Two Enantiocomplementary Ephedrine Dehydrogenases from *Arthrobacter sp.* TS-15 with Broad Substrate Specificity

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ABSTRACT: The recently identified pseudoephedrine and ephedrine dehydrogenases from *Arthrobacter sp.* TS-15, PseDH and EDH, are NADH-dependent members of the oxidoreductase superfamily of short-chain dehydrogenases/reductases (SDRs). They are specific for the enantioselective oxidation of (+)-(*S*) *N*-(pseudo)ephedrine and (-)-(*R*) *N*-(pseudo)ephedrine, respectively. Anti-Prelog stereospecific PseDH and Prelog-specific EDH catalyse the regio- and enantiospecific reduction of 1-phenyl-1,2-propanedione to (*S*)-phenylacetylcarbinol and (*R*)-phenylacetylcarbinol with full conversion and enantiomeric excess of >99%. Moreover, they perform the reduction of a wide range of aryl aliphatic carbonyl compounds, including keto amines, ketoesters and haloketones, to the corresponding enantiopure alcohols. The highest stability of PseDH and EDH was determined to be at a pH range of 6.0-8.0 and 7.5-8.5, respectively. PseDH was more stable than EDH at 25 °C, with half-lives of 279 h and 38 h respectively. However, EDH is more stable at 40 °C with a two-fold greater half-life than at 25 °C. The crystal structure of the PseDH-NAD+ complex, refined to a resolution of 1.83 Å, revealed a tetrameric structure, which was confirmed by solution studies. A model of the active site in complex with NAD+ and 1-phenyl-1,2-propanedione suggested key roles for S143 and W152 in recognition of the substrate and positioning for the reduction reaction. The wide substrate spectrum of these dehydrogenases, combined with their regio- and enantioselectivity, suggests high potential for the industrial production of valuable chiral compounds.

**KEYWORDS:** *alcohol dehydrogenase, asymmetric hydrogen transfer, chiral carbonyl reduction, enantioselective alcohol synthesis, Phenylacetylcarbinol.*

**Introduction**

Chiral secondary alcohols are major synthons in the production of active pharmaceutical intermediates.1 A commonly practiced biotransformation for their synthesis under mild reaction conditions is the asymmetric transfer hydrogenation of prochiral ketones using NAD(P)H-dependent dehydrogenases,2-4 which exhibit high optical selectivity.5,6 Dehydrogenases are divided into three structural superfamilies: Short- (SDR), medium- (MDR) and long-chain (LDR) dehydrogenases/reductases.7 According to genomic analyses, about 25% of all dehydrogenases belong to the superfamily of SDRs.8 Their structural versatility offers a wide functional spectrum,9,10 which is exploited in several industrial applications. The NAD(P)H-dependent SDRs from *Lactobacillus brevis* (*Lb*ADH), *Lactobacillus kefir* DSM 20587 (*Lk*ADH), *Aromatoleum aromaticum* EbN1 (Phenylethanol dehydrogenase [PED]) and *Ralstonia sp.* DSM 6428 (RasADH), are well-known examples of biocatalysts used in the production of non-racemic aromatic alcohols such as (*R*)- and (*S*)-phenylethanol.11-14 The crystal structure of these four dehydrogenases have been determined.15-18 Although bioreductions of 1,2-diaryl-1,2-diketones have been reported using wild-type strains of yeasts such as *Pichia glucozyma*,19 , recombinantly expressed, isolated enzymes for the regio- and enantioselective reduction of aryl aliphatic 1,2‐diketones such as 1-phenyl-1,2-propanedione, to make (*S*)-phenylacetylcarbinol [(*S*)-PAC], are currently not available.

We have recently described the isolation of two enantiocomplementary dehydrogenases from *Arthrobacter sp.* TS-15 (DSM 32400).20 The enzymes, Pseudoephedrine dehydrogenase (PseDH) and Ephedrine dehydrogenase (EDH) catalyse asymmetric hydrogen transfer to 1-phenyl-1,2-propanedione (**Scheme 1**), producing (*S*)-PAC and (*R*)-PAC respectively.



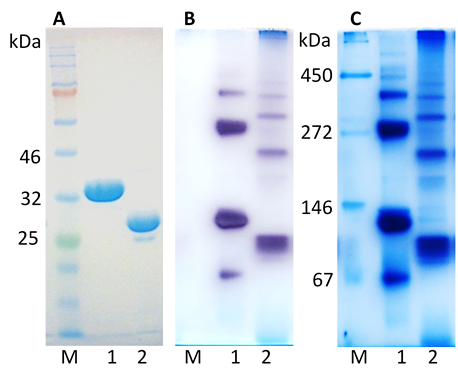
Scheme 1. Catalytic reduction of 1-phenyl-1,2-propanedione 1a.

The identification of such dehydrogenases is advantageous, as (*S*)-PAC can serve as a precursor in the synthesis of many pharmaceuticals, such as (+)-(*S*,*S*)-pseudoephedrine.

Here, we provide a detailed insight into both the functional and structural characteristics of PseDH and EDH, including definition of their wide and synthetically useful substrate spectra and stereoselectivity, studies on kinetics and stability, and an X-ray crystallographic structure of PseDH in complex with NAD+, refined to a resolution of 1.83Å.

**Results and Discussion**

**Molecular mass.** The calculated molecular masses based on the amino acid sequences of the native enzymes of a single subunit were 28.17 kDa and 25.93 kDa for PseDH and EDH, respectively (**Figure 1**). The relative molecular mass of the recombinant enzyme of a single subunit was estimated by SDS-PAGE to be about 33 kDa and 27 kDa for PseDH and EDH, respectively. According to the results of the Blue native PAGE, the smallest oligomeric state seemed to be a dimer and a tetramer form for PseDH and EDH, respectively. The predominant oligomeric states were determined to be the tetramer and octamer for PseDH and a tetramer in the case of EDH. In agreement with the results of the In-gel activity assay, the EDH tended to form higher oligomeric states, up to a hexadecamer. The largest band observed with PseDH had the size of a dodecamer, whereas the smallest active oligomer was a dimer.



