Nicotinoprotein (NADH-containing) alcohol dehydrogenase from *Rhodococcus erythropolis* DSM 1069: an efficient catalyst for coenzyme-independent oxidation of a broad spectrum of alcohols and the interconversion of alcohols and aldehydes

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Extracts from benzyl-alcohol-grown *Rhodococcus erythropolis* DSM 1069 showed NAD(P)-independent, *N,N*-dimethyl-4-nitrosoaniline (NDMA)-dependent alcohol dehydrogenase activity. The enzyme exhibiting this activity was purified to homogeneity and characterized. It appears to be a typical nicotinoprotein as it contains tightly bound NADH acting as cofactor instead of coenzyme. Other characteristics indicate that it is highly similar to the known nicotinoprotein alcohol dehydrogenase (np-ADH) from *Amycolatopsis methanolica*: it is a homotetramer of 150 kDa; N-terminal amino acid sequencing (22 residues) showed that 77% of these amino acids are identical in the two enzymes; it has optimal activity at pH 7·0; it lacks NAD(P)H-dependent aldehyde reductase activity; it catalyses the oxidation of a broad range of (preferably) primary and secondary alcohols, either aliphatic or aromatic, and formaldehyde, with the concomitant reduction of the artificial electron acceptor NDMA. NDMA could be replaced by an aldehyde, but not formaldehyde, the substrate specificity of the enzyme for the aldehydes reflecting that for the corresponding alcohols. The latter also applied to the low aldehyde dismutase activity displayed by the enzyme. From this, together with the results of the induction studies, it is concluded that np-ADH functions as the main alcohol-oxidizing enzyme in the dissimilation of many, but not all, alcohols by *R. erythropolis* and may also catalyse coenzyme-independent interconversion of alcohols and aldehydes under certain circumstances. It is anticipated that the enzyme may be of even wider significance since structural data indicate that np-ADH is also present in other (nocardioform) actinomycetes.

Keywords: nicotinoprotein alcohol dehydrogenase, *Rhodococcus erythropolis*

INTRODUCTION

The Gram-positive bacterial genus *Rhodococcus*, belonging to the nocardioform actinomycetes, is known for its ability to metabolize a great variety of natural compounds as well as xenobiotics (Finnerty, 1992; Warhurst & Fewson, 1994; Bell *et al.*, 1998). Often, one of the steps in the degradation pathway of such compounds is the oxidation of an alcohol into the corresponding aldehyde or ketone. Several alcohol dehydrogenases have been (partly) characterized from species belonging to this genus, e.g. NAD-dependent coniferyl alcohol dehydrogenase (Jaeger *et al.*, 1981; Jaeger, 1988), NAD/mycothiol-dependent for-

maldehyde dehydrogenase (Eggeling & Sahm, 1984, 1985; van Ophem *et al.*, 1992; van Ophem & Duine, 1994; Misset-Smits *et al.*, 1997) and methanol: *N*,*N*-dimethyl-4-nitrosoaniline (NDMA) oxidoreductase (MNO) from *R. erythropolis* DSM 1069 (van Ophem *et al.*, 1993); MNO from *R. rhodochrous* LMD 89.129 (van Ophem *et al.*, 1993) and from *Rhodococcus* sp. NI86/21 (Nagy *et al.*, 1995), which has since been identified as *R. erythropolis* (de Schrijver *et al.*, 1997); NAD-dependent propan-2-ol dehydrogenase from *R. rhodochrous* PNKb1 (Ashraf & Murrell, 1990, 1992); NADH-dependent carbonyl reductase from *R. erythropolis* DSM 743 (Peters *et al.*, 1992, 1993; Zelinski *et al.*, 1994); NAD-dependent long-chain secondary alcohol dehydrogenase from *R. erythropolis* ATCC 4277 (Ludwig *et al.*, 1995); NAD-dependent secondary alcohol dehydrogenase from *Rhodococcus* sp. GK1 (Krier *et al.*, 1998). Although not clear for every case, it appears from this that the functional role of these enzymes is confined to the oxidation of one particular alcohol and that an alcohol dehydrogenase with a broad induction and substrate specificity pattern and kinetic parameter values compatible with a broad physiological role in dissimilatory alcohol oxidation has not been found so far in rhodococci.

MNO (EC 1.1.99.–) is a member of the group of nicotinoprotein alcohol dehydrogenases, i.e. enzymes which contain tightly bound, non-exchangeable NAD(P)(H) in the active site acting as cofactor instead of coenzyme. MNO was detected in *Amycolatopsis methanolica* NCIB 11946 (grown on methanol, ethanol, butan-1-ol, hexan-1-ol or acetate) and *Mycobacterium gastri* MB 19 (grown on methanol, propan-1-ol, propan-2-ol or glycerol) (Bystrykh *et al.*, 1993a, b; van Ophem *et al.*, 1993), i.e. Gram-positive, methylotrophic actinomycetes closely related to (non-methylotrophic) rhodococci. It is a 490–500 kDa homodecameric class III alcohol dehydrogenase, each subunit containing a tightly, but not covalently bound NADP(H) cofactor (Bystrykh *et al.*, 1993a). Since no transfer of reducing equivalents takes place to externally added NAD(P), MNO is not active in the common assays for NAD(P)-dependent alcohol dehydrogenases. Its alcohol dehydrogenase activity can be demonstrated, however, by using the artificial electron acceptor NDMA. In such an assay, the enzyme acts as a coenzyme-independent alcohol: NDMA oxidoreductase and obeys a ping-pong mechanism. Moreover, MNO catalyses the dismutation of formaldehyde and the NADH-dependent reduction of aldehydes (Bystrykh *et al.*, 1993b). Gene-disruption mutants of *A. methanolica* lacking a functionally active gene encoding MNO were unable to grow on methanol, ethanol, propan-1-ol or butan-1-ol as sole carbon source (Hektor & Dijkhuizen, 1996; Hektor, 1997). It seems, therefore, that MNO is crucial for oxidation of these C_1–C_4 alcohols.

