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ORIGINAL PAPER

Biochemical characterization of a recombinant short-chain NAD(H)-dependent dehydrogenase/reductase from *Sulfolobus acidocaldarius*

Angela Pennacchio · Assunta Giordano · Biagio Pucci · Mosè Rossi · Carlo A. Raia

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Abstract The gene encoding a novel alcohol dehydrogenase that belongs to the short-chain dehydrogenases/reductases (SDRs) superfamily was identified in the aerobic thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* strain DSM 639. The *saadh* gene was heterologously overexpressed in *Escherichia coli*, and the protein (SaADH) was purified to homogeneity and characterized. SaADH is a tetrameric enzyme consisting of identical 28,978-Da subunits, each composed of 264 amino acids. The enzyme has remarkable thermophilicity and thermal stability, displaying activity at temperatures up to 75°C and a 30-min half-inactivation temperature of ~90°C, and shows good tolerance to common organic solvents. SaADH has a strict requirement for NAD(H) as the coenzyme, and displays a preference for the reduction of alicyclic, bicyclic and aromatic ketones and α-keto esters, but is poorly active on aliphatic, cyclic and aromatic alcohols, and shows no activity on aldehydes. The enzyme catalyses the reduction of α-methyl and α-ethyl benzoylformate, and methyl *o*-chlorobenzoylformate with 100% conversion to methyl (*S*)-mandelate [17% enantiomeric excess (ee)], ethyl (*R*)-mandelate (50% ee), and methyl (*R*)-*o*-chloromandelate (72% ee), respectively, with an efficient in situ NADH-recycling system which involves glucose and a thermophilic glucose dehydrogenase. This study provides further evidence supporting the critical role of the D37 residue in discriminating NAD(H) from NAD(P)H in members of the SDR superfamily.

Keywords Alcohol dehydrogenase · *Sulfolobus acidocaldarius* · Short-chain dehydrogenases/reductases · Archaea · Bioreduction

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A. Pennacchio · M. Rossi · C. A. Raia (✉)
Istituto di Biochimica delle Proteine, CNR, Via P. Castellino 111, 80131 Naples, Italy
e-mail: ca.raia@ibp.cnr.it

A. Giordano
Istituto di Chimica Biomolecolare, CNR, Via Campi Flegrei 34, 80078 Pozzuoli, Naples, Italy

B. Pucci
Centro Ricerche Oncologiche Mercogliano (CROM), Via A. Bianco, 83013 Mercogliano, Avellino, Italy

Introduction

Dehydrogenases/reductases are enzymes which are found throughout across a wide range of organisms, where they are involved in a broad spectrum of metabolic functions (Jörnvall 2008). A system of short-, medium- and long-chain dehydrogenase/reductases has been recently identified based on molecular size, sequence motifs, mechanistic features and structural comparisons (Kavanagh et al. 2008; Persson et al. 2009). Many studies have been addressed to characterize alcohol dehydrogenases (ADHs) from thermophiles and hyperthermophiles, mainly to understand their evolution and structure/function/stability relationship (Radianingtyas and Wright 2003) and develop their biotechnological potential mainly in the synthesis of chiral alcohols (Hummel 1999; Kroutil et al. 2004). Early applications in asymmetric reduction involved horse liver and yeast ADHs (Jones and Beck 1976), followed by *Thermoanaerobacter brockii* ADH (TbADH) (Keinan et al. 1986). Since then, new ADHs from various species of microorganisms displaying distinct substrate specificity, good efficiency and high enantioselectivity have been described.

Representative examples include the NADP-dependent (*R*)-specific ADH from *Lactobacillus brevis* (LB-RADH) (Schlieben et al. 2005), the NAD-dependent (*S*)-specific 1-phenylethanol dehydrogenase from the denitrifying bacterium strain EbN1 (PED) (Höffken et al. 2006), the NAD-dependent ADH from *Leifsonia* sp. strain S749 (LSADH) (Inoue et al. 2006), and the NADP-dependent carbonyl reductase from *Candida parapsilosis* (Nie et al. 2007). These enzymes originate from mesophilic microorganisms and belong to the short-chain dehydrogenases/reductases (SDRs) superfamily (Kavanagh et al. 2008) which is characterized by ~250 residue subunits, a Gly-motif in the coenzyme-binding regions, and a catalytic tetrad (Filling et al. 2002; Schlieben et al. 2005; Persson et al. 2009).

Representative examples of ADHs from thermophilic microorganisms are medium-chain enzymes, including TbADH (Korkhin et al. 1998), the ADH from *Bacillus stearothermophilus* (Ceccarelli et al. 2004) and two archaeal enzymes, the ADH from *Aeropyrum pernix* (Guy et al. 2003) and *Sulfolobus solfataricus* (Giordano et al. 2005). However, two archaeal short-chain ADHs have been identified in *Pyrococcus furiosus*, an NADP(H)-dependent SDR (AdhA) (van der Oost et al. 2001; Machielsen et al. 2008), and an NAD(H)-preferring ADH, that belongs to the aldo–keto reductase superfamily (Persson et al. 2009), recently characterized (Machielsen et al. 2006; Zhu et al. 2006, 2009). Furthermore, an NAD(H)-dependent ADH (TtADH) that belongs to the SDR superfamily, identified in *Thermus thermophilus* HB27, has been purified and characterized (Pennacchio et al. 2008).

In order to find a dehydrogenase/reductase that is both stable and NAD^+ dependent, we have focused our attention on the genomes of thermophilic organisms containing genes encoding putative ADHs belonging to the SDR superfamily, and applied the criteria used for TtADH to select enzymes with a high probability of being NAD^+-dependent (Pennacchio et al. 2008). An open reading frame coding for a protein belonging to the SDR superfamily with relatively high sequence identity to that of most representative ADHs (PED, LSADH and TtADH) was found in the genome of *Sulfolobus acidocaldarius*, an aerobic thermoacidophilic crenarchaeon which grows optimally at 80°C and pH 2 (Chen et al. 2005). Noteworthy, the amino acid sequence revealed the presence of an aspartate residue at position 42. This residue plays a critical role in determining the preference of SDRs for NAD(H) (Schlieben et al. 2005). Further evidence of this role is provided by LSADH (Inoue et al. 2006), PED (Höffken et al. 2006) and TtADH (Pennacchio et al. 2008), since these three SDRs are strictly NAD-dependent and have an aspartate residue at the same position within the sequence. Therefore, the putative dehydrogenase/reductase from *S. acidocaldarius* (SaADH) was expected to display a preference for NAD(H) rather than the more expensive NADP(H). This preference as well as an intrinsic thermostability and tolerance of organic solvents are the features that make an oxidoreductase more attractive from an application perspective (Hummel 1999; Kroutil et al. 2004; Zhu et al. 2006).