**Figure 1:** SDS-PAGE and blue native PAGE of purified PseDH (1) and EDH (2) (both recombinant from *E. coli*) with the molecular marker (M). A) SDS-PAGE analysis of PseDH and EDH with a molecular mass of 33 kDa and 27 kDa, respectively. B) In-gel activity assay based on NBT activity staining before the addition of Coomassie blue. Both enzymes show different active high oligomeric states. C) Blue native PAGE of the enzymes after Coomassie blue staining to highlight all protein bands including the marker.

**Effect of temperature on the stability and activity.** The influence of the temperature on both dehydrogenases was studied spectrophotometrically at 340 nm based on the consumption of NADH in the reduction of 1-phenyl-1,2-propanedione. The increase of the temperature between 4 and 25 °C showed 4-fold higher initial activity of PseDH, whereas EDH demonstrated twice the activity at 25 °C comparing to the activity at 4 °C (**Figure 2**). PseDH and EDH reached their maximal initial activity at 35 and 70 °C, respectively. Interestingly, PseDH exhibited 1.5-fold higher activity in the range of 25 to 35 °C, whereas EDH demonstrated 14-fold higher initial activity with an increase of the temperature from 25 to 70 °C. These results suggest higher thermal stability for EDH in comparison to PseDH.



**Figure 2:** Temperature optima of PseDH and EDH measured spectrophotometrically at 340 nm by the reduction of 1-phenyl-1,2-propanedione, using NADH in potassium phosphate buffer at pH 7.5 (100 mM).

In order to investigate the thermal stability, the half-lives (*t*1/2) of the enzymes were determined in the temperature range between 5 and 60 °C (**Figure 3**). PseDH exhibited a reasonable stability in the range of 15 to 25 °C (*t*1/2 of 379.4 and 279.7 h, respectively). Rapid enzyme deactivation was observed at 40 °C (*t*1/2 of 0.78 h).

**Figure 3:** Storage stability of PseDH and EDH in the temperature range of 5 to 60 °C using 100 mM potassium phosphate buffer pH 7.5. The enzymatic activity was determined spectrophotometrically at 340 nm following the consumption of NADH and after the addition of the substrate 1-Phenyl-1,2-propanedione. All stability curves are presented in Supporting Information **Figure S1**.

The other dehydrogenase EDH demonstrated an unusual behaviour concerning its thermal stability with a short *t*1/2 of 2.6 h at 5 °C and a higher stability at 40 °C (*t*1/2 of 82.8 h). In order to verify these results, the experiments were carried out twice, using enzyme from different cultivations, in triplicate, and each gave the same results. The cold denatured protein of EDH generated insoluble aggregates, which might be explained by its tendency to form higher oligomeric states as described in the previous paragraph. The *t*1/2 of EDH at 25 °C was lower than of PseDH (half-life time 38.3 h).

**Effect of pH on the stability and activity.** Reductions with PseDH and EDH displayed pH-optima of 6.5 and 6.0, respectively. However, EDH exhibited a higher tolerance than PseDH at acidic pH values in the reduction reaction. In contrast, PseDH demonstrated a broader range of pH optimum than EDH in the oxidation direction. (**Figure 4**). The stability of PseDH and EDH regarding the pH of the buffer was studied in a range between pH 5.0 and 9.5 (**Figure 5**). Depending on the pH of the storage buffer PseDH was stable in a pH-range between 6.0 and 8.0 (*t*1/2113-193 h). Outside this pH-range the stability of PseDH dropped rapidly, to under 10 h. Compared to PseDH, the stability of EDH was shifted toward basic pH-values between 7.5 and 8.5 (*t*1/234-21 h). Outside this range the stability of EDH dropped quickly.



**Figure 4:** PseDH and EDH activity (SA) as a function of pH in reduction and oxidation reactions using the following buffer solutions at a concentration of 100 mM. Symbols: (●) citrate-phosphate buffer, (■): potassium phosphate buffer and (▲): glycine-NaOH buffer. The activities were followed using 1-phenyl-1,2-propanedione for the reduction assay and using (+)-(*S,S*)-Pseudoephedrine and (-)-(*R,S*)-Ephedrine as substrates for PseDH and EDH, respectively.

Based on the results illustrated in **Figure 5.** the optimal storage pH was 7.5, at which both dehydrogenases had the highest stability. Regarding the activity and the stability of both dehydrogenases, within a reasonable pH range they are useful for sequential redox-reaction cascades, which require different pH values appropriate to those of PseDH and EDH.

**Figure 5:** The stability of the PseDH and EDH based on different pH values between 5.0-9.5 at 25 °C employing 100 mM of following buffer systems. Symbols: (●): citrate-phosphate buffer, (■): potassium phosphate buffer and (▲): glycine-NaOH buffer. The enzymatic activity was determined spectrophotometrically using the reductive assay after adding the substrate 1-phenyl-1,2-propanedione. Deactivation curves are given in Supporting Information **Figure S2**.

**Substrate specificity and stereoselectivity of PseDH and EDH.** The cofactor preference for both PseDH and EDH was examined using the asymmetric reduction of aryl-aliphatic ketones and by the oxidation of isomers of ephedrine as model reactions both dehydrogenases displayed no apparent activity with NADP/(H) and full catalytic activity with NAD/(H). The preference for NADH makes the utilization of both dehydrogenases PseDH and EDH more attractive for industrial applications, since NADH is more stable and less expensive than NADPH under operational reaction conditions.21

The biotransformation of various aryl-aliphatic ketones was explored separately with both recombinant dehydrogenases in 100 mM phosphate buffer at pH 7.5 and 25 °C. As a model substrate we chose the aromatic α-diketone 1-phenyl-1,2-propanedione **1a**, due to its high reactivity and appropriate solubility in water. Furthermore, its stereoselectively reduced products are important building blocks in industrial applications. For example, (*S*)-PAC was obtained with up to *ee* 97% and 78% yield purity and with predominant impurities of benzaldehyde and the regioisomer of PAC, 2-hydroxypropiophenone (HPP), *via* an enzymatic C–C coupling of benzaldehyde and pyruvic acid using variants of pyruvate decarboxylase from *Acetobacter pasteurianus*.22,23

Hence, the results in the presented study indicated that both enzymes performed the reduction of aromatic ketones with a strict regioselectivity for the first carbonyl function on the α-position close to the aromatic ring. After full conversion, PseDH reduced **1a** to (*S*)-phenylacetylcarbinol [(*S*)-PAC] with excellent enantioselectivity (*ee* >99%), whereas (*R*)-phenylacetylcarbinol [(*R*)-PAC] resulted with high enantioselectivity (*ee* >99%) when EDH was employed (**Scheme 1**).