Surprisingly, apart from MNO, *A. methanolica* (grown on methanol, ethanol, butan-1-ol, hexan-1-ol or acetate) contains a second nicotinoprotein alcohol: NDMA oxidoreductase (EC 1.1.99.–) (van Ophem *et al.*, 1993) for

which the name nicotinoprotein alcohol dehydrogenase (np-ADH) has been proposed (Piersma *et al.*, 1998). The enzyme is quite different from MNO with respect to protein structure and catalytic performance. np-ADH is a homotri- or tetrameric class I alcohol dehydrogenase of 120 kDa, each 39 kDa subunit containing firmly, non-covalently bound NADH as cofactor. It catalyses the NAD(P)-independent oxidation of various primary alcohols (but not methanol) with the concomitant reduction of NDMA, according to a ping-pong mechanism. However, in contrast to MNO it catalyses neither the dismutation of formaldehyde nor the NADH-dependent reduction of aldehydes. Despite the fact that MNO and np-ADH are simultaneously induced during growth on certain alcohols (methanol, ethanol, butan-1-ol or hexan-1-ol), and ethanol, propan-1-ol and butan-1-ol are good substrates for both enzymes *in vitro*, the physiological role of MNO in *A. methanolica* cannot be taken over by np-ADH, as judged from the inability of mutants lacking the MNO-encoding gene to grow on these alcohols. Since such studies using mutants lacking a functionally active gene encoding np-ADH have not been performed so far, the physiological role of np-ADH remains to be elucidated.

During our earlier work on novel types of alcohol dehydrogenases involved in the kinetic resolution of racemic alcohols (Geerlof *et al.*, 1994), strong indications were obtained that a nicotinoprotein alcohol dehydrogenase found in certain rhodococci is involved in resolving enantiomeric mixtures of alcohols. To study this in more detail, *R. erythropolis* DSM 1069 was chosen as a model organism. From these studies it appeared that this strain is able to produce MNO (characterization to be published elsewhere) as well as np-ADH. Here we report on the induction, purification and characterization of np-ADH.

METHODS

Chemicals. Yeast extract and bactopeptone were obtained from Difco. NAD(P) and NAD(P)H were from Boehringer Mannheim. All other chemicals used in this study were of the highest quality available from Sigma-Aldrich.

Cultivation and induction experiments. *Rhodococcus erythropolis* DSM 1069 was precultivated on a medium containing 2% (w/v) D-glucose, 1% (w/v) bactopeptone, 0·5% (w/v) NaCl and 0·05% (w/v) yeast extract in a rotary shaker incubator (200 r.p.m.) for 48 h at 30 °C. For induction studies, 25 ml precultures were used to inoculate 2 l Erlenmeyer flasks, containing 0·5 l mineral salt medium (Eggeling & Sahm, 1984), 0·05% (w/v) yeast extract, and growth substrate (see legend of Table 1). The flasks were shaken (at 200 r.p.m., at 30 °C) and the cells were harvested in the late exponential growth phase (after approximately 48 h, except for growth on citronellol, which required 168 h) by centrifugation (20 min at 16 300 *g*, at 4 °C) and washed twice with 10 mM potassium phosphate buffer, pH 7·0, at 4 °C. An aliquot of cell paste (typically 1–2 g) was resuspended in an equal volume of the same buffer, sonicated (using an MSE sonicator for 4 × 10 s on ice, at high power, amplitude 2, 10 μm peak to peak), and centrifuged (30 min at 20 000 *g*, at 4 °C). The supernatant was

used as the cell-free extract and assayed for various alcohol dehydrogenase activities.

To produce cells as starting material for isolation of np-ADH, growth was carried out in a fermenter (model Bio Bench 20, Applikon Dependable Instruments) containing 16 l mineral salt medium (Eggeling & Sahm, 1984) with benzyl alcohol as growth substrate (added to the cell suspension in three portions resulting in a concentration of 10 mM at time 0, 24 and 48 h). The culture was stirred at 400 r.p.m. and aerated at 5 l min^{-1}. During growth, the pH was controlled at $7 \cdot 0 \pm 0 \cdot 2$ by automatic titration with 4 M NaOH and 2 M H$_2$SO$_4$. After 72 h of growth, the cell suspension was concentrated to approximately 1·5 l by ultrafiltration. The cells were harvested by centrifugation (20 min at 16 300 **g**, at 4 °C), washed with 10 mM potassium phosphate buffer, pH 7·0, and stored frozen at −40 °C.

Enzyme purification. All purification steps were performed at room temperature unless indicated otherwise. The fractions were assayed for the presence of np-ADH, MNO or NAD(P)-dependent benzyl alcohol dehydrogenase activities. Thawed cell paste (50 g) was resuspended in an equal volume of 10 mM potassium phosphate buffer, pH 7·0, at 4 °C. After adding bovine DNase I, the suspension was passed three times through a French pressure cell under a pressure of 27·6 MPa. Centrifugation (60 min at 16 300 **g**, at 4 °C) yielded the cell-free extract. The extract was dialysed for 1 h against 1 M potassium phosphate buffer, pH 7·0, at 4 °C, and centrifuged (20 min at 16 300 **g**, at 4 °C). Aliquots (10 ml) of the supernatant were applied to a 40 ml Phenyl-Sepharose FF hydrophobic interaction column (Pharmacia Biotech) equilibrated with 1 M potassium phosphate buffer, pH 7·0, at 4 °C. A linear gradient of potassium phosphate (1 M–10 mM, pH 7·0) was applied for 45 min at a flow rate of 2 ml min^{-1}. Fractions showing np-ADH activity (eluting in the final part of the gradient) were concentrated by centrifugation (3000 **g**) using a 30 kDa cutoff filter (Centriprep 30, Amicon). Concentrate obtained from five separate runs on Phenyl-Sepharose was applied to a Poros HQ (1 × 10 cm) anion-exchange column (Boehringer Mannheim) equilibrated with 20 mM potassium phosphate buffer, pH 7·2. A linear gradient of potassium chloride (0–1·5 M) in the same buffer was applied for 60 min at a flow rate of 2 ml min^{-1}. The np-ADH activity, eluting at 35 mM KCl, was concentrated as above and applied in 200 μl portions to a Superdex 200 (1 × 30 cm) gel-filtration column (Pharmacia) equilibrated with 10 mM potassium phosphate buffer, pH 7·0, containing 0·2 M KCl, at a flow rate of 0·5 ml min^{-1}. Fractions with np-ADH activity from 10 separate runs were pooled and stored frozen at −80 °C.

