β HADs).^[6] In recent work, we engineered the β HAD from *Arabidopsis thaliana* (β HAD_At) into imine-reducing enzymes by a single amino acid exchange.^[7] The exchange of the proton-donor described lysine (K170) in β HAD_At by aspartic acid, the most common amino acid at this position in *R*-selective IREDs, led to a 12-fold increase in activity for the model substrate 2-methylpyrroline. At the same time, the activity for the natural substrate glyoxylic acid is reduced 885-fold, resulting in a total of 8200-fold change in catalytic activity through the exchange of an amino acid. At the same time, highly decreased soluble expression has been observed by exchanging asparagine at position 174 (N174) with leucine. We thus hypothesized, that the aspartic acid residue (D239) in near proximity to N174 will stabilize the underlying α -helix. Consequently, replacement of D239 with alanine should result in soluble expression of variants containing the N174 mutations. We generated variants K170D/D239A, N174L/D239A and K170D/N174L/D239A, and tested them on imine reduction of test substrates 2-methylpyrroline, 3,4-dihydroisoquinoline and 6-phenyl-2,3,4,5-tetrahydropyridine. Due to loss of essential cofactor and precipitation of purified proteins during purification procedure, activities of variants were determined using cell lysates. Notably, variants N174L/D239A and K170D/N174L/D239A demonstrated soluble expression and imine-reducing activities of up to 98 mU per mg of variant.

2. Methods

Plasmid construction

Based on the protein sequence of the β -hydroxyacid dehydrogenase from *Arabidopsis thaliana* (β HAD-At), which is deposited as glyoxylate reductase with accession number NP_566768.1 in the Genbank database, a synthetic gene codon-optimized for expression in *Escherichia coli* (*E. coli*) was sourced from BioCat (Heidelberg, Germany). Gibson assembly was applied for the cloning of the expression constructs. Gibson assembly was applied for the cloning of the expression constructs. The synthetic DNA with the target genes (already containing homology regions to the pBAD33 plasmid) and the pBAD33 backbone were amplified by PCR. After purification using the DNA Clean & ConcentratorTM-5 kit from Zymo Research (Irvine, USA), the PCR products of vector backbone and target gene were mixed in a ratio of 1:3. Isothermal Gibson assembly was performed according to literature using 15 μ l Gibson-Mix (containing T5 exonuclease (10 U/ μ l), Phusion HF DNA polymerase (2 U/ μ l), Taq DNA ligase (40 U/ μ l) and ISO reaction buffer) and 5 μ l of the 1:3 ratio of DNA fragments for 1 h in a PCR machine at 50°C.^[8] After cooling to 8°C, 5 μ l of the assembly products were transformed in chemically competent *E. coli* DH5 α cells (competent cells were prepared according to the rubidium-chloride method^[9]). Cells were plated on LB agar with 34 μ g/ml chloramphenicol and grown overnight

at 37°C. The analyzed mutants were constructed by site-directed mutagenesis according to the QuikChange™ procedure of the commercially-available kit from Agilent.^[10] Therefore, the PfuUltra II Fusion HS polymerase with the corresponding buffer was used (Thermo Fisher Scientific, Schwerte, Germany). Appropriate primers were purchased from Metabion (Metabion GmbH, Planegg, Germany). All used primers are listed in Supplementary Table A.1. To confirm the correct plasmid assembly and amino acid substitutions, GATC Biotech (Konstanz, Germany) performed DNA sequencing.

Site-directed mutagenesis

The analyzed mutants were constructed by site-directed mutagenesis according to the QuikChange™ procedure of the commercial-available kit from Agilent. Therefore, the PfuUltra II Fusion HS polymerase with the corresponding buffer was used (Thermo Fisher Scientific, Schwerte, Germany). Appropriate primers were purchased from Metabion (Metabion GmbH, Planegg, Germany). All used primers are listed in Table S1. To confirm the correct plasmid assembly and amino acid substitutions, GATC Biotech (Konstanz, Germany) performed DNA sequencing using sequencing primer pTRCHis-RP and pTRCHis-RP.