The reduction with EDH followed the Prelog rule, whereas PseDH exhibited a strict anti-Prelog enantioselectivity. The catalysed reduction of diketones using both enzymes was irreversible. Moreover, there was no further reduction to the diols. Both enzymes are active on broad range of different aryl-alkyl ketones e.g. haloketones, ketoamines, diketones and ketoesters. Furthermore, they accepted various several types of aryl group including phenyl-, pyridyl-, thienyl-, and furyl-rings. The calculated activity of the catalysed reduction of ketones **1a**-**26a** was determined by monitoring NADH depletion and is presented in **Table 1**. The resulting products were identified chromatographically via HPLC and GC (See **Figure S3** in Supporting Information). The explored ketones are representative of symmetric and asymmetric aryl-aryl diketones, asymmetric aryl-aryl ketones, and aryl-alkyl ketones. The results revealed that the presence of an aromatic ring is essential for the activity of both dehydrogenases. However, the smallest aryl-alkyl ketone acetophenone **26a** was not accepted as substrate with either enzymes. However, the presence of halogens in the halogenated acetophenones **15a** and **16a** resulted in activity with EDH. The longer propyl chain of **11a** resulted in a lower activity with EDH compared to the model substrate. Further substrates, such as aryl *β*-diketone **12a** and aryl ketonitrile **22a**, were reduced only by EDH.

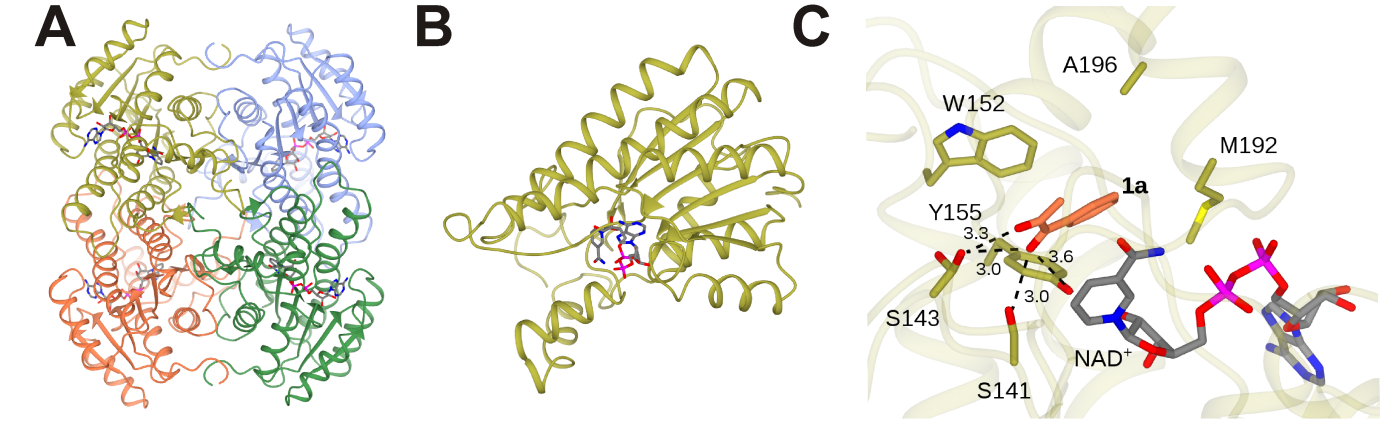
In the case of PseDH there was no activity with substrates **14a**, **15a**, **11a**, **12a** or **22a,** suggesting that the presence of a functional group on the alkyl chain, such as an amine, certain halogen, or ketone, is crucial for the activity of this enzyme. Thus, PseDH could reduce various halogenated substrates, **3a**, **4a**, **5a** and **16a**. In the case of double halogenated substrate **5a** PseDH reduced it with a 0.2-fold lower specific activity than for **1a**. The ketoamine **10a** was reduced to the corresponding (*S*)- and (*R*)-α-alcoholamines with 0.17-fold and 0.40-fold lower specific activity than the model substrate, using PseDH and EDH respectively.