Enzyme assays. All measurements were performed in duplicate at 20 °C. np-ADH activity was measured by following the reduction rate of NDMA at 440 nm with benzyl alcohol as electron donor. The reaction mixture contained (total volume 1 ml): benzyl alcohol, 20 μM; NDMA, 28 μM; potassium phosphate buffer, pH 7·0, 10 mM. The reaction was started by the addition of an appropriate amount of enzyme. Specific activities were calculated using a molar absorption coefficient for NDMA of 35 400 M^{-1} cm^{-1} at 440 nm (Dunn & Bernard, 1971). In order to determine whether extracts exhibited NDMA-dependent alcohol oxidation activity for the alcohol used as growth substrate, benzyl alcohol in the assay was replaced by this alcohol. The optimal concentration of the latter (as indicated by the values in parentheses in Table 1), yielding an apparent maximal rate, was determined by varying each substrate in the range from 5 μM to 25 mM (or up to saturation in the case of alcohols with low solubility). MNO

activity was measured in 50 mM potassium phosphate buffer, pH 6·3, by following the reduction of NDMA (28 μM, final concentration), starting the reaction by the addition of methanol (25 mM, final concentration) (Bystrykh *et al.*, 1993b). NAD(P)-dependent alcohol dehydrogenase activities were determined in 0·1 M sodium pyrophosphate buffer, pH 9·0 (Duine *et al.*, 1984) by measuring the formation rate of NAD(P)H at 340 nm in the presence of the alcohol used as growth substrate (apparent maximal rates were determined as indicated above). Assays for aldehyde dismutase activity were carried out in a 1·5 ml reaction vessel containing (final concentrations) 50 mM potassium phosphate buffer, pH 7·0, aldehyde (formaldehyde, 5 mM; acetaldehyde, 2 mM; propionaldehyde, 0·1 mM; methylglyoxal, 5 mM; benzaldehyde, 5 mM; phenylacetaldehyde, 0·2 mM) and an appropriate amount of enzyme (typically 0·67 μM) in a total volume of 1·0 ml. The reaction was started by the addition of enzyme. Samples (100 μl) were taken at regular intervals and the reaction was stopped by the addition of 2 μl formic acid (100 %) [in the case of formaldehyde, aqueous acetic acid (10 %) was used instead]. After centrifugation (2 min at 20 000 **g**), the remaining substrate and the products were determined by injecting 1·0 μl of supernatant into the HP-Innowax GC-column (see below). However, in the case of benzaldehyde and phenylacetaldehyde, analysis was done according to the HPLC method described below for the veratryl alcohol/methylglyoxal interconversion. Formaldehyde solutions were prepared by heating paraformaldehyde in water for 8 h at 100 °C.

Substrate specificity. Values for V'_{max}, K'_m and K'_i of the NDMA-linked dehydrogenase activity of np-ADH were determined by varying the concentration of the alcohol or aldehyde substrate (in the range from 1 μM to 200 mM, or up to saturation) in the assay mixture at a constant concentration of NDMA (28 μM). The apparent kinetic parameter values of the alcohol:carbonyl oxidoreductase activity of np-ADH were determined by varying the concentration of the aldehyde or ketone substrate (from 1 μM up to saturation) at a fixed concentration of veratryl alcohol (1 mM) and measuring the initial rates of veratraldehyde formation at 310 nm ($\varepsilon_{310} = 9300$ M^{-1} cm^{-1}). The steady-state kinetic data were analysed by non-linear regression using an equation derived from Michaelis–Menten kinetics (with a single substrate), including a substrate-inhibition term (Cornish-Bowden, 1995), using Igor Pro software (WaveMetrics) on a Power Macintosh G3 computer (Apple).

Interconversion of the veratryl alcohol/methylglyoxal combination. The np-ADH-catalysed oxidation of veratryl alcohol to veratraldehyde with the concomitant reduction of methylglyoxal was carried out at 20 °C in a quartz cuvette. The reaction mixture (total volume 2 ml) consisted of 10 mM potassium phosphate buffer, pH 7·0, 0·15 mM veratryl alcohol, 1·0 mM methylglyoxal and 0·1 μM np-ADH. The progression of the conversion with time was monitored by measuring veratraldehyde formation at 310 nm. Samples (25 μl) were taken at regular intervals and the reaction was quenched by adding 50 μl of the HPLC column eluent (aqueous 30 %, v/v, acetonitrile containing 1 %, v/v, acetic acid), and centrifuging (2 min at 20 000 **g**). An aliquot of the supernatant (20 μl) was applied to a Nova-pak C$_{18}$ reversed-phase column (150 × 3·9 mm, Waters) followed by isocratic elution at a flow rate of 0·8 ml min^{-1} (Piersma, 1998). The eluate was monitored at 236 nm, 278 nm and 310 nm using a UV/VIS photodiode-array detector (Hewlett Packard, model 1040 A). Baseline separation could be achieved with this setup for veratryl alcohol, veratraldehyde and veratric acid, allowing reliable

integration of the peak areas of the compounds using Chemstation chromatography software (Hewlett Packard). Quantitative data were obtained by using calibration curves of the authentic compounds chromatographed in the same way.

