Supplementary material

Structural insights into alcohol dehydrogenases catalyzing asymmetric reductions

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Supplementary Figure 1. Amino acid sequence alignment of various SDRs. Amino acid full-length sequences of SDRs (PDB IDs : 1FJH; 1NXQ; 2Q2W; 3AFM; 3AK4; 3CTM; 3E9N; 3O03; 3PQD) were aligned using MAFFT online service (1). ESPript V3 (http://espript.ibcp.fr) was used to shade residues red or yellow that indicated the conserved amino acids (2). Numbering corresponds to 1FJH. Secondary structure elements of the 1FJH crystal structure are displayed. The NADP(H) binding motif (Thr-Gly-X-X-Gly-Gly) and an active site position (Tyr-X-X-X-Lys) are shown in the figure.
Supplementary Figure 2. Structural alignment of various SDRs. The backbone structures are shown in different colors with the PDB IDs (1FJH: cyan; 1NXQ: purple; 2Q2W: yellow; 3AFM: salmon; 3AK4: gray; 3CTM: slate; 3E9N: orange; 3O03: green; 3PQD: forest). Protein structures were retrieved from the RCSB PDB. PyMOL was used for 3D structural analysis and visualization.
Supplementary Figure 3. Amino acid sequence alignment of various MDRs. Amino acid full-length sequences of MDRs (PDB IDs: 1CDO; 1JVB; 1PED; 1RJW; 1YKF; 4W6Z; 3JV7; 3MEQ) were aligned using MAFFT online service (1). ESPript V3 (http://espript.ibcp.fr) was used to edit residues (2). Numbering corresponds to 1CDO. Identical and similar amino acids are highlighted in red and yellow, respectively. Secondary structure elements of the 1CDO crystal structure are displayed. The substrate-binding domain and the cofactor-binding domain are indicated by green and purple bars, respectively.
Supplementary Figure 4. Structural alignment of various MDRs. The backbone structures are shown in different colors with the PDB IDs (1CDO; 1JVB; 1PED; 1RJW; 1YKF; 4W6Z; 3JV7; 3MEQ). Protein structures were retrieved from the RCSB PDB. PyMOL was used for 3D structural analysis and visualization.
**Supplementary Figure 5.** (A) Reduction of ethyl 3,3-dimethyl-2-oxobutyrate by the carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR). (B) Functional sites involved in the formation of substrate-binding pocket of SSCR (PDB ID: 1Y1P) with the ligands including ethyl 3,3-dimethyl-2-oxobutyrate and the cofactor NADH. The carbonyl oxygen atom of a substrate forms hydrogen bonds with both Tyr177 and Ser133 residues, and it is protonated from the Tyr177 residue, followed by the attacking of a hydride from C4 atom of NADPH to the carbonyl carbon atom of the substrate. PyMOL was used for 3D structural analysis and visualization.
Supplementary Figure 6. (A) Reduction of 4-hydroxyacetophenone by the 1-(4-hydroxyphenyl)-ethanol dehydrogenase from strain EbN1 (HPED). (B) Functional sites involved in the formation of substrate-binding pocket of HPED (PDB ID: 2EWM) with the ligands including 4-hydroxyacetophenone and the cofactor NADH. PyMOL was used for 3D structural analysis and visualization.
Supplementary Figure 7. (A) Conversion of (6R)-2,2,6-trimethyl-1,4-cyclohexanedione to (4R)-hydroxy-(6R)-2,2,6-trimethylcyclohexanone by levodione reductase (LVR) from Corynebacterium aquaticum M-13. (B) Functional sites involved in the formation of substrate-binding pocket of LVR (PDB ID: 1IY8) with the ligands including (6R)-2,2,6-trimethyl-1,4-cyclohexanedione and the cofactor NADH. PyMOL was used for 3D structural analysis and visualization.
Supplementary Figure 8. Functional sites involved in cofactor binding of L-2,3-butanediol dehydrogenase (L-BDH, PDB ID: 3A28). Protein structure was retrieved from the RCSB PDB. The picture was performed with the LigPlot program.
Supplementary Figure 9. Functional sites involved in cofactor binding of the alcohol dehydrogenases from Clostridium beijerinckii (CBADH, PDB ID: 1KEV). Protein structure was retrieved from the RCSB PDB. The picture was performed with the LigPlot program.
**Supplementary Table 1** Industrial application example of asymmetric synthesis of important chiral pharmaceutical intermediates catalyzed by carbonyl reductase (3-7)