The current report describes the cloning and heterologous expression of the *S. acidocaldarius saadh* gene, which encodes the SDR SaADH. The purified and characterized enzyme was found to be a thermophilic and thermostable NAD(H)-dependent archaeal short-chain ADH tolerant to organic solvents and active on alicyclic alcohols, aromatic ketones, diketones, and α- and β-keto esters.

Materials and methods

Chemicals

$NAD(P)^+$ and NAD(P)H were obtained from AppliChem (Darmstadt, Germany). Alcohols, aldehydes, ketones and keto esters were obtained from Sigma-Aldrich. MES {2-(*N*-morpholino)ethanesulfonic acid} was obtained from Sigma Chemical Co. (St. Louis, MO). Aldolase was obtained from Amersham Biosciences. Dimethylsuberimidate (DMS) and dimethyladipimate (DMA) chemical cross-linkers were from Pierce. Methyl *o*-chlorobenzoylformate was a kind gift from Tadashi Ema. 1-Phenylethanol-d_9 [$C_6D_5CD(OH)CD_3$] was obtained from Ricci Chimica (Perugia, Italy). Other chemicals were A grade substances from AppliChem. Solutions of NADH and NAD^+ were prepared as previously reported (Raia et al. 2001). All solutions were made up with MilliQ water.

Amplification and cloning of the *saadh* gene

Chromosomal DNA was extracted by caesium chloride purification as described by Sambrook et al. (1989). Ethidium bromide and caesium chloride were removed by repeated extraction with isoamyl alcohol and extensive dialysis against 10 mM Tris–HCl pH 8.0, 1 mM EDTA, respectively. DNA concentration was determined spectrophotometrically at 260 nm, and the molecular weight was checked by electrophoresis on 0.8% agarose gel in 90 mM Tris–borate pH 8.0, 20 mM EDTA, using DNA molecular size markers. The *saadh* gene was amplified by polymerase chain reaction (PCR) using oligonucleotide primers based on the *saadh* sequence of *Sulfolobus acidocaldarius* strain DSM 639 (GenBank accession no. YP_256716.1). The following oligonucleotides were used: 5′-GGTTGG CATATGGACATTGATAGGCTCTTTTCAGTA-3′ as the forward primer (the *Nde*I restriction site is underlined in the sequence) and the oligonucleotide 5′-GGTTGG GAATTCCTACACCTTTGGGTCATATCTACCATCTA-3′

as the reverse primer. This latter oligonucleotide introduces a translational stop following the last codon of the ADH gene, followed by an *Eco*RI restriction site, which is underlined in the sequence shown. The PCR product was cloned into the expression vector pET29a (Novagen, Madison, WI, USA) and digested with the appropriate restriction enzymes to create pET29a-*sa*ADH. The insert was sequenced in order to verify that mutations had not been introduced during PCR.

Expression and purification of recombinant SaADH

Recombinant protein was expressed in *E. coli* BL21(DE3) cells (Novagen) transformed with the corresponding expression vector. Cultures were grown at 37°C in 2 l of LB medium containing 30 μg ml^{-1} kanamycin. When the A_{600} of the culture reached 1.4, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 1.0 mM. The bacterial culture was incubated at 37°C for a further 24 h. Cells were harvested by centrifugation, and the pellet was stored at −20°C until use. The cells obtained from 2 l of culture were suspended in 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and were lysed using a French pressure cell (Aminco Co., Silver Spring, MD) at 2,000 psi (1 psi = 6.9 kPa). The lysate was centrifuged, and the supernatant was incubated in the presence of DNase I (50 μg per ml of solution) and 5 mM $MgCl_2$ for 30 min at 37°C, followed by protamine sulphate (1 mg per ml of solution) at 4°C for 30 min. The nucleic acid fragments were removed by centrifugation, and the supernatant was incubated at 75°C for 15 min. The host protein precipitate was removed by centrifugation. The supernatant was dialysed overnight at 4°C against 20 mM Tris–HCl, pH 8.4 (buffer A) containing 1 mM PMSF. The dialysed solution was applied to a DEAE-Sepharose Fast Flow (1.6 × 12 cm) column equilibrated in buffer A. After washing with 1 bed volume of the same buffer, elution was performed with a linear gradient of 0–0.06 M NaCl (80 ml of each concentration) in buffer A, at a flow rate of 60 ml h^{-1}. The active pool was dialysed against buffer A, concentrated fivefold with a 30,000 MWCO centrifugal filter device (Millipore), and applied to a Sephadex G-75 (1.6 × 30 cm) column equilibrated in buffer A containing 0.15 M NaCl. The active pool was dialysed against buffer A and concentrated to obtain 2.5 mg protein ml^{-1} as described previously. SaADH was stored at −20°C, without loss of activity following several months of storage. SDS-PAGE and non-denaturing PAGE were carried out according to the Laemmli method (1970), with minor modifications (Raia et al. 2001). The subunit molecular mass value was determined by electrospray ionization mass spectrometry (ESI-MS) with a QSTAR Elite instrument (Applied Biosystems, USA).

The protein concentration was determined with a Bio-Rad protein assay kit using BSA as a standard.

Size-exclusion chromatography

Molecular masses were determined by size-exclusion chromatography using a Superdex 200 10/300 GL column (Amersham Biosciences), equilibrated with 50 mM Tris–HCl, pH 9.0, containing 0.15 M NaCl, at a flow rate of 0.5 ml min^{-1}. The following molecular mass standards were used for calibration: vitamin B_{12} (1,350 Da), horse myoglobin (17.5 kDa), chicken ovalbumin (44 kDa), beef γ-globulin (158 kDa), and thyroglobulin (670 kDa) from Bio-Rad. In order to calculate the distribution coefficient, the void and total volumes of the column were determined with blue dextran and tryptophan, respectively.

Chemical cross-linking

Cross-linking reactions of SaADH with DMA and DMS were conducted for 1 h at 25°C in a 25-μl volume of 0.1 M triethanolamine–HCl, pH 8.5 at 0.20 mg ml^{-1} protein, and 0.1 mg ml^{-1} bifunctional reagent. The molecular mass of cross-linked SaADH species was estimated by SDS-PAGE according to the procedure described by Davies and Stark (1970).