Molecular mass determinations. The subunit molecular mass of np-ADH was determined by SDS-PAGE (Laemmli, 1970) using 8–25 % polyacrylamide gradient gels on a Phast-System (Pharmacia) according to the manufacturer's instructions. A low-molecular-mass marker kit (Pharmacia) was used for calibration and Coomassie brilliant blue R250 for protein staining. The molecular mass of native np-ADH was determined by gel filtration on a Superdex 200 (30×1 cm) column equilibrated with 10 mM potassium phosphate buffer, pH 7·0, containing 0·2 M KCl, at a flow rate of 0·5 ml min^{-1}. Blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (BSA, 67 kDa) were used as molecular mass references.

Protein determination. Protein concentrations were estimated using the bicinchoninic acid/CuSO$_4$ method (Pierce) with desalted BSA as a standard (Smith *et al.*, 1985).

N-terminal sequence analysis. The N-terminal amino acid sequence of the enzyme (the blotted band obtained with SDS-PAGE) was determined by automated Edman degradation using a Procise 494 sequenator (Applied Biosystems Division, Perkin Elmer) running in pulsed-liquid mode.

Determination of bound NADH. The enzyme-bound chromophore was dissociated from the enzyme using a slightly modified urea-extraction procedure (Bystrykh *et al.*, 1993a). For that purpose, an aliquot of enzyme in 0·1 M Tris/HCl, pH 8·5, was brought to 6 M urea by adding a saturated urea solution in the same buffer. The mixture was heated for 2 min at 85 °C. After cooling and centrifugation (5 min at 20 000 *g*), the supernatant was applied to a MonoQ (0.5×5 cm) column equilibrated with 10 mM Tris/HCl, pH 8·5, containing 6 M urea. Elution was done at a flow rate of 1 ml min^{-1} with a linear gradient of 0–1 M KCl in the same buffer. The eluate was monitored with the UV/VIS photodiode-array detector. The resulting elution profile and the absorption spectra of the compounds corresponding to the peaks in the chromatogram were compared with those of urea-treated authentic NAD, NADH, NADP and NADPH.

Fluorescence spectroscopy. Fluorescence spectra were obtained at room temperature using a Shimadzu spectrofluorimeter, model RF-5001PC. Fluorescence emission spectra were recorded from 350 to 600 nm using a fixed excitation wavelength of 334 nm (bandwidth 3 nm). Fluorescence excitation spectra were obtained by scanning from 250 to 400 nm and recording the fluorescence emission at a fixed wavelength of 430 nm (bandwidth 3 nm).

RESULTS

Induction studies

R. erythropolis DSM 1069 utilized a large number of aliphatic and aromatic primary alcohols as sole carbon and energy source. During growth on these compounds, both NAD- and NDMA-dependent alcohol dehydrogenase activities were induced (Table 1), the latter suggesting the presence of one or more nicotinoprotein alcohol dehydrogenases. Attempts to detect the presence of an alcohol oxidase or an NADP-dependent alcohol dehydrogenase gave negative results (except for significant NADP-dependent coniferyl alcohol dehydrogenase activity after growth on this alcohol; see below). In extracts of cells grown on acetate + methanol, ethanol, propan-1-ol, veratryl alcohol or cyclohexanol, NDMA-dependent methanol dehydrogenase activity was observed. Purification and characterization revealed that this activity derives from MNO (to be published elsewhere), in agreement with earlier indications of the presence of MNO in rhodococci (van Ophem *et al.*, 1993; Nagy *et al.*, 1995). Growth on each of the other alcohols, in particular on benzyl alcohol, 3-phenylpropan-1-ol and butan-1-ol, induced significant NDMA-dependent benzyl alcohol dehydrogenase activity, whereas NDMA-dependent methanol dehydrogenase activity was absent, suggesting the presence of an NDMA-dependent alcohol dehydrogenase other than MNO. When benzyl alcohol in the assay was replaced by the alcohol on which the corresponding cells had grown, significant NDMA-dependent alcohol dehydrogenase activities were detected, showing apparent maximal velocities at low substrate concentrations (micromolar range, as indicated by the values given in parentheses in Table 1). In general, the NAD-dependent alcohol dehydrogenase activities in the extracts were very low, and maximal activities were obtained only at relatively high substrate concentrations (millimolar range), except in extracts of coniferyl-alcohol-grown cells. It is very likely that the coniferyl alcohol dehydrogenase activity derives from the NAD(P)-dependent coniferyl alcohol dehydrogenase already described for this organism (Jaeger *et al.*, 1981; Jaeger, 1988).

Purification and structural properties

The induction studies indicated that benzyl alcohol as growth substrate gives rise to the highest level of NDMA-dependent alcohol dehydrogenase activity without inducing MNO or NAD(P)-dependent alcohol dehydrogenase activities. Therefore, this alcohol was chosen as a growth substrate to produce cells as starting material for the purification of np-ADH. The data for the purification protocol of a typical batch are presented in Table 2. SDS-PAGE of the final preparation revealed a single band corresponding to an M_r of 38 000 ($\pm 5 \%$). Gel-filtration chromatography of native enzyme yielded a single peak, which corresponded to an M_r of 150 000 ($\pm 10 \%$). Since overlaying of the chromatograms taken at 205, 280 and 330 nm showed a single, symmetrical peak with the same retention time, it is concluded that the final enzyme preparation was homogeneous. A single N-terminal amino acid sequence was detected (Fig. 1), showing high sequence identity to that of np-ADH from *A. methanolica* (van Ophem *et al.*, 1993), i.e. 77 % identical amino acid residues out of 22. This suggested that the enzyme purified is an np-ADH which has, according to the data given above, a homotetrameric structure.