Table S1. Site-directed mutagenesis primers for β HAD_{At}

Mutation	Forward primer [5' - 3']	Reverse primer [5' - 3']
K170D	GGCGCGAAGATGGATCTGATCGTTAA C	GTTAACGATCAGATCCATCTTCGC GCC
N174L	CTGATCGTTCTGATGATTATGGGTAGC ATGATG	CATCATGCTACCCATAATCATCAG AACGATCAG
D239A	GCACCAGCAAAAAGCGATGCGTCTGG CGCTGGC	GCCAGCGCCAGACGCATCGCTTTT TGCTGGTGC

Expression and cell lysis

The pBAD33-based expression constructs were transformed via heat shock (42°C, 30 sec) into chemically competent *E. coli* JW5510 cells (competent cells were prepared according to the rubidium-chloride method^[9]). Cells were plated on LB agar supplemented with 34 µg/ml chloramphenicol and were grown overnight. As preculture, 5 ml LB media with 34 µg/ml chloramphenicol was inoculated with a single colony and incubated at 37°C and 180 rpm overnight. Cultivation conditions in shake flask cultures were as follows: 200 ml TB medium (12 g/l tryptone, 24 g/l yeast extract, 5 g/l glycerol, 2.13 g/l KH₂PO₄, 12.54 g/l K₂HPO₄, pH 7.2) in 2-l baffled flasks supplemented with antibiotic (34 µg/ml chloramphenicol). The culture was inoculated with 1% (v/v) preculture (2 ml) and incubated on a rotary shaker (InforsHT, Bottmingen, Switzerland) at 37°C and 180 rpm. For inducible expression of the enzymes, cells were grown to an OD_{600 nm} of 0.6–0.8, followed by the induction with 0.03% v/v (1.33 mM) L-(+)-arabinose. Cells were incubated at 25°C and 150 rpm for 18 h and harvested by centrifugation at 4°C, 9800 × g for 30 min (centrifuge Avanti J 26 S XP, Beckmann Coulter, Krefeld, Germany). The cell pellet was suspended in 2 ml working buffer (50 mM sodium phosphate buffer pH 7.5) per gram cell pellet. Cell disruption was performed by high-pressure

homogenization (3 cycles, 750–1000 bar) with an EmulsiFlex C-5 (Avestin, Canada). The lysate was cleared by centrifugation for 30 min at 4°C and 8000 × g (centrifuge Avanti J-26 S XP, Beckmann Coulter, Krefeld, Germany). Validation of enzyme expression and solubility was done by SDS-PAGE using 12% gels (ExpressPlus™ PAGE, GenScript, Hölzel Diagnostika, Cologne, Germany).

Protein purification

The lysates were purified by His₆-tag affinity chromatography using His GraviTrap™ TALON® columns according to the instruction manual (GE Healthcare, Freiburg, Germany). After sample loading, the columns were washed with five column volumes of binding buffer (50 mM sodium phosphate buffer pH 7.5 containing 300 mM NaCl and 2% w/v L-sorbitol). Non-specifically bound proteins were eluted with five column volumes of 10 mM imidazole in the 50 mM sodium phosphate buffer pH 7.5 containing 300 mM NaCl and 2% w/v L-sorbitol. His-tagged β-HAD-At proteins were eluted with 300 mM imidazole in the 50 mM sodium phosphate buffer pH 7.5 containing 300 mM NaCl and 2% w/v L-sorbitol (two-column volumes). The eluted enzyme solution was desalted and concentrated in 50 mM sodium phosphate buffer pH 7.5 using ultrafiltration spin columns (Vivaspin, MWCO: 10 kDa, PES membrane, Sartorius, Göttingen, Germany). Analysis of enzyme purity was done by SDS-PAGE using 12% gels (ExpressPlus™ PAGE, GenScript, Hölzel Diagnostika, Cologne, Germany). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions (Randall and Lewis, 1951) with bovine serum albumin as protein standard in concentrations of 25–2000 µg/ml. The enzymes were stored at –80°C until further use.