Table 1: Substrate spectrum of PseDH and EDH for the reduction of several aromatic ketones with the determined specific activity (SA) and enantiomeric excess.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Substrate |  | PseDH | | EDH | |
|  |  |  | SA (Unit mg-1) | *ee* % | SA (Unit mg-1) | *ee* % |
| 1a | 1-Phenyl-1,2-propanedione |  | 55.47 | 99 (*S*) | 64.02 | 99 (*R*) |
| 2a | Methyl benzoylformate |  | 160.12 | 99 (*S*) | 46.56 | 98 (*R*) |
| 3a | Ethyl 2-chlorobenzoylacetate |  | 16.13 | n.d. | 35.21 | n.d. |
| 4a | 2-Chloro-1-phenyl-1-propanone |  | 5.41 | n.d. | 8.00 | n.d. |
| 5a | 2-Bromo-1-(3-chlorophenyl)-1-propanone |  | 12.58 | n.d. | 5.10 | n.d. |
| 6a | 1,2-Indandione |  | 9.52 | n.d. | 50.05 | n.d. |
| 7a | 1,2-Naphthoquinone |  | 21.96 | n.d. | 13.89 | n.d. |
| 8a | Isatin |  | 66.99 | 89 (*S*) | 7.84 | 52 (*R*) |
| 9a | 2-Aminoacetophenone |  | 9.49 | 99 (*S*) | 25.79 | 99 (*R*) |
| 10a | 4-Chlorobenzil |  | 5.26 | n.d. (*S*) | 6.50 | 95 (*R*) |
| 11a | 1-Phenyl-1-propanone |  | n.a. | n.a. | 1.55 | 99 (*R*) |
| 12a | 1-Phenyl-1,3-butanedione |  | n.a. | n.a. | 13.64 | 98 (*R*) |
| 13a | Phenylglyoxal |  | 5.51 | n.d. | 12.10 | 99 (*R*) |
| 14a | 3-Chloropropiophenone |  | n.a. | n.a. | 4.45 | 99 (*R*) |
| 15a | 2-Chloroacetophenone |  | n.a. | n.a. | 8.71 | 98 (*R*) |
| 16a | 2-Bromoacetophenone |  | 3.75 | 37 (*S*) | 9.44 | 85 (*R*) |
| 17a | Phenyl-2-pyridinylmethanone |  | 0.04 | 5 (*S*) | 2.81 | 24 (*R*) |
| 18a | (4-Chlorophenyl)-2-pyridinylmethanone |  | 0.20 | 60 (*R*) | 1.74 | 70 (*S*) |
| 19a | Benzil |  | 2.40 | 99 (*S*) | 47.06 | 99 (*R*) |
| 20a | 2,2'-Furil |  | 0.66 | 99 (*S*) | 19.95 | 99 (*R*) |
| 21a | 2,2'-Thenil |  | 0.58 | 99 (*S*) | 46.77 | 99 (*R*) |
| 22a | 2-Thenoylacetonitrile |  | n.a. | n.a. | 6.87 | 99 (*R*) |
| 23a | 4,4′-Difluorobenzil |  | 1.50 | 99 (*S*) | 48.20 | 99 (*R*) |
| 24a | 2,2′-Dichlorobenzil |  | 10.08 | 97 (*S*) | 27.91 | 94 (*R*) |
| 25a | 4,4′-Dimethylbenzil |  | 2.93 | 99 (*S*) | 3.12 | 99 (*R*) |
| 26a | Acetophenone |  | n.a. | n.a. | n.a. | n.a. |

Both PseDH and EDH reduced the aryl-alkyl *α*-ketoester **2a** with a higher rate than **1a**. Asymmetric aryl-aryl ketones **17a** and **18a**, and symmetric bulky-bulky α-diketones **19a**, **20a**, **21a**, **23a**, **24a** and **25a** could be also reduced by both dehydrogenases. Compared to other dehydrogenases, such as the NADPH-dependent RADH from *Ralstonia sp.*,24 PseDH is strictly regiospecific for the α-diketone and therefore there is no need to use the alpha-hydroxy ketone as a starting material to obtain the desired (*S*)-alcohol as the product. There is, to our knowledge, no other reported ADH with the activity described here, which transforms 1-phenyl-1,2-propanedione to (*S*)-PAC. While there is a considerable number of alcohol dehydrogenases for the reduction of prochiral ketones to secondary alcohols,25-28 they do not usually exhibit such diverse substrate spectra as those observed for PseDH and EDH. Those two novel NADH dependent carbonyl reductases with broad substrate specificity suggest that they have excellent potential for the production of key materials in the pharmaceutical industry, including (+)-Pseudoephedrine, Ezetimibe, Doluxitine and Merabegron.

Although oxidation of the hydroxyl group using either PseDH or EDH was not be detected for α-hydroxyketone [(*R*)-PAC], both enzymes could stereoselectively oxidise α- alcoholamines such as the isomers of ephedrine.

Moreover, the novelty of those two dehydrogenases is demonstrated through their strict regio- and enantioselectivity in the reduction of α-diketones such as **1a** to (*S*)-PAC, (*ee* >99%) and (*R*)-PAC (*ee* >99%) using PseDH and EDH, respectively. No isomers, such as 2-hydroxypropiophenone, or any diols were observed after full conversion. Aromatic *α*-ketoamines could also be reduced to the corresponding (*S*)- and (*R*)-alcoholamines with enantioselectivity of99% using PseDH and EDH, respectively (**Table 1**). In the case of *α*-haloketones, the enantioselectivity was more dependent on the nature of the halogen atom. Whereas the enantiomeric excess of 1-phenyl-2-chloroethanol was *ee* >99% for both enzymes, 1-phenyl-2-bromoethanol was produced with a lower enantioselectivity *ee* 37% and *ee* 85% with PseDH and EDH, respectively. According to this result there is presumably a steric or electronic hindrance with both enzymes using the larger halogen atom. A ketoreductase from *Bacillus sp.* ECU0013 also exhibited contrary behaviour toward chlorinated and brominated aryl ketones, resulting in higher enantiomeric excess with brominated ketones.29 The bulky-bulky symmetric *α*-diketones and *α*-ketoesters were reduced enantioselectively with *ee*>99% with both enzymes. The absence of a functional group on the alpha position to the carbonyl group did not affect the enantioselectivity with EDH, which was still high, with *ee*s of 99%, 98%, and 99% observed for (*S*)-1-phenyl-1-propanol, (*S*)-4-hydroxy-4-phenyl-2-butanone, and (*S*)-3-(2-thienyl)-3-hydroxypropanenitrile, respectively. By comparison, the ketoreductase KRED1-Pglu from *Pichia glucozyma* displayed lower selectivity than EDH for the reduction of *β*-ketonitrile **22a**.30 The reduction of the asymmetric bulky-bulky ketone **10a** revealed more details about the affinity toward halogenated aromatic rings. Based on the reference materials, EDH had a significant affinity for non-halogenated aromatic rings, whereas PseDH accepted both rings without significant preference. In the case of non-symmetrical aryl-aryl ketone **17a** both PseDH and EDH displayed higher affinity towards the benzene ring than the pyridine ring. The substitution of the benzene ring with a chloride atom inverted the substrate-protein interaction by both dehydrogenases resulting in a higher preference toward the pyridine ring. Thus, (*R*)-alcohol resulted from using PseDH (*ee* 60%) for the reduction of **18a**. In contrast, EDH reduced the same substrate **18a** to the (*S*)-alcohol with *ee* 70%, which was higher than that reported for a commercial ketoreductase from Merck Research Laboratories (*ee* 60%).31

The functional versatility and strict enantioselectivity of PseDH and EDH are noteworthy, since different sterically demanding ketones are reduced to the corresponding secondary alcohols with high regio- and enantiomeric excesses.