The absorption spectrum of the final preparation (Fig. 2a, solid line) showed that the enzyme contains a

Table 1. Alcohol dehydrogenase activities exhibited by extracts of *R. erythropolis* DSM 1069

Cell-free extracts were assayed for NDMA-linked np-ADH and MNO activities (with 20 μM benzyl alcohol, at pH 7·0, and with 25 mM methanol, at pH 6·3, respectively), and for NDMA- and NAD-linked 'growth-substrate' ('GS') dehydrogenase activities (at pH 7·0 and 9·0, respectively).

Growth substrate*	Specific alcohol dehydrogenase activity†			
	NDMA-linked			NAD-linked 'GS'‡
	Benzyl alcohol	'GS'‡	Methanol	
Methanol§ (0·5%, v/v)	<1	3·5 (25)	3·5	0
Ethanol (1%, v/v)	1	1 (0·8)	1·1	<1 (21)
Propan-1-ol (0·2%, v/v)	3	ND	<1	2 (13)
Butan-1-ol (0·2%, v/v)	35	25 (0·04)	0	6 (16)
Hexan-1-ol (0·1%, v/v)	9	9 (0·04)	0	4 (16)
Citronellol (1 mM)	7	5 (0·009)	0	<1 (1)
Benzyl alcohol (10 mM)	54	54 (0·02)	0	0
Veratryl alcohol (10 mM)	8	7 (0·3)	<1	16 (6)
Vanillyl alcohol (5 mM)	9	5 (0·9)	0	4 (2)
Coniferyl alcohol (1 mM)	4	3 (0·01)	0	52 (0·4)
2-Phenylethanol (10 mM)	4	5 (0·03)	0	2 (0·7)
3-Phenylpropan-1-ol (10 mM)	50	38 (0·06)	0	11 (5)
Cyclohexanol (10 mM)	16	8 (0·4)	<1	<1 (22)
Sodium acetate (0·5%, w/v)	0	ND	0	ND
D-Glucose (0·5%, w/v)	0	0	0	<1 (19)

ND, Not determined.

* Compounds used as sole carbon and energy source; final concentrations are given in parentheses.

†Expressed in nmol NDMA consumed or NADH formed min^{-1} (mg protein)$^{-1}$.

‡ Values in parentheses indicate an estimate of the concentration (in mM) for optimal activity (see Methods).

§ Methanol (not a growth substrate) was added as a cosubstrate (0·5%, v/v) to 0·5% (w/v) sodium acetate.

Table 2. Summary of the purification of np-ADH from benzyl-alcohol-grown *R. erythropolis*

Purification step	Total protein (mg)	Specific activity* (μmol min^{-1} mg^{-1})	Yield (%)	Purification (-fold)
Cell-free extract	1635	0·3	100	1
Phenyl-Sepharose	311	1·3	82	4
Poros HQ	58	3·4	40	11
Superdex 200	30	4·0	24	13

* Specific activities were determined with the np-ADH assay using benzyl alcohol (20 μM, final concentration) as a substrate, and expressed in μmol NDMA reduced min^{-1} (mg protein)$^{-1}$.

chromophore with an absorption maximum at 330 nm. Fluorescence excitation and emission spectra (Fig. 2b, dashed and solid lines, respectively) of the enzyme displayed excitation maxima at 286 nm and 334 nm, and an emission maximum at 431 nm, characteristics in line with the presence of a pyridine nucleotide. Extraction and chromatography showed that the chromophore is indeed identical to NADH, the same cofactor which has been found in np-ADH from *A. methanolica* (van Ophem et al., 1993). Addition of acetaldehyde to the enzyme preparation bleached the absorbance of enzyme-bound NADH, which could be reversed by subsequent addition of ethanol (Fig. 2a, dashed and dotted lines, respectively). Thus, the nicotinoprotein alcohol dehydrogenase from *R. erythropolis* is an np-ADH, containing tightly, but not covalently bound NADH acting as cofactor.

Catalytic performance and substrate specificity

To get insight into the catalytic properties of np-ADH, its performance with respect to the forward and reverse reactions and its substrate specificities were determined.

				5				10				15				20
R. erythropolis np-ADH	M K T K A A V L F E	T H	K P F E I V	E L E L
A. methanolica np-ADH	M K T K A A V L H S	A G	K P F E I E	E L E L
M. tuberculosis AdhD	M K T T A A V L F E	A G	K P F E L M	E L D L

Fig. 1. N-terminal amino acid sequence of np-ADH from *R. erythropolis*. The sequence is compared to that of np-ADH from *A. methanolica* (van Ophem *et al.*, 1993), and to that of the AdhD gene product corresponding to the *adhD* gene from *Mycobacterium tuberculosis* H37Rv, which encodes a putative alcohol dehydrogenase according to Cole *et al.* (1998). Amino acid residues identical to those of np-ADH from *R. erythropolis* are boxed.
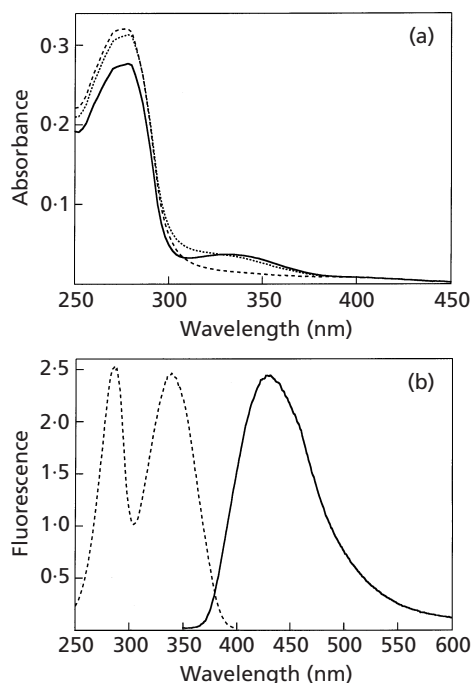
Fig. 2. (a) UV/visible absorption spectra of np-ADH. The final preparation (0·4 mg protein ml⁻¹) in 10 mM potassium phosphate buffer, pH 7·0, was measured as isolated (solid line), after the addition of acetaldehyde (5 mM, dashed line), and subsequently ethanol (5 mM, dotted line). Spectra were corrected for volume changes. (b) Fluorescence spectra of np-ADH. Excitation (dashed line) and emission (solid line) spectra were measured in 10 mM potassium phosphate buffer, pH 7·0.