| Product                        | Catalyst                        | Yield  | e.e.%  | Scale  | Company          |
|--------------------------------|---------------------------------|--------|--------|--------|------------------|
|                                | KRED                            | >95%   | >99.9% | >200 kg| Merck            |
|                                | KRED/HHDH                        | n.d.   | >99.9% | n.d.   | Codexis          |
|                                | *Lactobacillus brevis* (cell extract) | 96%     | >99.8% | 400 kg | Wacker, Chemie   |
|                                | *Zygosaccharomyces rouxii*       | 96%     | >99.9% | 300 L  | Eli Lilly        |
|                                | *Neurospora crassa*              | >85%   | >98%   | Multi ton | AstraZeneca |
|                                | *Staphylococcus epidermidis*     | 91%     | >99.9% | n.d.   | Ciba            |
|                                | *Staphylococcus epidermidis* (isolated DHs) | 99%     | >99.0% | Multi kg | Pfizer          |
|                                | *Lactobacillus brevis* (cell extract) | 96%     | >99.8% | 35 ta\(^1\) | Wacker, Chemie |
|                                | *Geotrichum candidum*            | 95%     | >99%   | Multi kg | Bristol-Myers, Squibb |
|                                | *Candida sorbophilia*            | 82.5%   | >98%   | Multi kg | Merck           |
|                                | *Nocardia salmonicolor*          | 96%     | >99.8% | n.d.   | Bristol-Myers, Squibb |