**Figure 6**. Structure of PseDH determined to a resolution of 1.83 Å. **A**: Tetrameric structure of PseDH, with monomers shown in ribbon format in gold, blue, coral and green; **B**: Structure of monomer of PseDH in complex with NAD+ in cylinder format with carbon atoms in grey; **C**: Structure of PseDH-NAD+ complex modelled with **1a** (carbon atoms in coral) in the active site. The -carbonyl group is shown making interactions with the side chains of S141, S143 and Y155 at distances of 3.0, 3.0 and 3.6 Å respectively. The -carbonyl group is shown interacting with the side chains of S143 at a distance of 3.3 Å. The side chain of S143 is in two conformations.

**Crystal structure of PseDH.** PseDH was crystallised in the presence of both NAD+ and substrate **1a**, and the structure was solved in complex with the cofactor using a monomer of a gluconate 5-dehydrogenase from *Thermotoga maritima* (PDB code 1VL8, 37% sequence ID) as the model. The crystals were in space group *C*2221 and there were six monomers, representing one-and-a-half tetramers, in the asymmetric unit. The tetramer is shown in **Figure 6A** and the monomer in **Figure 6B.** Analysis of the monomer structure using the DALI server32 suggested that the most similar structures were a short-chain dehydrogenase gox2181 from *Gluconobacter oxydans* (PDB code 3AWD;33; 34% sequence ID; rmsd 1.6 Å over 257 C-α atoms) and the human retinal dehydrogenase (PDB code 1YDE;34; 30% sequence ID rmsd 1.3 Å over 254 C-α atoms). Superimposition of the PseDH monomer with 3AWD revealed few significant differences in secondary or tertiary structure, save for an extended loop in the region A197 to S207. As expected, PseDH contained the typical catalytic tetrad of the superfamily SDR consisting of N113, S141, Y155 and K159 residues.35-37 K159 interacts with the nicotinamide ribose of the cofactor and decreases the *pK*a of the -OH group of Y155 initiating the hydrogen transfer. Y155 acts as catalytic base and S141 stabilizes the substrate. N113 interacts with K159 *via* a water molecule to create a network of hydrogen bonds between cofactor, catalytic side-chains and water molecules.38 Although peaks of electron density were observed in the difference map adjacent to the nicotinamide ring of NAD+, these were not sufficient for modelling the substrate **1a**. **1a** was therefore docked into the active centre of PseDH using Autodock Vina. The model shows the expected interactions between the α-carbonyl group to be reduced and the side chains of Y155, which is thought to be the proton donor to the nascent alkoxide in the reduction reactions, and to S141 which appear to anchor the carbonyl group in the correct orientation for reduction.35-38 The β-carbonyl group also makes an interaction with the side chain of S143 (**Figure 6C**).

The poor activity of PseDH with non-functionalised acetophenone derivatives such as 1-phenyl-1-propanone **11a** could be a consequence of the inhibited interaction between the β-substituent and the side chain of S143. **1a** is positioned within the active site such that delivery of the pro-(*S*) hydride from NADH will be to the (*re*)-face, resulting in the (*S*)-enantiomer of Phenylacetylcarbinol being formed as the product, as determined experimentally.

The side chain of W152 forms a stacking interaction with the aromatic ring of **1a**. Although the active site residues that determine enantioselectivity in PseDH and in EDH have not yet been determined, the amino acid sequence alignment of PseDH and in EDH shows that, while Y155 and S141 are conserved, as may be expected, and also S143 as well as W152 are substituted in the (*R*)-selective EDH by an alanine and a tyrosine residue respectively (**Figure S5, S6** inSupporting Information).

A comparison with 30 structures of SDRs from bacterial species in the PDB suggests that S143 is most commonly a small hydrophobic residue, such as G, A or V, whereas W152 is most commonly an H, M or L, although in SDRs from *Burkholderia vietnamiensis* (5IF3) and *Mycobacterium smegmatis* (3PK0) the residue is W.39 However, there are no reports of chiral ketone reductions by either of these enzymes that might confirm a W in this position as essential for (*S*)-PAC stereopreference.

Further comparison of both dehydrogenases with other well-studied anti-Prelog specific ADHs, for example*Lb*ADH and *Lk*ADH, as well as the Prelog specific ADHs, PED and RasARH, is provided in Supporting Information (**Table** **S1**, **S2**). Among these ADHs, the amino acid sequence of PseDH demonstrates the highest relevance to EDH and RasADH (Id 32% and 31.5%), respectively. EDH shares higher identity with PED (47%). Interestingly, S143 in PseDH is replaced with polar residue E in *Lb*ADH and *Lk*ADH. The other prelog specific ADHs share a hydrophobic residue A analogous to this position. Moreover, D149 is conserved among the anti-Prelog specific ADHs, whereas this position is filled with hydrophobic residues, such as I and L in the Prelog specific ADHs (**Figure S5**).