As shown in Table 3(a), using NDMA as electron acceptor np-ADH catalysed the oxidation of a large number of aliphatic, aromatic or branched primary alcohols (except methanol, ethanolamine, hydroxy-acetone), secondary alcohols and polyols (except ethylene glycol, *trans*-cyclohexane-1,2-diol and cyclohexane-1,4-diol), but not aldehydes (except formaldehyde). On inspecting the specificity constants, it appears that primary alcohols are generally preferred over secondary alcohols and diols. In accordance with its nicotinoprotein character, steady-state experiments showed that the enzyme has a ping-pong kinetic mechanism, as judged from the parallel lines in a Lineweaver–Burk plot for the couples ethanol/NDMA and veratryl alcohol/acetaldehyde (not shown). Using high concentrations of enzyme, very low but detectable aldehyde dismutase activities were measured for (turn-over numbers in parentheses) formaldehyde (0·074 s⁻¹), acetaldehyde (0·034 s⁻¹), propionaldehyde (0·094 s⁻¹), methylglyoxal (1·5 s⁻¹), benzaldehyde (0·42 s⁻¹) and phenylacetaldehyde (0·60 s⁻¹).

np-ADH catalysed the reduction of various aldehydes (except formaldehyde) in the presence of an alcohol (Table 3b) such as ethanol, benzyl alcohol or veratryl alcohol (the latter was used in most cases because its oxidation to veratraldehyde can be measured conveniently at 310 nm). When comparing both electron donor and acceptor substrate specificities (Table 3a, b), it appears that when np-ADH catalyses the oxidation of a certain alcohol, it also reduces the corresponding aldehyde or ketone. However, the specificity constants for carbonyl reduction are generally some orders of magnitude lower than those for the oxidation of the corresponding alcohol. np-ADH did not catalyse NAD(P)H-dependent reduction of aldehydes or ketones.

From the apparent kinetic parameter values in Table 3(a, b), it follows that variation in the specificity constant values is mainly caused by the K'_m values, not by the V'_{max} values. The best substrates are primary alcohols containing a phenyl group (compare methanol and ethanol with benzyl alcohol and 2-phenylethanol) or an aliphatic chain of a certain length (pentan-1-ol up to heptan-1-ol). Substitution of the phenyl ring (with hydroxyl and/or methoxy groups) results in less favourable substrates, unless the aliphatic chain is enlarged somewhat. Branching of the aliphatic chain also gives improvement (compare butan-1-ol with 3-methylbutan-1-ol). Introduction of a double bond has a negative effect (compare propan-1-ol with 2-propen-1-ol) but this can be compensated by substitution with a phenyl group (compare 2-propen-1-ol with cinnamyl alcohol).

In many cases, severe substrate inhibition was observed (often in the submillimolar range), hampering the determination of kinetic parameter values, especially when there were small differences in K'_m and K'_i values. This indicates that in such cases the substrate binds nearly equally well to the oxidized and the reduced enzyme forms.

Table 3. Substrate specificity of np-ADH with (a) NDMA and (b) veratryl alcohol as electron acceptor and donor, respectively

| Substrate	Apparent kinetic constants			
	V'_{max} (µmol min^{-1} mg^{-1})	K'_m* (mM)	K'_i (mM)	V'_{max}/K'_m
(a) NDMA as electron acceptor				
Primary alcohols				
Ethanol	2·0	0·10	105	19
Propan-1-ol	2·5	0·0037	3·8	668
Butan-1-ol	5·8	0·010	0·068	567
Pentan-1-ol	2·0	<0·001	0·36	>1950
Hexan-1-ol	2·2	<0·001	0·24	>2180
Heptan-1-ol	2·7	0·0011	0·042	2452
Octan-1-ol	3·5	0·059	0·13	59
2-Propen-1-ol	3·0	0·028	8·1	108
Isobutanol	3·3	0·0072	1·9	453
3-Methylbutan-1-ol	3·1	0·0028	0·053	1136
Citronellol	1·9	<0·001	0·11	>1925
Benzyl alcohol	4·9	0·0018	0·081	2734
4-Hydroxybenzyl alcohol	0·79	0·0047	6·1	168
Vanillyl alcohol	2·0	0·39	6·0	5·2
Veratryl alcohol	1·8	0·16	5·9	12
2-Phenylethanol	2·6	0·0070	2·2	391
2-Phenylpropan-1-ol	1·8	0·056	2·6	33
3-Phenylpropan-1-ol	2·4	0·014	0·58	170
3-(4-Hydroxyphenyl)propan-1-ol	2·7	0·0019	0·92	420
Cinnamyl alcohol	3·4	0·0036	0·18	947
Coniferyl alcohol	0·92	0·0055	4·3	168
2-Phenylbutan-1-ol	1·3	0·20	1·9	6·5
4-(4-Methoxyphenyl)butan-1-ol	4·0	0·0059	0·039	670
Secondary alcohols				
Propan-2-ol	0·16	6·0	∞	0·027
Butan-2-ol	0·81	4·1	13	0·20
Pentan-2-ol	2·0	1·3	4·7	1·5
Hexan-2-ol	2·6	0·83	2·3	3·1
Heptan-2-ol	1·1	0·070	4·9	15
Octan-2-ol	0·75	0·12	∞	6·2
3-Methylheptan-4-ol	6·6	9·1	∞	0·73
4-Methylheptan-3-ol	0·58	0·28	∞	2·1
1-Phenylethanol	1·2	2·8	14	0·44
1-Phenylpropan-1-ol	0·84	0·55	22	1·5
1-Phenylpropan-2-ol	1·9	1·6	4·6	1·2
1-Phenylbutan-2-ol	1·5	0·55	1·4	2·7
Cyclohexanol	1·9	0·19	1·6	9·9
Diols				
Propane-1,2-diol	2·3	35	∞	0·065
Propane-1,3-diol	3·0	4·1	194	0·72
3-Butene-1,2-diol	1·6	8·3	388	0·19
Pentane-1,2-diol	2·0	0·20	47	9·9
1-Phenylethane-1,2-diol	1·4	3·1	99	0·46
Aldehydes				
Formaldehyde	3·4	0·93	5·4	3·6