n.d.: not disclosed.
| Organism                          | Enzyme name                                  | PDB ID   | Ligand *                               | Resolution (Å) | Classification | Stereo-configuration | Reference |
|----------------------------------|----------------------------------------------|----------|----------------------------------------|----------------|----------------|----------------------|-----------|
| *Agrobacterium tumefaciens*      | Quinuclidinone reductase                     | 3AK4     | NAD                                    | 2.00           | SDR            | —                    | Not published |
| *Bacillus subtilis*              | Lactate dehydrogenase                        | 3PQD     | NAD, FBP                               | 2.38           | SDR            | L-specific           | Not published |
| *Brucella suis*                  | Alcohol dehydrogenase                        | 3MEQ     | NAI, EDO, ZN                           | 2.00           | MDR            | —                    | Not published |
| *Candida parapsilosis*           | Carbonyl Reductase                           | 3CTM (Apo)| —                                      | 2.69           | SDR            | Anti-prelog          | 8         |
| *Clostridium beijerinckii*       | Alcohol dehydrogenase                        | 1PED     | NDP, ZN                                | 2.15           | MDR            | —                    | 9         |
| *Comamonas testosteroni*         | 3-α-Hydroxysteroid dehydrogenase             | 1FJH     | NAD                                    | 1.68           | SDR            | —                    | 10        |
| *Corynebacterium glutamicum*     | Putative short-chain dehydrogenase/Reductase | 3E9N (Apo)| —                                      | 2.40           | SDR            | —                    | Not published |
| *Equus caballus*                 | Horse liver alcohol dehydrogenase            | 1YE3     | MPD                                    | 1.59           | MDR            | (4S)-MPD             | 11-14     |
|                                  |                                              | 1HLD     | BRB, NAD, PFB, ZN                       | 2.10           |                |                      |           |
|                                  |                                              | 1CDO     | NAD, ZN                                | 2.05           |                |                      |           |
|                                  |                                              | 3BTO     | NAD, SSB, ZN                           | 1.66           |                | (1S,3S)-SSB          |           |
|                                  |                                              | 1LDE     | FPI, NAD, ZN                           | 2.50           |                |                      |           |
| Organism                        | Enzyme                                      | Chain ID | Coenzyme(s) | K_M (M) | Enzyme(s) | α (M) | β (M) | γ (M) | δ (M) |
|--------------------------------|---------------------------------------------|----------|-------------|---------|-----------|-------|-------|-------|-------|
| *Escherichia coli*             | 6-Phosphogluconate dehydrogenase           | 1BTO     | NAD, SSB, ZN| 2.00    | LDR       |       |       |       |       |
| *Geobacillus stearothermophilus*| Alcohol dehydrogenase                       | 1RJW     | ETF         | 2.35    | MDR       |       |       |       |       |
| *Haloferax mediterranei*       | Glucose dehydrogenase                      | 1RJW     | ETF         | 2.35    | MDR       |       |       |       |       |
| *Lactobacillus brevis*         | R-specific alcohol dehydrogenase           | 1NXQ, 1ZK4| MG         | 1.79    | SDR       | R-specific |       |       |       |
| *Pseudomonas fluorescens*      | Mannitol dehydrogenase                     | 1LJ8     | NAD         | 1.70    | LDR       | D-specific |       |       |       |
| *Pseudomonas putida*           | β-D-hydroxybutyrate dehydrogenase          | 1LJ8     | NAD         | 1.70    | LDR       | D-specific |       |       |       |
| *Ralstonia eutropha*           | 2-Dehydropanoate 2-reductase               | 3HWR     | BCN, MRD, NDP| 2.15   | MDR       | R-specific | Not published |
| *Rhodococcus ruber*            | Secondary alcohol dehydrogenase            | 3JVT     | ACY, MPD, NAD, ZN | 2.00 | MDR       | S-specific |       |       |       |
| *Rhodococcus sp.*              | L-phenylalanine dehydrogenase              | 2XAA, 1BW9, 1BXG | EDO, IPA, PPY, NAD | 2.80 | MDR       | L-specific |       |       |       |
| Organism                  | Enzyme Activity                      | Protein ID | Coenzymes | K_m (mM) | r (μM/min) |
|--------------------------|--------------------------------------|------------|-----------|----------|------------|
| **Saccharomyces cerevisiae** | Alcohol dehydrogenase I              | 1C1X, 1C1D | HFA, IPA, PH | 1.25     | 1.40       |
|                          |                                      | 2HCY       | E, NAD    |          |            |
| **Shewanella denitrificans** | Iron-containing alcohol dehydrogenase | 3RF7       | 8ID, ETF  | 2.44     | MDR ~      |
|                          |                                      |            |           |          |            |
| **Sphingomonas sp.**     | Carbonyl reductase                    | 3AFM, 3AFN | NAP, TBU  | 1.65     | SDR ~      |
|                          |                                      |            |           |          |            |
| **Sporobolomyces salmonicolor** | Aldehyde reductase II                | 1Y1P       | ACT, AMP, | 1.60     | SDR S-specific |
|                          |                                      | 1ZZE, 1UJM | NMN       | 1.80     |            |
|                          |                                      |            |           |          |            |
| **Streptococcus suis**  | Gluconate 5-dehydrogenase            | 3O03, 3CXR | GCO, NAP  | 1.90     | SDR ~      |
|                          |                                      |            |           |          |            |
| **Sulfolobus solfataricus** | NAD-dependent alcohol dehydrogenase | 1JV, 1NVG | NAP, ZN, ETX | 1.94     | MDR S-specific |
|                          |                                      | 1R37, 1NT |           |          |            |
|                          |                                      | 3H4C       |           |          |            |
|                          |                                      | 2NVB, 3FSR |           |          |            |
|                          |                                      | 3FPC, 3FTN |           |          |            |
| **Thermoanaerobacter brockii** | NADP-dependent alcohol dehydrogenase | 1YKF, 1BXZ | NAP, ZN, SBT, MG | 2.50     | MDR ~      |
|                          |                                      | 1YKF, 1BXZ | SBT, MG   | 2.99     |            |
|                          |                                      | 2NVB, 3FSR | NAP, ZN, EDO, ZN | 2.80     |            |
|                          |                                      | 3FPL, 3FPC | PGE, ZN, ZN, EDO | 1.90     |            |
|                          |                                      | 3FTN       | OXY, ACT  | 1.40     |            |
|                          |                                      |            |           |          |            |
| **Thermotoga maritima**  | L-lactate                            | 1A5Z       | FBP, NAD, | 2.10     | SDR L-specific |

- MDR: Maximum Daily Rate
- SDR: Specific Daily Rate
- S-specific: Specific for S-lactate
- L-specific: Specific for L-lactate
- Not published: Information not available in the published literature.
|                | dehydrogenase | OXM       |       |       |              |              |
|----------------|---------------|-----------|-------|-------|--------------|--------------|
| Zymomonas mobilis | Alcohol       | 3OWO      | FE2,  | 2.07  | MDR          | --           |
|                | dehydrogenase 2 | 3OX4      | NAD   | 2.00  |              |              |