Table 2: Steady-state kinetic parameters of different substrates for PseDH and EDH. Non-linear regressions of the kinetic data are given in Supporting Information Figure S4.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Substrate | |  | PseDH |  |  | EDH |  |
|  | *Km*[mM] | | Ki [mM] | kcat [s-1] | Km [mM] | Ki [mM] | kcat [s-1] |
| 1a | 3.12±0.23 | |  | 34.24±1.02 | 0.06±0.01 |  | 30.43±1.18 |
| 2a | 7.01±0.23 | |  | 109.18±2.34 | 0.19±0.02 | 1.46±0.22 | 40.05±3.05 |
| 3a | 3.94±0.37 | |  | 9.89±0.39 | 0.06±0.01 | 0.16±0.04 | 37.38±6.73 |
| 4a | 8.74±0.66 | |  | 3.8±0.21 | 0.02±0.00 | 0.1±0.02 | 7.31±1.02 |
| 5a | 12.71±1.25 | | | 9.52±0.53 | 0.00±0.00 |  | 2.54±0.05 |
| 6a | 0.93±0.15 | |  | 6.55±0.43 | 2.29±0.05 |  | 31.11±2.66 |
| 7a | 0.05±0.01 | |  | 12.76±0.64 | 0.1±0.02 |  | 7.94±0.45 |
| 8a | 0.81±0.07 | |  | 43.95±1.43 | 0.51±0.07 |  | 4.36±0.22 |
| 9a | 0.14±0.01 | | 23.05±3.49 | 5.44±0.22 | 0.6±0.2 | 1.53±0.8 | 27.13±8.13 |
| 10a | 0.05±0.00 | |  | 3.75±0.22 | 0.17±0.01 |  | 3.48±0.11 |
| 11a |  | |  |  | 0.01±0.00 |  | 0.77±0.01 |
| 12a |  | |  |  | 0.2±0.17 | 0.26±0.27 | 15.37±11.74 |
| 13a |  | |  |  | 5.67±1.08 |  | 9.49±0.83 |
| NADH |  | |  |  | 0.07±0.01 |  | 15.74±0.67 |

**Kinetic parameters of PseDH and EDH.** As a basis for future reaction design, kinetic parameters for the target substrates **1a**-**13a** were determined (**Table 2**). The results of the kinetic studies demonstrate a dependence of Michaelis-Menten constants (*Vmax* and *Km*) on the steric and electronic properties of the tested substrates.

According to these data EDH mostly exhibited lower *K*m and *Vmax* values compared to PseDH. The *K*m values of the model substrate **1a** were 3.12 mM and 0.06 mM for PseDH and EDH, respectively, indicating weaker affinity of PseDH for this substrate. Furthermore, EDH exhibited substrate inhibition with some substrates, including **2a**, **3a**, **4a** and **9a**.

These substrate inhibitions were also observed during the oxidation of (-)-(*R,S*)-ephedrine and (-)-(*R,R*)-pseudoephedrine with EDH, and may have a significant physiological role in the feedback regulation by the redox-reactions in the metabolism of ephedrine (data not published yet). However, both enzymes exhibited high substrate affinity for the tested ketones, since the *K*m values were between 0.01 mM and 8.74 mM. The maximal reaction velocity was observed in reductions of the aryl α-ketoester **2a** with both enzymes.

**Conclusions**

The novel dehydrogenases PseDH and EDH are promising tools for green chemistry, as they permit for the first time the oxidation of the isomers of ephedrine and the regio- and enantioselective reduction of sterically demanding substrate **1a** to give the desired (*S*)-PAC and(*R*)-PAC. Both PseDH and EDH belong to the family of SDRs and use NADH as cofactor. Based on the original substrate ephedrine the substrate spectrum of these enzymes was extended to arylaliphatic haloketones, diketones, ketoesters, aryl-aryl ketones and bulky-bulky α-diketones. (**Scheme 2**). Their excellent stereoselectivity, coupled with their broad substrate specificity, makes them especially attractive biocatalysts for industrial applications.



Scheme 2: Asymmetric hydrogen transfer for a wide range of substrates.

Due to the observed inhibition by some substrates, a suitable reaction design should be carried out to achieve the full potential of the enzymes. Moreover, the determined enzymes’ stabilities are a good starting point for further stabilisation studies, crystal structure of PseDH also provides a valuable platform on which to base engineering studies directed towards the further broadening of substrate specificity and improving the stability of this enzyme.

**Material and Methods**

**Chemicals, media and bacterial strains.** Unless otherwise stated, all chemicals and oligonucleotides were purchased from Sigma-Aldrich Merck (St Louis, MO, USA). Molecular biotechnological tools and *E. coli* strains were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany). Cofactors and media components were purchased from Carl Roth GmbH & Co. (Karlsruhe,Germany).

**Steady-state kinetic assays.** Initial activities were determined photometrically at 340 nm for the reduction of ketones using a Cary 60 UV-Vis spectrophotometer (Agilent, Waldbronn, Germany) following the absorbance decrease of NADH. Kinetic parameters were measured at 25 °C in 1 mL of 100 mM potassium phosphate buffer pH 7.5. The reduction assay mixture contained 250 µM NADH, variable concentrations of candidate ketones and an appropriate concentration of the enzymes. Initial rate data for cofactor NADH could be determined only for EDH by fixing the concentration of the diketone 1-phenyl-1,2-propanedione (600 µM). Kinetic parameters (*Km* and *Vmax*) were calculated by fitting to experimental data using nonlinear regression in GraphPad Prism 8 (ε340 0.00622 L μmol−1 cm−1). All substrates were diluted in an appropriate amount of DMSO. The concentration of the purified enzymes was determined at 280 nm using a NanoDrop® ND-1000 (ThermoFisher Scientific, Bremen, Germany).

**Enzyme preparation.** The genes coding for PseDH and EDH were overexpressed in E. coli T7 Shuffle (DE3) after insertion into plasmid pET19b. Resulting enzymes were equipped with an N-terminal 10X histidine –tag.

**Gene overexpression and protein purification.** The main cultures of 500 mL TB medium containing 100 µg mL-1 ampicillin were inoculated with an overnight starter culture and incubated at 30 °C with shaking at 200 rpm, until an optical density of 0.8 (measured at 600 nm) was reached. Subsequently, the cultures were induced for gene overexpression with 100 µM and 30 µM Isopropyl-*β*-D-thiogalactoside (IPTG) for PseDH and EDH, respectively. After overnight overexpression at 20 °C with shaking at 180 rpm, the cells were harvested by centrifugation (4 °C, 8000 *x g*, 10 min) and stored at -20 °C. The cell pellets were resuspended with 20 mL of 25 mM potassium phosphate buffer pH 7.5 containing 500 mM NaCl and 50 mM imidazole.