Enzyme assay

SaADH activity was assayed spectrophotometrically at 65°C by measuring the change in absorbance of NADH at 340 nm using a Cary 1E spectrophotometer equipped with a Peltier effect-controlled temperature cuvette holder. The standard assay for the reduction reaction was performed by adding 5–25 μg of the enzyme to 1 ml of preheated assay mixture containing 25 mM ethyl benzoylformate (EBF) and 0.3 mM NADH in 37.5 mM MES–NaOH, pH 5.5. The standard assay for the oxidation reaction was performed using a mixture containing 25 mM cycloheptanol and 5 mM NAD^+ in 25 mM sodium phosphate, pH 8.0. Screening of the substrates was performed using 1 ml of assay mixture containing either 10 mM alcohol and 5 mM NAD^+ in 25 mM sodium phosphate, pH 8.0, or 10 mM carbonyl compound and 0.1 mM NADH in 37.5 mM MES–NaOH, pH 5.5. One unit of SaADH represented 1 μmol of NADH produced or utilized per min at 65°C, on the basis of an absorption coefficient of 6.22 mM^{-1} cm^{-1} for NADH at 340 nm.

Effect of pH on activity

The optimum pH values for the reduction and oxidation reactions were determined at 65°C under the conditions

used for EBF and cycloheptanol, respectively, except that different buffer systems were used. The pH was controlled in each assay mixture at 65°C.

Kinetics

The SaADH kinetic parameters were calculated from measurements determined in duplicate or triplicate and by analysing the kinetic results using the program GraFit (Leatherbarrow 2004). The turnover value (k_{cat}) for SaADH was calculated on the basis of a molecular mass of 29 kDa, assuming that the four subunits are catalytically active.

Thermophilicity and thermal stability

SaADH was assayed in a temperature range of 30–95°C using standard assay conditions and 22 μg of protein ml^{-1} of assay mixture. The stability at various temperatures was studied by incubating 0.2–mg ml^{-1} protein samples in 50 mM Tris–HCl, pH 9.0 at temperatures between 25 and 95°C for 30 min. Each sample was then centrifuged at 5°C, and the residual activity was assayed as described above. Long-term stability was studied by incubating protein samples (0.2 mg ml^{-1}) in 50 mM Tris–HCl, pH 9.0, at 50 and 70°C, or in 50 mM Tris–H_3PO_4, pH 7.0 at 50 and 60°C and the residual activity was assayed after 6 and 24 h as described above.

The effect of chelating agents on enzyme stability was studied by measuring the activities before and after exhaustive dialysis of the enzyme against buffer A, containing 1 mM EDTA, and then against buffer A alone. An aliquot of the dialysed enzyme was then incubated at 70°C in the absence and presence of 1 mM EDTA or *o*-phenanthroline, and the activity was assayed at different times.

Effects of compounds on enzyme activity

The effects of salts, metal ions, and chelating agents on SaADH activity were investigated by assaying the enzyme in the presence of an appropriate amount of each compound in the standard assay mixture used for the oxidation reaction.

The effects of organic solvents were investigated by measuring the activity in enzyme samples (0.5 mg ml^{-1} in 100 mM MES, pH 6.0, 5 mM 2-mercaptoethanol) immediately after the addition of organic solvents at different concentrations, and 6 and 24 h of incubation at 50°C. The percentage activity for each sample was calculated by comparison with the value measured prior to incubation. The volume of the solution in a tightly capped test tube did not change during incubation.

Enantioselectivity

The enantioselectivity of SaADH was determined by examining the reduction of bicyclic ketones and α-keto esters using an NADH regeneration system consisting of glucose and glucose dehydrogenase from *Thermoplasma acidophilum* (TaGDH) (Sigma, St. Louis, MO). The reaction mixture contained 1 mM NAD^+, 5–10 mM carbonyl compound, 50 mM glucose, 125 μg SaADH, and 25 μg of TaGDH in 1 ml of 50 mM Tris–H_3PO_4, pH 7.0. The reactions were carried out at 50°C at different times in a temperature-controlled water bath. Upon termination of the reaction, the reaction mixture was extracted twice with ethyl acetate. The conversion yield and enantiomeric purity of the product were determined on the basis of the peak areas of ketone substrates and alcohol products by HPLC, on a Chiralcel OD-H column (Daicel Chemical Industries Ltd., Osaka, Japan). The absolute configuration of product alcohols was identified by comparing the chiral HPLC data with the standard samples. Products were analysed with isocratic elution, under the following conditions: hexane/2-propanol (9:1) (mobile phase), flow rate of 1 ml min^{-1}, detection at 210 nm for bioconversions of methyl and ethyl benzoylformate and for methyl *o*-chlorobenzoylformate; hexane/2-propanol (95:5), flow rate of 1 ml min^{-1}, detection at 217 nm for 1-indanone. Retention times were the following: 5.7, 8.1 and 13.4 min for methyl benzoylformate, methyl (*S*)-mandelate and methyl (*R*)-mandelate; 5.5, 7.4 and 12.1 min for ethyl benzoylformate, ethyl (*S*)-mandelate and ethyl (*R*)-mandelate; 6.9, 8.8 and 9.7 min for methyl *o*-chlorobenzoylformate, methyl (*R*)-*o*-chloromandelate and methyl (*S*)-*o*-chloromandelate; 8.8, 9.7, 10.8 min for 1-indanone and (*S*)- and (*R*)-1-indanol, respectively.

Coenzyme stereospecificity determination

The SaADH stereospecificity was investigated by NMR analysis of NADD obtained from the reaction of NAD^+ with 1-phenylethanol-d_9. The reaction mixture contained 2.0 mmol 1-phenylethanol-d_9, 30 μmol NAD^+, 26 units of SaADH and 0.25 mmol sodium phosphate buffer (pH 8.0) in a total volume of 5 ml. The reaction proceeded at 50°C while monitoring at 340 nm. When the A_{340} of the mixture reached a maximum (after 4 h), the solution was cooled and then applied to a DEAE-FF Sepharose column (1.6 × 8 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0). The column was washed enough with the same buffer and then NADD was eluted with 20 mM sodium phosphate buffer (pH 8.0) containing 20 mM NaCl. Fractions with value of A_{260}/A_{340} ratio $\leq$2.3 were collected and the pool (8.2 μmol of NADD) was lyophilized for ^{1}H-NMR analysis. ^{1}H-NMR spectra were

Table 1 Purification of recombinant SaADH

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	294	750	2.5	100	1
Thermal step	184	618	3.3	82.4	1.3
DEAE-FF	119	578	4.8	77.0	1.9
Gel filtration	68	452	6.6	60.3	2.6

The data are for a 2-l culture. Activities were measured at 65°C as described in "Materials and methods", using cycloheptanol as substrate

recorded on a Bruker cryoprobe^{TM} Avance DRX instrument operating at 600 MHz. Samples were dissolved in 0.5 ml of D_2O. Chemical shifts were given in ppm (δ) scale; the HDO signal was used as the internal standard (δ 4.68).