NADPH-depletion assay determining activity using cell lysate

To check the activity of the generated βHAD variants for the imine substrates 2-methylpyrroline, 3,4-dihydroisoquinoline, and 6-phenyl-2,3,4,5-tetrahydropyridine, NADPH depletion assays were performed by monitoring the change in absorbance for NADPH at 340 nm. Activity for substrate 3,4-dihydroisoquinoline was monitored at 340 nm and 370 nm due to self-absorbance of this substrate at 340 nm. Reactions contained 17 mg/ml cell lysate, 300 µM nicotinamide cofactor, and 1 mM of the substrate (dissolved in H₂O) in 50 mM sodium phosphate buffer pH 7.5. Reactions were performed in a 200-µl scale in a 96-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany) at 30°C and started by the addition of the cofactor. One unit of enzyme activity was defined as the amount of protein that oxidizes 1 µmol NADPH per minute.

3. Results

Activity using purified variants

Purification of variants proved to be difficult because protein precipitation occurred during purification or re-buffering. In addition, colorless protein solutions were obtained during purification, indicating the loss of essential nicotinamide cofactor. Moreover, testing variants in the NADPH-depletion assay at 340 nm indicating the consumption of cofactor in the

reduction process of imine substrates demonstrated inactive variants. Optimization of purification procedure, along with the addition of stabilizing additives to enzyme solutions prior to purification, proved to be ineffective. Consequently, cell lysates of variants were utilized for activity testing.

Activity using cell lysates of variants

SDS-PAGE gel analysis of lysed variants showed different expression levels (Figure S1). While variant 1A (K170D/D239A) was overexpressed with high amounts of recombinant protein formed, 1.9-fold and 2.8-fold less soluble protein was detected with variants 2A (N174L/D239A) as well 4A (K170D/N174L/D239A), respectively.

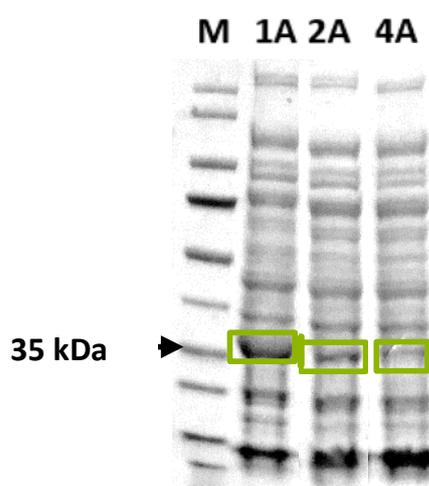
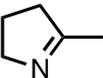
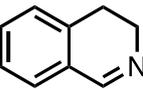
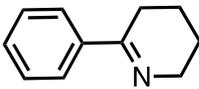


Figure S1. SDS-PAGE analysis of recombinant expression of variants with aminoterminal His₆ tag (35 kDa). M = marker

The imine-reducing activity of variants was determined spectrophotometrically by monitoring the decrease of the NADPH cofactor at 340 nm using cell lysate of variants. Three different imine substrates were used for spectrophotometric activity analysis. The results are shown in Table S2.

Table S2: Activities measured for asymmetric reduction of substrates 2-methylpyrroline, 3,4-dihydroisoquinoline and 6-phenyl-2,3,4,5-tetrahydropyridine at 340 nm.

Imine substrates	Activity of variants [mU/mg]		
	K170D/D239A ^a	N174L/D239A	K170D/N174L/D239A
 2-methylpyrroline	-	33 ± 1	97 ± 3
 3,4-dihydroisoquinoline	-	65 ± 5	93 ± 1
 6-phenyl-2,3,4,5-tetrahydropyridine	-	53 ± 1	98 ± 1

a) Soluble expression of double variant K170D/D239A was observed; however, no imine-reducing activity was monitored using this variant in the NADPH-depletion assay.

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