The disruption of the cells was carried out on ice by ultrasonication for 3 x 1 min with 1 min breaks (Sartorius Labsonic M, Settings:Cycle 0.6, Amplitude 100%). After addition of 1 µg mL-1 DNAseI (AppliChem GmbH,Darmstadt, Germany) the crude extracts were centrifuged twice at 20,000 *x g* for 30 min at 4 °C.

The clear lysate was loaded onto a 5 mL Histrap FF column charged with nickel to purify the enzymes. The column was washed with 120 mM imidazole for 5 column volumes, after which the enzymes were eluted with a linear gradient up to 500 mM imidazole.

The elution buffer was then exchanged with storage buffer of 100 mM potassium phosphate buffer pH 7.5 using a desalting column (PD-10, Sephadex G-25 M, GE Healthcare).

**SDS- and blue Native-PAGE.** SDS-PAGE was performed by using the procedure of Laemmli40 with a 12% separation gel. The SDS-PAGE electrophoresis was carried out for 1 h at 25 °C and 180 V. Blue Native-PAGE was conducted according to a modified protocol of Schägger41 with a gradient gel (4-16%). The electrophoresis was performed for 4 h at 15 °C and 120 V. The loaded protein amounts on all gels were ca. 13 µg. The molecular masses of subunits and oligomers were determined by the Rf values driven from the marker proteins. The calibration curves were obtained using molecular mass marker ColorPlus™ Prestained Protein Ladder (New England Biolabs) and Serva native marker (SERVA Electrophoresis) for SDS-PAGE and Blue Native-PAGE, respectively.

**In-gel activity assay.** A modified protocol of Wittig42 was performed to show the active migrated bands of Native-PAGE. The gel was washed twice for 30 min at 25 °C with 50 mL of 100 mM phosphate buffer pH 7.5. For staining, the gel was incubated overnight at 25 °C in 50 mL 100 mM phosphate buffer pH 7.5 containing 2.5 mM NAD+, 0.5 mM (-)-ephedrine, 0.5 mM (+)-pseudoephedrine and 1.5 mM nitrotetrazolium blue chloride (NTB).

**Product analytics.** The extraction of phenylethanolamine was performed under basic conditions and with equal volumes of ethyl acetate. The resulting stereoisomers of phenylethanolamine were separated using a chiral column CycloSil-B 30 m length, ID 0.25 mm, film thickness 0.25 µm (Agilent Inc. USA) on a gas chromatography system (Shimadzu GC2010) with a flame ionisation detector and nitrogen as carrier gas, applying a temperature gradient for 15 min from 130-190 °C with a ramp of 4 °C min-1. Retention times for 2-aminoacetophenone, (*S*)-(+)- phenylethanolamine and (*R*)-(-)- phenylethanolamine were 11.5 min, 13.1 min, and 13.3 min, respectively.

All other products were extracted with n-hexane and analysed using HPLC (Knauer GmbH, Berlin, Germany) with a normal-phase CHIRALPAK IB-column 4.6×250 mm, particle size: 5 µm (Daicel Chemical Industries, Ltd). The mobile phase *n*-hexane:isopropanol was applied with appropriate ratios and a flow rate of 1 mL min-1. The absorbance of the products was detected at wavelengths between 220-270 nm. For further details about HPLC methods see **Table S3** in the Supporting Information. Chiral separations are demonstrated in **Figure S3A-AZ**. The absolute configurations of the products were determined via polarimeter model 343 (PerkinElmer, Inc., USA) and by analogy to other authentic standards of *S*- and *R*-Benzoin and -Furoin. In order to determine specific rotation values of the reduced products (4-chlorophenyl)-2-pyridinylmethanone and phenyl-2-pyridinylmethanone at 586 nm and 25 °C in dichloromethane at concentration of 10 mg mL-1. The specific values are presented in **Table S4** (Supporting Information).

The identification of the reduced products (*S*)-/(*R*)-phenylacetylcarbinol was performed using GC-MS after 40 mL reaction assay (Agilent 6890N GC / HP 5973N MSD equipped with an Optima 35 MS column). A constant temperature program at 130 °C for 30 min was applied. substrate and product peaks were detected at 9.89 min and 11.08 min, respectively. (**Figure S7, S8** Supporting Information).

1H-NMR spectroscopy (400 MHz, Bruker DRX-500) was used to analyse the reaction products (*S*)-phenylacetylcarbinol and (*R*)-phenylacetylcarbinol using chloroform-d (1H: δ7.26). Resulting spectra are provided in the Supporting Information (**Figure S9**, **S10**).

**Crystallisation of Pseudoephedrine dehydrogenase.** For the purposes of crystallisation, pure PseDH was generated in the following way: *E. coli* BL21 (DE3) cells were first transformed with the plasmid pET19b containing the PseDH gene. The cultures were spread-plated onto LB agar containing 30 mg mL-1 ampicillin and grown overnight at 37 °C. Single colonies were used to inoculate 10 mL starter cultures of LB medium containing 30 mg mL-1 ampicillin. These starter cultures were incubated at 37 °C overnight with shaking and each was then used to inoculate 500 mL cultures of LB medium containing 30 mg mL-1ampicillin, which were then incubated at 37 °C with shaking at 180 rpm until the optical density A600 reached 0.5. At this point gene expression was induced by the addition of 1 mM IPTG, after which the cultures were incubated at 16 °C overnight. Cells from 2 L combined culture were harvested by centrifugation at 4225 *x g* for 20 min using a Sorvall RC5B centrifuge and resuspended in 50 mL of 50 mM Tris buffer (pH 7.0), containing 100 mM NaCl, 20% (v/v) glycerol and 0.1% (v/v) Tween 20 with 20 mM imidazole (‘buffer’). The cells were disrupted using ultrasonication at an amplitude of 14,000 microns for 3 x 1 min with 1 min breaks at 4°C, after which the suspension was centrifuged at 26,892 *x g* for 30 min to yield a clear lysate. The lysate was loaded onto a 5 mL HisTrap™ FF column charged with nickel. PseDH was eluted using a gradient of 50 – 500 mM imidazole over 10 column volumes. Peak fractions containing PseDH, as determined by SDS-PAGE were pooled and concentrated to a volume of 2 mL. The concentrated protein was loaded onto a HiLoad 16/60 Superdex™ 75 Prep Grade gel filtration column and eluted using the buffer. Fractions containing PseDH were pooled, concentrated to 20 mg mL-1 and stored at 4 °C for crystallisation trials.