Results and discussion

Expression and protein purification

Analysis of the *Sulfolobus acidocaldarius* genome (Chen et al. 2005) for genes encoding short-chain ADHs resulted in identification of a putative oxidoreductase gene. The oxidoreductase protein, named SaADH, was expected to be NAD(H)-dependent on the basis of the presence of an aspartic residue at position 42, whose critical role in determining the preference for NAD(H) (Kallberg et al. 2002) has been shown through kinetic and structural studies of the LB-RADH G37D mutant (Schlieben et al. 2005), and recently supported by kinetic studies (Pennacchio et al. 2008) and structural characterization of TtADH (Asada et al. 2009).

SaADH is a 28,978-Da protein whose sequence shows the greatest identity to four typical SDRs, LB-RADH (30% identity), LSADH (29%), PED (28%), and TtADH (27%), as well as the archaeal AdhA (25% identity). The *saadh* gene was successfully expressed in *E. coli* cells, yielding an active enzyme accounting for more than 20% of the total protein content of the cell extract (Table 1). Host protein precipitation at 75°C was found to be the most effective purification step. An overall purification of 2.6-fold was achieved from crude, cell-free extracts with an overall yield of 60%. The enzyme was shown to be homogeneous by denaturing and non-denaturing PAGE (data not shown).

Protein separation by SDS-PAGE resulted in a single band corresponding to a molecular mass of ~30 kDa (see below). Gel filtration performed in 50 mM Tris–HCl buffer, pH 9.0 containing 150 mM NaCl yielded a profile consisting of a single peak corresponding to molecular mass ~65 kDa (data not shown). The quaternary structure

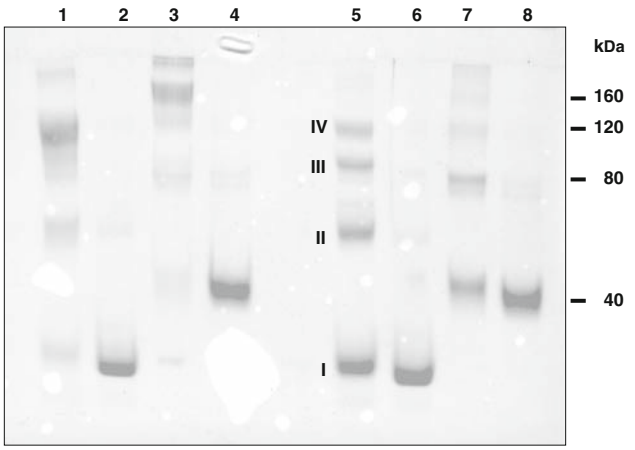

Fig. 1 SDS-PAGE analysis of cross-linked SaADH. *Lanes 1* and *5* DMS- and DMA-cross-linked and denatured enzyme, respectively. *Lanes 2* and *6* non-cross-linked denatured enzyme. *Lanes 3* and *7* DMS- and DMA-cross-linked and denatured aldolase, respectively. *Lanes 4* and *6* non-cross-linked denatured aldolase. The kDa values refer to Mr of monomer, dimer, trimer, and tetramer of cross-linked aldolase, used as molecular mass markers. *I*, *II*, *III*, and *IV* indicate the monomer, dimer, trimer, and tetramer of DMS-cross-linked SaADH. Cross-linking reaction and SDS-PAGE were carried out as described in "Materials and methods"

of SaADH was further investigated by chemical cross-linking using dimethyladipimate (DMA, N–N distance 8.6 Å) and dimethylsuberimidate (DMS, N–N distance 11.0 Å) as bifunctional reagents. SDS-PAGE of the DMA-cross-linked enzyme resulted in the appearance of four bands decreasing in intensity from monomer to tetramer (Fig. 1). However, the longer cross-linking reagent, DMS, resulted in a pattern predominantly consisting of cross-linked tetramer and smaller amounts of the other species (Fig. 1). These data suggest that SaADH has a tetrameric structure which adopts a more compact structure in the presence of salt, resulting in greater permeation into the packing pores of the gel matrix. However, it is not excluded that a tetramer to dimer dissociation occurs in the presence of relatively high salt concentration.

The molecular mass of the subunit determined by ESI-MS analysis proved to be 28,978.0 Da (average mass), in agreement with the theoretical value of the sequence.

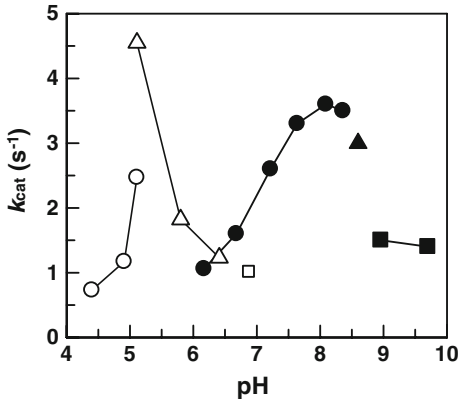

Fig. 2 SaADH activity as a function of pH in the reduction and oxidation reactions. The following 50 mM buffer solutions were used: sodium acetate (*open circles*), MES (*open triangles*), and sodium phosphate (*open square*) in the reduction reaction, and sodium phosphate (*filled circles*), Tris–HCl (*filled triangle*), and glycine–NaOH (*filled squares*) in the oxidation reaction. The mixture for the reduction reaction contained 25 mM methyl benzoylformate and 0.1 mM NADH, and the mixture for the oxidation reaction contained 25 mM cycloheptanol and 5 mM NAD^+. The assays were performed at 65°C under the conditions described in "Materials and methods"