* 6PG, 6-phosphogluconic acid; 8ID, nicotinamide-8-iodo-adenine-dinucleotide; AC0, 1-phenylethanone; ACT, acetate ion; ACY, acetic acid; AMP, adenosine monophosphate; ATR, 2′-monophosphoadenosine-5′-diphosphate; BCN, bicine; BGC, beta-D-glucose; BMD, butyramide; BRB, para-bromobenzyl alcohol; BU1, 1,4-butanediol; EDO, 1,2-ethanediol; EPE, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; ETF, trifluoroethanol; ETX, 2-ethoxyethanol; FBP, beta-fructose-1,6-diphosphate; FE, Fe (iii) ion; FPI, n-formylpiperidine; GCO, gluconic acid; GLO, D-glucose in linear form; HFA, alpha-hydroxy-beta-phenyl-propionic acid; IPA, isopropyl alcohol; LGC, (3S,4R,5S,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-one; MG, magnesium ion; MPD, (4S)-2-methyl-2,4-pentanediol; MRD, (4R)-2-methylpentane-2,4-diol; NAD, nicotinamide-adenine-dinucleotide; NAP, NADP nicotinamide-adenine-dinucleotide phosphate; NDP, NADPH dihydro-nicotinamide-adenine-dinucleotide phosphate; NI, nickel (ii) ion; NMN, beta-nicotinamide ribose monophosphate; OXM, oxamic acid; OXY, oxygen molecule; PEG, di(hydroxyethyl) ether; PFB, 2,3,4,5,6-pentafluorobenzyl alcohol; PHE, phenylalanine; PPY, 3-phenylpyruvic acid; SBT, 2-butanol; SSB, 3-butylthiolane 1-oxide; TBU, tertiary-butyl alcohol; ZN, zinc ion.
### Supplementary Table 3 Properties of various stereospecific alcohol dehydrogenases*

| Microorganism | Enzyme | Mr (kDa) | Oligomer | Cofactor | Optimum pH/T (ºC) | Substrate | Product config. | Reference |
|---------------|--------|----------|----------|----------|------------------|-----------|-----------------|-----------|
| **Ancylobacter aquaticus** | FDH | 90 | Dimer | NAD* | 6.3/50 | Aldehydes/carboxylic acids | — | 38 |
| **Candida macedoniensis** | MR | 45 | Monomer | NADPH | 6.5/40 | COBE | S | 39, 40 |
| **Candida magnoliae** | R | 33 | Monomer | NADPH | 7.0/40 | COBE/ketoesters/aldehydes | R | 41, 42 |
| | S1 | 77 | Dimer | | 5.5/55 | | | |
| | S4 | 86 | Trimer | | 6.0/50 | | | |
| **Candida parapsilosis** | RCR | 35 | Monomer | NADH | 6.0/45 | Secondary alcohols/ketones | R | 43 |
| **Candida parapsilosis** | CPADH | 30 | Monomer | NADPH | 4.5/35 | Ketones | S | 44 |
| **Candida parapsilosis** | SADH | 140 | Tetramer | NAD* | 6.0/— | BDO/secondary alcohols | R | 45 |
| | C1 | 38 | Monomer | NADPH | 7.5/50 | ketopantoyl lactone, conjugated polyketone | D | 46, 47 |
| | C2 | 36 | Monomer | NADPH | 7.0/40 | | | |
| **Corynebacterium sp.** | PAR | 155 | Tetramer | NADH | 6.0-6.5/— | 2-alkanones, aromatic ketones | S | 48 |
| **Lactobacillus kefir** | ADH | — | — | NADPH | 7.0/— | Acetophenone, ketones | R | 49 |
| **Nocardia fusca** | ADH | 150 | Tetramer | NAD* | 5.5-6.5/65 | PTO/secondary alcohols | R | 50 |
| **Penicillium citrinum** | AKR | 37 | Monomer | NADPH | 6.5/— | Aldehydes/ketones | S | 51 |
| **Pseudomonas fluorescens** | ADH | 32 | Monomer | NADPH | 8.0/20 | Alcohols | R | 52 |
| **Rhodococcus erythropolis** | ALDH | 162 | Trimer | NAD* | 9.5-10/47 | Aldehydes | — | 53 |
| **Rhodotorula glutinis** | CR | 40 | Monomer | NADPH | 5.6/40-50 | Ketones | R | 54 |
| **Sporobolomyces salmonicolor** | AR I | 37 | Monomer | NADPH | 7.0/60 | COBE, aldehyde, ketoesters | R | 55; |
| | AR II | 34 | Monomer | NADPH | 5.5/40 | | S | 56, 57 |
| | AR III | 37 | Monomer | —/— | | | R | |
| **Thiothrix sp.** | FDH | 90 | Dimer | NAD* | 5.6/58 | Aldehydes/carboxylic acids | — | 58 |
| Zygosaccharomyces rouxii | KR | 42 | Monomer | NADPH | 6.6-6.8/37 | MDA | S | 59 |
|-------------------------|----|----|---------|-------|------------|-----|---|----|