Pure PseDH, at a concentration of 2 mg mL-1, was subjected to crystallisation trials using a range of commercially available screens in 96-well plates using 300 nL drops with 150 nL protein solution and 150 nL of precipitant solution. The most promising hits were obtained in conditions containing 0.1 M Bis-tris buffer pH 5.5 and 25% (w/v) PEG 3350 and 200 mM magnesium chloride. Larger crystals were grown using the hanging-drop vapour diffusion method in 24-well plate Linbro dishes and using crystallisation drops of 1 µL enzyme solution (at a concentration of 20 mg mL-1) plus 1 µL mother liquor. Superior crystals were obtained in drops containing 0.1 M Bis-tris buffer pH 5.5 and 25% (w/v) PEG 3350 and 200 mM magnesium chloride with 5 mM NADH and in the presence of 5 mM **1a**. Crystals were flash-cooled in liquid nitrogen without the addition of a cryoprotectant, and tested for diffraction using a Rigaku Micromax-007HF fitted with Osmic multilayer optics and a MARRESEARCH MAR345 imaging plate detector. The crystals had the space group C2221. Crystals that displayed diffraction of better than 3 Å resolution were retained for data collection at the synchrotron.

**Data collection and processing, structure solution and refinement for PseDH.** A dataset for a crystal of PseDH in complex with NADH was collected on beamline I04 at the Diamond Light Source Synchrotron in Oxford, U.K. Data, which were collected to 1.83 Å, were processed and integrated using XDS43 and scaled using SCALA44 as part of the Xia2 processing system.45 Data collection statistics are given in **Table 3**. The structure was solved with the program MOLREP,46 using a monomer of the gluconate 5-dehydrogenase from *Thermotoga maritima* (PDB code 1VL8) as a model. The solution contained six molecules in the asymmetric unit, consisting of one-and-a-half tetramers. The structure was built and refined using iterative cycles within the programs COOT47 and REFMAC48 respectively. After building the protein and water molecules, clear density was observed for the cofactor NADH in five out of the six monomers. The structure was refined to Rcryst and Rfree values of 16.1 and 18.1% respectively, and was validated upon deposition within the Protein DataBank (PDB). Refinement statistics are presented in Table 1. The Ramachandran plot for the structure showed 96.6% of residues to be situated in preferred regions, 3.0% in allowed regions and 0.4 % outliers. Coordinates for the PseDH NADH complex structure have been deposited in the PDB with the accession code 6QHE.

Table 3. Data Collection and Refinement Statistics for PseDH. Numbers in brackets refer to data for highest resolution shells.

|  |  |
| --- | --- |
|  | PseDH |
| Beamline | Diamond I04 |
| Wavelength (Å) | 0.97950 |
| Resolution (Å) | 52.92-1.83 (1.86-1.83) |
| Space Group | *C*2221 |
| Unit cell (Å) | a = 80.73; b = 119.04;  c = 348.58   =  =  = 90.00° |
| No. of molecules in the asymmetric unit | 6 |
| Unique reflections | 147773 (7240) |
| Completeness (%) | 100.0 (100.0) |
| *R*merge (%) | 0.06 (0.75) |
| *R*p.i.m. | 0.04 (0.42) |
| Multiplicity | 8.1 (8.2) |
| <I/(I)> | 16.8 (2.6) |
| Overall *B* factor from Wilson plot (Å2) | 25 |
| CC1/2 | 1.00 (0.84) |
| Rcryst/ Rfree (%) | 16.2/18.3 |
| r.m.s.d 1-2 bonds (Å) | 0.013 |
| r.m.s.d 1-3 angles (o) | 1.60 |
| Avge main chain B (Å2) | 29 |
| Avge side chain B (Å2) | 34 |
| Avge water B (Å2) | 37 |
| NAD+ B (Å2) | 29 |

**Docking**

Automated docking was carried out using AUTODOCK VINA 1.1.2.49 The structure of PseDH was prepared using the AUTODOCK utility script. The coordinates for **1a** were prepared using ACEDRG within ccp4.50 A monomer model of PseDH was used, with the appropriate pdbqt file prepared in AUTODOCK Tools. The active site of PseDH was contained in a grid of 24 x 24 x 24 with 0.375Å spacing, centred around the NAD+ nicotinamide ring and was generated using AutoGrid in the AUTODOCK Tools interface. The number of runs for the genetic algorithm was set to 10 and other docking parameters were set to default values. Docking was performed by VINA, and so the posed dockings were below 2Å rmsd. The results were visualised in AUTODOCK Tools 1.5.6, where ligand conformations were ranked based on VINA energy, but also criteria established by the known mechanism of short-chain ADHs7 and also the experimentally-determined enantioselectivity of PseDH for substrate **1a**. These dictated that the only poses considered were those in which the carbonyl of **1a** interacted with the phenolic hydroxyl of the catalytic Y155 and the conserved active site serine S141, and in which the (*re*)-face of the carbonyl of **1a** was presented to the C4 atom of the nicotinamide ring of NAD+, and thus resulting in the experimentally observed (*S*)-PAC product.

ASSOCIATED CONTENT

**Supporting Information**. HPLC product detection list. HPLC, GC, NMR and GC-MS chromatograms, raw spectrophotometric data of stability, and kinetic parameters. Polarimetric data. Chart of ion effect. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes  
The authors declare no competing financial interest.

International patent application WO 2019/002459 A1 (published on 3rd January 2019) includes protection of enzymes EDH and PseDH for commercial exploitation.

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**Graphical abstract:**