Table 3 (*cont.*)

Substrate	Apparent kinetic constants			
	V'_{max} (µmol min^{-1} mg^{-1})	K'_m* (mM)	K'_i (mM)	V'_{max}/K'_m
	(b) **Veratryl alcohol as electron donor**			
Aldehydes				
Acetaldehyde	2·1	4·95	∞	0·42
Benzaldehyde	3·7	0·083	0·035	45
2-Phenylpropionaldehyde	2·38	0·088	∞	27
3-Phenylpropionaldehyde	8·32	0·14	0·41	59
3-Phenylbutyraldehyde	8·52	0·12	0·31	73
Glyoxal	1·0	242	∞	0·004
Methylglyoxal	4·5	3·2	5·2	1·4
Phenylglyoxal	5·1	0·21	1·3	24
Ketones				
Hydroxyacetone	0·15	38	∞	0·004
Cyclohexanone	0·097	1·6	∞	0·06
Nitrosoanilines				
NDMA†	1·7	0·0041	∞	425

∞ Substrate inhibition could not be observed within the concentration range tested.

* The lower limit of the determination of K_m with NDMA as electron acceptor was 0·001 mM.

† Veratryl alcohol (1 mM, final concentration) in the assay (see Methods) was replaced by ethanol (0·5 mM, final concentration) and initial rates of the reduction of NDMA were measured at 440 nm.
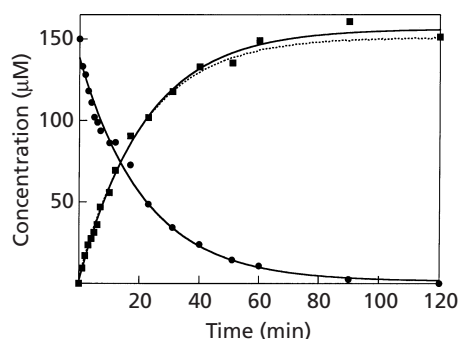
Fig. 3. Interconversion of veratryl alcohol and methylglyoxal by np-ADH. The progression of the conversion was monitored (dotted line) by following the rise in absorbance at 310 nm (due to formation of veratraldehyde) as well as by HPLC, measuring the veratryl alcohol (●) and veratraldehyde concentrations (■). Fitting (solid lines) of the data was carried out using a single exponential equation.

Conversion experiments

For reasons of analytical simplicity, most enzyme-catalysed conversion experiments were carried out with the couple veratryl alcohol/methylglyoxal. As shown in Fig. 3, a small amount of np-ADH catalysed the complete oxidation of veratryl alcohol into veratraldehyde with a stoichiometric amount of methyl-glyoxal as electron acceptor, in line with the fact that no veratric acid was found in the final reaction mixture. Complete conversion was also achieved using the couple veratraldehyde/ethanol, although the reaction proceeded approximately 10-fold slower than that for the couple veratryl alcohol/methylglyoxal. These observations indicate that np-ADH is stable for several hours in mixtures of alcohols and aldehydes during catalysis. Rapid, irreversible inactivation of np-ADH was observed (typically within a few minutes) when performing alcohol oxidation in the presence of NDMA (not shown). This phenomenon could be partly suppressed by adding DTT (or other thiols) to the assay mixture. Incubating the enzyme with solely NDMA, an alcohol, or an aldehyde did not lead to any inactivation (unpublished results). It seems, therefore, that the inactivation of np-ADH in the assay mixture is caused by products generated by the reduction of NDMA, an observation which has also been made in the case of horse liver alcohol dehydrogenase (Dunn & Bernard, 1971).

DISCUSSION

From the results described here, it is concluded that benzyl-alcohol-grown *R. erythropolis* DSM 1069 produces np-ADH as the sole alcohol dehydrogenase. In theory, it is possible that benzyl alcohol is oxidized by another alcohol dehydrogenase, requiring unusual assay conditions, and that np-ADH has a role in dissimilation

Table 4. Presence in various alcohol dehydrogenases of amino acid residues regarded as characteristic for np-ADH (from *A. methanolica*)

Amino acid residues identical to those in np-ADH from *A. methanolica* are shown in bold. Non-identical, but analogous residues are in normal type. Non-identical and non-analogous residues are in italics. Abbreviations: np-ADH *A. met.*, nicotinoprotein alcohol dehydrogenase from *A. methanolica*; AdhD and AdhB *M. tub.*, gene products from *M. tuberculosis* H37Rv encoded by the *adhD* and *adhB* genes, respectively, which have been assigned as putative alcohol dehydrogenases by Cole *et al.* (1998); hyp-ADH *R. rho.*, gene product from *R. rhodochrous*, whose corresponding gene is found in a plasmid-located operon related to haloalkane dehalogenation, which has been assigned as a hypothetical alcohol dehydrogenase by Kulakova *et al.* (1997). Data from Eklund *et al.* (1990) for the corresponding residues of NAD-dependent alcohol dehydrogenase from horse liver (the ethanol-active subunit: E-type) are included for comparison.