*a* FDH: formate dehydrogenase; MR, Menadione reductase; AR, aldehyde reductase; RCR, (R)-specific carbonyl reductases; CPADH, *Candida parapsilosis* alcohol dehydrogenase; SADH, secondary alcohol dehydrogenase; C1, conjugated polyketone reductase 1; C2, conjugated polyketone reductase 2; PAR, phenylacetaldehyde reductase; ADH: alcohol dehydrogenase; AKR: aldo-keto reductase; ALDH: aldehyde dehydrogenase; KR, ketone reductase; COBE: ethyl 4-chloro-3-oxobutyrate, MOB, Methyl 3-oxobutanoate; BDO, 1,3-butanediol; PTO, 3-pentyn-2-ol; MDA, 3,4-methylene-dioxyphenyl acetone.
**Supplementary Table 4** Organic solvent tolerance and thermostable ADHs

| Enzyme | Source                   | Cofactor | Additional information                                                                                                                                                                                                 | Reference |
|--------|--------------------------|----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| TBADH  | *Thermoanaerobacter* brockii | NADP(H)  | TBADH reversibly catalyzes the oxidation of secondary alcohols to the corresponding ketones. It exhibited good retention of activity in organic solvents: acetone was tolerated at up to 50% concentration and in two-phase systems with hexane and octane, up to 80% activity was conserved. The half-life of the activity is lost after 1 h of incubation is 93°C, and the melting temperature is 98°C. | 60, 61    |
| CBADH  | *Clostridium beijerinckii* | NADP(H)  | It is thermostable (half-life of 1 h at 63.8°C). It can reduce acetoin to (R,R)-2,3-butanediol (92 g/L, ee 90%, 56 h).                                                                                                      | 62        |
| TeSADH | *Thermoanaerobacter* ethanolicus | NADP+    | The wild-type enzyme is in general (S)-selective (except 2-butanone) and not active towards any aromatic compounds or more sterically demanding substrates. It retains 90%, 100%, 80% and 68% activity after a 3-h incubation in 100% n-dodecane, n-octane, toluene and pyridine, respectively. It is optimally active near 90°C, thermostable (half-life of 1.7 h at 90°C). | 63, 64    |
| TKADH  | *Thermococcus* kodakarensis KOD1 | NAD+     | The substrates are secondary alcohols and accepted various ketones and aldehydes. For example, TkADH could also reduce 2,2,2-trifluoroacetophenone to (R)-2,2,2-trifluoro-1-phenylethanol with high enantioselectivity (>99.6% ee). The enzyme showed high resistance to organic solvents and was particularly highly active in the presence of H2O–20% 2-propanol and H2O–50% n-hexane or n-octane. It was highly thermostable with an optimal temperature of 90°C and a half-life of 4.5 h at 95°C. | 65        |
| ADH-‘A’| *Rhodococcus ruber*       | NADH     |                                                                                                                                                                                                                       | 66, 67    |
It was applied to ketone–alcohol conversions in both the oxidative and reductive directions. High tolerance toward organic solvents, particularly acetone (up to 50%, v/v), 2-propanol (up to 80%, v/v), and hexane (up to 99%, v/v).

| Enzyme | Organism | Coenzyme |
|--------|----------|----------|
| PFADH  | *Pyrococcus furiosus* | NADH |
| Pcal_1311 | *Pyrobaculum calidifontis* | NAD+ |
| HvADH2 | *Haloferax volcanii* | NAD+ |
| ChnA   | *Azoarcus* sp. EbNl | NAD(P)+ |
| Ebn2   | *Azoarcus* sp. EbNl | NAD(P)+ |

It catalyzes the reduction of various ketones including aryl ketones, a- and b-ketoesters, which usually display not only an extreme stability at a high temperature (a half-life of 130 min at 100 °C) and high pressure, but also a high tolerance of chemical denaturants such as organic solvent.

Pcal_1311 catalyzed the NAD(H)-dependent oxidation of various alcohols and reduction of aldehydes, with a marked preference for substrates with functional group at the terminal carbon. Pcal_1311 was highly stable and retained more than 90% activity even after incubation of 180 min at 90 °C.

HvADH2 showed an unusually broad substrate specificity, with good activity with medium-chain alcohols, modest activity with secondary alcohols and also significant activity with benzyl alcohol. The HvADH2 showed remarkable stability and catalysed the reaction in aqueous–organic medium containing dimethyl sulfoxide (DMSO) and methanol (MeOH).

The alcohol dehydrogenases ChnA and Ebn2 accept various simple alcohols as reducing agents. They are oxidized to the corresponding carbonyl compounds. Simple alcohols which are suitable for regenerating NADH or NADPH are iso-propanol, butan-2-ol and pentan-2-ol.
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