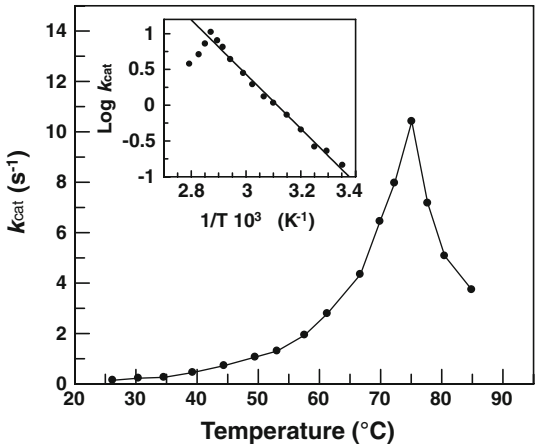

Fig. 3 Temperature dependence of the SaADH activity. The assay was carried out as described in "Materials and methods", using cycloheptanol as the substrate. The *inset* shows the Arrhenius plot from the same data. The value of the activation energy was 72.8 ± 2.0 kJ mol^{-1}

Optimal pH and thermophilicity

Figure 2 shows the pH dependence of SaADH in the reduction and oxidation reactions. The SaADH activity was found to be closely dependent on pH in the reduction reaction, displaying a narrow peak of maximum activity at approximately pH 5.1. The oxidation reaction showed a less marked dependence on pH, displaying a peak with a maximum at around pH 8.2.

The effect of temperature on SaADH activity is shown in Fig. 3. The reaction rate increases up to 75°C and then decreases rapidly due to thermal inactivation. This optimal temperature value is similar to that of TtADH (73°C) (Pennacchio et al. 2008), lower than that of thermophilic medium-chain, zinc-containing ADHs such as SsADH (88°C) (Raia et al. 2001) and TbADH (85°C) (Korkhin et al. 1998), and significantly lower than that of *Pyrococcus furiosus* aldo–keto reductase (100°C) (Machielsen et al. 2006). The activation energy for oxidation is 72.8 ± 2.0 kJ mol^{-1}, which is higher than that determined for TtADH (62.9 ± 2.6 kJ mol^{-1}) (Pennacchio et al. 2008) or SsADH (46.7 kJ mol^{-1}) (Raia et al. 2001), and lower than that reported for TbADH (94.1 kJ mol^{-1}) (Korkhin et al. 1998).

Coenzyme and substrate specificity

The enzyme showed no activity with NADP(H) and full activity with NAD(H). This coenzyme preference further supports the evidence that an aspartate residue at position 37 of an SDR enzyme (numbering of LB-RADH) plays a critical role in discriminating NAD(H) from NADP(H) (Filling et al. 2002; Kallberg et al. 2002, Pennacchio et al. 2008). The specificity of SaADH for various alcohols, aldehydes and ketones was examined (Table 2). The enzyme showed no activity on aliphatic linear and branched alcohols (not shown), except for a poor activity on 2-propyn-1-ol, 3-methyl-1-butanol and 2-pentanol; however, it showed a discrete activity on aliphatic cyclic and bicyclic alcohols. Benzyl alcohol and 4-bromobenzyl alcohol were not found to be substrates. In contrast, in view of the great influence that the strong electron-donating methoxy group exerts on the *para* position of the benzene ring, only the 4-methoxybenzyl alcohol was found to be an active substrate. (*S*)-1-Phenylethanol was also found to be an active substrate, whereas the *S* and *R* enantiomers of α-(trifluoromethyl)benzyl alcohol and methyl and ethyl mandelates showed no apparent activity with SaADH (data not shown). Moreover, the enzyme showed relatively poor activity on (±)-1-phenyl-1-propanol, 1-(1-naphthyl)ethanol and the two enantiomers of 1-(2-naphthyl)ethanol. However, SaADH showed highest activity with 1-indanol and α-tetralol (Table 2).

The enzyme was not active on aliphatic and aromatic aldehydes, and on aliphatic linear, branched and cyclic ketones (data not shown) except for 3-methylcyclohexanone (Table 2). However, the enzyme showed a relatively high reduction rate with isatin, 2,2,2-trifluoroacetophenone, and with an aryl diketone such as 1-phenyl-1,2-propanedione, and a bicyclic ketone such as 1-decalone (Table 2). The electronic factor accounts for the discrete activity measured with (*S*)-1-phenylethanol and 2,2,2-trifluoroacetophenone, as compared to the apparent catalytic inactivity observed with acetophenone and (*S*)-α-(trifluoromethyl)benzyl

Table 2 Substrate specificity of SaADH in the oxidation and reduction reactions

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Alcohols[a]		Ketones[b]	
2-Propyn-1-ol	6	Cyclohexanone	0
3-Methyl-1-butanol	4	2-Methylcyclohexanone	0
(±)-2-Pentanol	5	3-Methylcyclohexanone	29
(*S*)-2-Pentanol	11	4-Methylcyclohexanone	0
(*R*)-2-Pentanol	0	2,6-Dimethylcyclohexanone	0
Cyclopentanol	5	Cycloheptanone	0
Cyclohexanol	8	1-Decalone	14
Cycloheptanol	37	Acetophenone	0
Cyclohexylmethanol[c]	0	2,2,2-Trifluoroacetophenone	100
2-Cyclohexylethanol	0	1-Indanone	0
3-Methylcyclohexanol[c]	14	α-Tetralone	0
3-Cyclohexyl-1-propanol[c]	0	1-phenyl-1,2-propanedione	70
cis-Decahydro-1-naphthol	11	Isatin[d]	300
Benzyl alcohol	0	2-Acetonaphthone	0
2-Methoxybenzyl alcohol	0	Keto esters[b]	
3-Methoxybenzyl alcohol	0	Ethyl pyruvate	43
4-Methoxybenzyl alcohol	5	Methyl benzoylformate	100
(*R*,*S*)-1-Phenylethanol[c]	6	Methyl *o*-chlorobenzoylformate	160
(*S*)-(−)-1-Phenylethanol	8	Ethyl benzoylformate	88
(*R*)-(+)-1-Phenylethanol	0	Ethyl 4-chloroacetoacetate	27
(±)-1-Phenyl-1-propanol[c]	8		
1-(1-Naphthyl)ethanol	5		
(±)-1-(2-Naphthyl)ethanol	6		
(*R*)-1-(2-Naphthyl)ethanol	5		
(*S*)-1-(2-Naphthyl)ethanol	5		
(*R*)-3-Chloro-1-phenyl-1-propanol	5		
(*R*)-1-Indanol[e]	62		
(*S*)-1-Indanol[e]	100		
(*R*)-α-Tetralol[c]	58		
(*S*)-α-Tetralol[c]	12		