Residue*	np-ADH *A. met.*	AdhD *M. tub.*	hyp-ADH *R. rho.*	AdhB *M. tub.*	ADH (E-type) horse liver
Inner					
48	**Ser**	**Ser**	**Ser**	**Ser**	**Ser**
93	**Phe**	**Phe**	**Phe**	**Phe**	**Phe**
140	**Met**	**Met**	**Met**	**Met**	*Phe*
141	**Cys**	**Cys**	**Cys**	*Thr*	*Leu*
Middle					
57	**Leu**	**Leu**	**Leu**	Ile	**Leu**
116	**Ala**	**Ala**	Gln	**Ala**	Leu
294	**Leu**	**Leu**	**Leu**	Ile	Val
318	**Leu**	**Leu**	**Leu**	Ile	Ile
Outer					
110	**Leu**	**Leu**	**Leu**	**Leu**	*Phe*
306	**Val**	*Asp*	*Asp*	*Met*	*Met*
309	**Leu**	**Leu**	**Leu**	**Leu**	**Leu**
Catalytic Zn					
46	**Cys**	**Cys**	**Cys**	**Cys**	**Cys**
67	**His**	**His**	**His**	**His**	**His**
174	**Cys**	**Cys**	**Cys**	**Cys**	**Cys**
NAD(H)-binding					
47	**His**	**His**	**His**	Arg	Arg
48	**Ser**	**Ser**	**Ser**	**Ser**	**Ser**
51	**His**	**His**	**His**	**His**	**His**
269	**Val**	**Val**	*Thr*	**Val**	Ile
271	**Val**	**Val**	Gly	Leu	*Arg*
369	**Arg**	**Arg**	**Arg**	**Arg**	**Arg**
Sequence length (aa)	371	368	370	375	375
Sequence identity (%)	100	78	51	46	35
Sequence similarity (%)	100	86	69	65	52

*Inner, middle and outer refer to different spheres of the substrate binding-pocket of the class 1 NAD-dependent alcohol dehydrogenase from horse liver (Eklund *et al.*, 1990; Piersma, 1998). Also included are the regions involved in the binding of the catalytic zinc ion and the pyridine nucleotide coenzyme.

of the product, benzaldehyde. However, since np-ADH shows no dehydrogenase activity and a very low dismutase activity for this compound, the latter possibility can be excluded. Since no other candidate functioning as electron acceptor for np-ADH is present during cultivation of the organism on benzyl alcohol, it seems logical that the organism's respiratory chain is the electron acceptor for np-ADH. Evidence has been provided for this in the case of MNO (Bystrykh *et al.*, 1997).

In view of the induction of np-ADH by many alcohols, as deduced from the activity exhibited by extracts in the assay with benzyl alcohol and NDMA, the absence or low activity of other alcohol dehydrogenases, and the broad substrate specificity of np-ADH, the enzyme may be the general alcohol-oxidizing enzyme for *R. erythropolis* DSM 1069. Since its assay is not standard practice, the significance of np-ADH may be even wider, as it may have been overlooked so far in other organisms. Accordingly, a search in the databases was made to see

whether structural evidence could be found. It appeared that the N-terminal amino acid sequence of the AdhD gene product of *Mycobacterium tuberculosis* H37Rv (Cole *et al.*, 1998), which was designated by the authors as a putative alcohol dehydrogenase, has 73 % identical amino acids, as shown in Fig. 1. Also, a significant sequence identity (51 %) was observed with a hypothetical alcohol dehydrogenase from *R. rhodochrous* NCIMB 13064, whose corresponding gene occurs in a dehalogenation operon (Kulakova *et al.*, 1997), and with the AdhB gene product (46 %) from *M. tuberculosis* (not shown). Recently, the complete amino acid sequence of np-ADH from *A. methanolica* has been determined and structural modelling was carried out using this sequence and the known three-dimensional structure of horse liver alcohol dehydrogenase (see Piersma, 1998). Taking the differences with respect to structural and catalytic aspects into account, this provided a number of amino acid residues which can be used as markers to distinguish between the two types of alcohol dehydrogenase (Table 4). Applying this to the sequences found in the databases, it appears that characteristic amino acid residues for np-ADH are present, most of them in the gene product of the *adhD* gene of *M. tuberculosis* and fewer in those of the hypothetical alcohol dehydrogenase from *R. rhodochrous* and the AdhB gene product of *M. tuberculosis* (Table 4). It can be anticipated, therefore, that np-ADHs may also be found in other nocardioform actinomycetes.

The ability of np-ADH to interconvert alcohols and aldehydes may be physiologically important under conditions where such a combination occurs, e.g. in the biodegradation of wood, where a complex interplay of aldehydes and alcohols is responsible for generating H_2O_2-activated peroxidase and reactive intermediates for attacking lignin (Kersten *et al.*, 1990). Benefits of using a nicotinoprotein alcohol dehydrogenase instead of an NAD(P)-dependent alcohol dehydrogenase for this could be that the interconversion is not directly affected by the redox balance of the cell (the activities of np-ADH are not affected by the intracellular NAD/NADH ratio). This property as well as the ability of np-ADH to catalyse these reactions in the absence of (added) coenzyme and, as shown here, its stability in this, could well be exploited in industrially relevant conversions.

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