[a] The assays were performed using 1 ml of mixture containing 10 mM alcohol and 5 mM NAD^+ in 25 mM sodium phosphate, pH 8.0, at 65°C for 1 min. Relative rates were calculated by setting the activity to be 100 for (*S*)-1-indanol

[b] The assays were performed using 1 ml of mixture containing 10 mM carbonyl compound and 0.1 mM NADH in 37.5 mM MES–NaOH, pH 5.5, at 65°C for 1 min. Substrates were dissolved in 2-propanol and added to the reaction mixture (10 mM in 2% (v/v) 2-propanol). The percent values refer to 2,2,2-trifluoroacetophenone for ketones, and to methyl benzoylformate for ketoesters

[c] Substrates were dissolved in 100% 2-propanol

[d] Substrate concentration, 1 mM; solvent, acetonitrile; the enzyme was present at a sevenfold lower concentration

[e] Substrate was dissolved in 100% acetonitrile

alcohol. In fact, although the CF_3 group is sterically more demanding than the CH_3 group (van der Waals volume = 42.7 and 24.5 $Å^3$, respectively) (Zhao et al. 2003), the electron-withdrawing character of fluorine favours hydride transfer, inductively decreasing electron density at the acceptor carbon C1. It is noteworthy that SaADH proved to be very effective in reducing aliphatic and aryl α-keto esters, halogenated aryl α-keto esters, and halogenated β-keto esters (Table 2).

These data show that SaADH is a strictly NAD(H)-dependent oxidoreductase that has discrete substrate specificity. As such, SaADH is more similar to the thermophilic TtADH (Pennacchio et al. 2008), than to the three SDR superfamily mesophilic ADHs (i.e., LSADH, LB-RADH, and PED) which are active on a variety of aliphatic as well as aromatic alcohols, ketones, diketones and keto esters.

Kinetic studies

The kinetic parameters of SaADH determined for the most active substrates are shown in Table 3. Based on the specificity constant (k_{cat}/K_m), this enzyme shows a fivefold greater preference for (*R*)- than (*S*)-α-tetralol, but a similar preference for the enantiomers of 1-indanol. (*R*)- and (*S*)-1-(2-naphthyl)ethanol are very poor substrates and display similar specificity constants. Alicyclic alcohols such as 3-methylcyclohexanol, cycloheptanol and *cis*-decahydro-1-naphthol were oxidized with relatively low efficiency. In the reduction reaction, isatin was preferred more than

Table 3 Steady-state kinetic constants of SaADH

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
3-Methylcylohexanol	3.4 ± 0.2	9.9 ± 0.5	0.34
Cycloheptanol	4.4 ± 0.1	9.3 ± 0.3	0.47
(±)-1-Indanol	6.6 ± 0.2	2.30 ± 0.02	2.86
(*R*)-Indanol	7.1 ± 0.2	2.39 ± 0.01	2.98
(*S*)-Indanol	13.7 ± 0.4	6.4 ± 0.3	2.14
cis-Decahydro-1-naphthol	0.98 ± 0.04	4.2 ± 0.3	0.23
α-Tetralol	1.2 ± 0.1	0.48 ± 0.09	2.5
(*R*)-α-Tetralol	13.5 ± 0.5	1.28 ± 0.03	10.5
(*S*)-α-Tetralol	2.1 ± 0.2	1.05 ± 0.08	2.0
(±)-1-(2-Naphthyl)ethanol	0.43 ± 0.01	3.7 ± 0.4	0.11
(*R*)-(+)-1-(2-Naphthyl)ethanol	0.54 ± 0.06	5.3 ± 0.7	0.10
(*S*)-(−)-1-(2-Naphthyl)ethanol	0.48 ± 0.01	5.4 ± 0.5	0.09
NAD^+	3.7 ± 0.1	0.44 ± 0.04	8.4
3-Methylcyclohexanone	3.8 ± 0.03	66.0 ± 3.1	0.06
2,2,2-Trifluoroacetophenone	10.0 ± 0.6	23.5 ± 1.3	0.42
1-Phenyl-1,2-propanedione	3.1 ± 0.3	12.1 ± 2.2	0.25
Methyl benzoylformate	6.2 ± 0.21	6.5 ± 0.4	0.95
Methyl *o*-chlorobenzoylformate	9.2 ± 0.9	2.8 ± 0.2	3.3
Ethyl benzoylformate	2.35 ± 0.07	5.3 ± 0.4	0.44
Isatin	22.0 ± 2.5	0.71 ± 0.11	31
NADH	28.0 ± 2.5	0.16 ± 0.02	175

The activity was measured at 65°C as described in "Materials and methods". Kinetic constants for NAD^+ and NADH were determined with 25 mM cycloheptanol and 5 mM isatin, respectively. The k_{cat} and K_m data are the mean ± standard deviations

methyl and ethyl benzoylformate, and the halogenated acetophenones as well as the aryl diketone 1-phenyl-1,2-propanedione. It is noteworthy that a chlorine substituent at position 2 of the phenyl ring of methyl benzoylformate enhances the reaction rate to 150%, due to the electron-withdrawing character of halogen; it also increases the affinity of the substrate, reflecting the hydrophobic character of the environment surrounding the phenyl group. Moreover, the specificity constant value is 21-fold higher for NADH than NAD^+.

These results suggest that the physiological direction of the catalytic reaction is reduction rather than oxidation. However, the natural substrate and function of SaADH are unknown. As far as the substrate stereospecificity of SaADH is concerned the enzyme does not seem discriminate between the enantiomer *S* and *R* of 1-indanol, α-tetralol and 1-(2-naphthyl)ethanol, based on the similarity in the K_m values.

Coenzyme stereospecificity

The ^1H NMR spectrum of the deuterium-labelled NADD obtained by the reaction of SaADH with 1-phenylethanol-d_9 and NAD^+ shows a single peak at 2.68 ppm corresponding to the *pro-R* hydrogen at the C-4 position of NADD in place of a double doublet centered at 2.62 ppm present in the NADH spectrum (data not shown). The presence of a *pro-S* deuterium atom at C-4 of the pyridine ring of the NADD produced indicates that SaADH transfers the deuteride to the *Si*-face of NAD^+, as illustrated in Fig. 4. Therefore, SaADH is a B-type (*pro-S* specific) dehydrogenase in agreement with the classification proposed by Schneider-Bernlöhr et al. (1986) that the SDRs enzymes are *pro-S*, whereas the medium-chain ADHs are *pro-R*, recently substantiated by the structural studies of Kwiecień et al. (2009). SaADH shares the same coenzyme stereospecificity with few other short-chain ADHs such as the thermophilic *T. thermophilus* ADH and the mesophilic enzymes *Leifsonia* sp. strain S749 ADH, 1-phenylethanol dehydrogenase from the denitrifying bacterium strain EbN1, and *Mucor javanicus* and *Drosophila* ADH (Pennacchio et al. 2009 and references therein).

Thermal stability

The thermal stability of SaADH was determined by measuring the residual enzymatic activity after 30 min of incubation over a temperature range from 25 to 95°C (Fig. 5). SaADH was shown to be quite stable up to a temperature of 80°C, above which its activity decreased abruptly, resulting in a $T_{1/2}$ value (the temperature of 50% inactivation) of ~88°C.

The residual activity measured after 24 h incubation in 50 mM Tris–HCl, pH 9.0, at 50 and 70°C was 109 and 76%, respectively. In 50 mM Tris–H_3PO_4, pH 7.0, i.e., the buffer used for the bioconversion studies, the residual

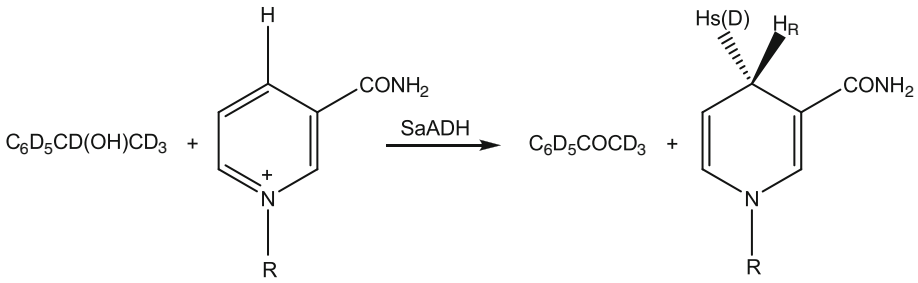

Fig. 4 Stereospecificity of hydrogen transfer catalysed by SaADH. *R* adenosine diphosphate ribose

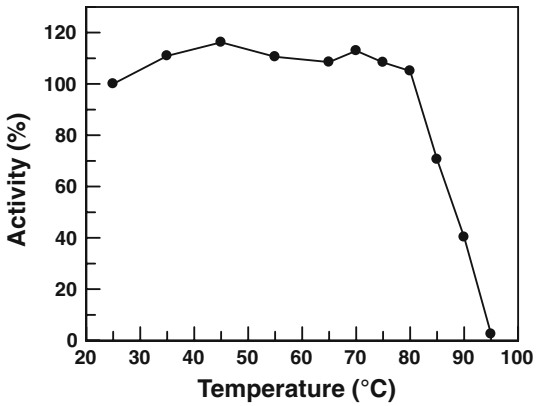

Fig. 5 Thermal denaturation of SaADH monitored by dehydrogenase activity. The stability was studied by incubating 0.2 mg ml^{-1} protein samples in 50 mM Tris–HCl, pH 9.0 for 30 min at the indicated temperatures. Activity measurements were carried out under the conditions of the standard assay using cycloheptanol as the substrate. The assay temperature was 65°C

activity measured after 6 and 24 h incubation at 50°C was 105 and 107%, respectively.

Effects of various compounds

The effects of salts, ions and reagents on SaADH activity were studied by adding each compound to the standard assay mixture (Table 4). The chlorides of Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect the enzyme's activity, whereas the sulphate of heavy metal ions such as Fe^{2+}, Co^{2+}, and Cu^{2+} caused a slight inactivation. Iodoacetate and Hg^{2+} did, to some extent, affect the activity, indicating that the only Cys residue per monomer, C67, may have a functional role.

The presence of metal-chelating agents did not affect enzyme activity, suggesting that either the protein does not require metals for its activity or the chelating molecule was not able to remove the metal under the assay conditions. Furthermore, the enzyme showed no loss in activity following exhaustive dialysis against EDTA. The EDTA-dialysed enzyme turned out to be quite stable at 70°C for 6 h, both in the absence and the presence of EDTA or *o*-phenanthroline (data not shown). These results suggest that SaADH does not require metals for its activity or structural stabilization. This is usual for the typically non-metal SDRs enzymes, although the well-known LB-RADH shows strong Mg^{2+} dependency (Niefind et al. 2003).

Stability in organic solvents

The effects of common organic solvents, such as 2-propanol, ethyl acetate, acetonitrile, *n*-hexane, *n*-heptane, *tert*-butylmethyl ether (TBME), methanol, and 1,4-dioxane, on SaADH were investigated at 50°C and at two different time points (Fig. 6). SaADH was activated up to 115% after incubation for 6 h in aqueous buffer and up to 120–125% after incubation for 25 h in the presence of 17% 2-propanol or 17% acetonitrile. Significant increases in enzyme activity occurred after 25 h incubation when ethyl acetate, *n*-hexane, *n*-heptane, TBME, and 1,4-dioxane were present at 30% concentration. Activity values ranged from 170 to 210% of the values determined prior to incubation. However, the presence of 2-propanol, acetonitrile, and 1,4-dioxane at a high concentration (30%) resulted in enzyme inactivation to 5–30% of the initial values following 5 h incubation at 50°C.

Standard assays performed in the presence of 0.1–0.5% 2-propanol and 0.15% TBME did not affect enzyme activity, suggesting that the enhancement in activity was not due to an immediate effect of solvent on the protein structure. A considerable body of literature exists which describes the activation of thermophilic enzymes by loosening of their rigid structure in the presence of protein perturbants (Fontana et al. 1998; Liang et al. 2004). To account for the observed enhancements of SaADH activity, the organic solvent is proposed to induce a conformational change in the protein molecule to a more relaxed and flexible conformation that is optimal for activity. Analogously, the activating effect following heating of SaADH in aqueous buffer at 50°C over a short period (Fig. 6) may be due to partial loosening of the overall structure, inducing an increased flexibility at the active site, and consequently an increased turnover.

Enantioselectivity

The enantioselectivity of SaADH was tested using α-methyl and α-ethyl benzoylformate, methyl *o*-chlorobenzoylformate,

Table 4 Effect of various compounds on SaADH

Compound	Concentration (mM)	Relative activity (%)
None		100
LiCl	1	110
NaCl	1	88
	10	95
KCl	1	99
	10	97
$MgCl_2$	1	94
	10	98
$CaCl_2$	1	100
	10	99
$MnCl_2$	1	95
$FeSO_4$	1	80
$CoSO_4$	1	89
$NiSO_4$	1	106
$CuSO_4$	1	89
$ZnSO_4$	1	91
$Hg(CH_3COO)_2$	1	63
2-Mercaptoethanol	5	112
Iodoacetate	4	88
o-Phenanthroline	1	110
EDTA	1	93
	10	107

Each compounds was added to the reaction mixture at the indicated concentration. The enzyme activity was measured at 65°C using cycloheptanol as substrate

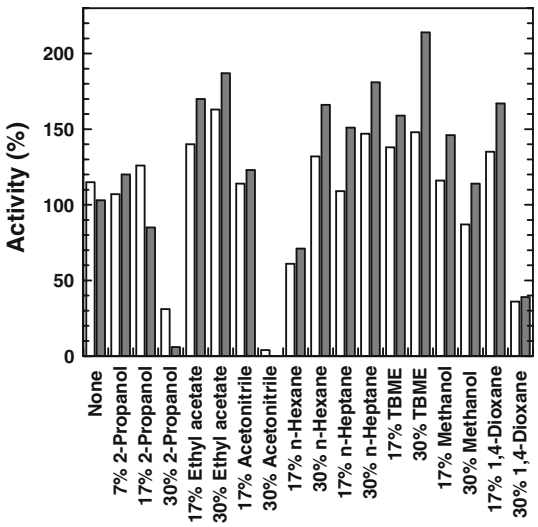

Fig. 6 Effect of organic solvents on SaADH. Samples of enzyme (0.5 mg ml^{-1}) were incubated at 50°C in the absence and presence of the organic solvents at the concentrations indicated, and assays were performed after 6 h (*white bars*) and 25 h (*grey bars*). The activity assays were performed as described in "Materials and methods" using (*R*)-α-tetralol as the substrate. The data obtained in the absence and presence of organic solvents are expressed as percentage of activity relative to the value determined prior to incubation

and 1-indanone as substrates, and an NADH regeneration system consisting of glucose and glucose dehydrogenase from *Thermoplasma acidophilum* (TaGDH), a thermophilic enzyme with dual coenzyme specificity, but with a marked preference for NADP(H) over NAD(H) (John et al. 1994). Tris–phosphate buffer pH 7.0 and 50°C was chosen as the pH and temperature conditions suitable for catalysis by the two archaeal enzymes. Thermal stability studies indicated that TaGDH activity dropped to ~70 and 35% following 24 h incubation at 50°C in Tris–phosphate buffer, pH 7.0, in the absence and presence of 10% acetonitrile, respectively (data not shown).

Bioconversions of EBF were carried out allowing the reactions to proceed for 1, 3, 6 and 24 h at 50°C. Figure 7 shows that conversion of EFB was completed in 6 h. However, reaction times as long as 24 h did not improve the enantiomeric excess (ee) of the biotransformation. SaADH preferably reduced this keto ester to ethyl (*R*)-mandelate with an ee of 50%, after 6 h of reaction. Methyl benzoylformate was reduced by SaADH to methyl (*S*)-mandelate with 100% conversion after 6 h of reaction and an ee of 17%, and methyl *o*-chlorobenzoylformate was reduced to methyl (*R*)-*o*-chloromandelate with >99% conversion and an ee of 72% (Table 5). These results show that enantioselectivity of SaADH is dependent upon the structure of both the phenyl group and alkyl substituent of the ester group of the substrate. 1-Indanone was reduced to the corresponding (*S*)-alcohol following a 6-h reaction at 50°C, with a conversion of 6% and an ee of 25%, reflecting the apparent inactivity under different assay conditions (see Table 2). It is noteworthy that the optically active alcohols produced are used as chiral building blocks in organic synthesis. Methyl (*R*)-*o*-chloromandelate is an intermediate

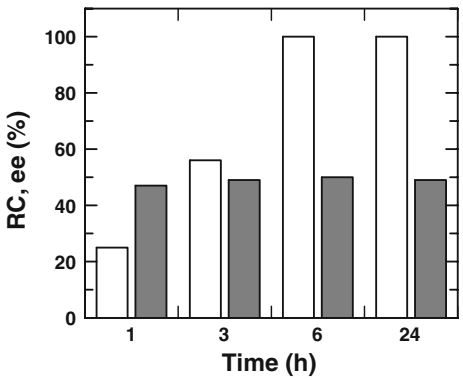

Fig. 7 Reduction of ethyl benzoylformate catalysed by SaADH. Biotransformations were carried out at 50°C at different reaction times as described in "Materials and methods". The reactions were stopped by addition of ethyl acetate at the times indicated. The dried extracts were analysed by chiral HPLC to determine the relative conversion (RC, *open bars*) and the enantiomeric excess (ee, *grey bars*)

Table 5 Asymmetric reduction of carbonyl compounds by SaADH

Substrate	Product	Conversion[a] (%)	ee[a] (%)	*R*/*S*
Methyl benzoylformate	Methyl mandelate	100	17	*S*
Ethyl benzoylformate	Ethyl mandelate	100	50	*R*
Methyl *o*-chlorobenzoylformate	Methyl *o*-chloromandelate	100	72	*R*

Reactions were performed at 50°C for 6 h as described in "Materials and methods"

[a] Conversion yields and enantiomeric excesses were determined by chiral HPLC analysis. Configuration of product alcohol was determined by comparing the retention time with that of standard samples as described in "Materials and methods"

for the platelet aggregation inhibitor clopidogrel (Ema et al. 2008), and (*R*)-ethyl mandelate is precursor for the synthesis of cryptophycins (Gardinier and Leahy 1997).

To our knowledge, this study represents the first biochemical characterization of an NAD(H)-dependent archaeal short-chain ADH. The remarkable activation of SaADH by organic solvents constitutes evidence of the sturdiness of this biocatalyst, and suggests exploratory investigations on conversions of poorly water-soluble prochiral substrates in a biphasic reaction media. Moreover, the importance of the critical role of the above-mentioned D37 in SDRs in discriminating NAD(H) from NADP(H) is further supported by this study.

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