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NAD$^+$-Dependent (*S*)-Specific Secondary Alcohol Dehydrogenase Involved in Stereoinversion of 3-Pentyn-2-ol Catalyzed by *Nocardia fusca* AKU 2123

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An NAD$^+$-dependent alcohol dehydrogenase was purified to homogeneity from *Nocardia fusca* AKU 2123. The enzyme catalyzed (*S*)-specific oxidation of 3-pentyn-2-ol (PYOH), *i.e.*, part of the stereoinversion reaction for the production of (*R*)-PYOH, which is a valuable chiral building block for pharmaceuticals, from the racemate. The enzyme used a broad variety of secondary alcohols including alkyl alcohols, alkenyl alcohols, acetylenic alcohols, and aromatic alcohols as substrates. The oxidation was (*S*)-isomer specific in every case. The K_m and V_{max} for (*S*)-PYOH and (*S*)-2-hexanol oxidation were 1.6 mM and 53 μmol/min/mg, and 0.33 mM and 130 μmol/min/mg, respectively. The enzyme also catalyzed stereoselective reduction of carbonyl compounds. (*S*)-2-Hexanol and ethyl (*R*)-4-chloro-3-hydroxybutanoate in high optical purity were produced from 2-hexanone and ethyl 4-chloro-3-oxobutanoate by the purified enzyme, respectively. The K_m and V_{max} for 2-hexanone reduction were 2.5 mM and 260 μmol/min/mg. The enzyme has a relative molecular mass of 150,000 and consists of four identical subunits. The NH_2-terminal amino acid sequence of the enzyme shows similarity with those of the carbonyl reductase from *Rhodococcus erythropolis* and phenylacetaldehyde reductase from *Corynebacterium* sp.

Key words: secondary alcohol dehydrogenase; stereoinversion; *Nocardia fusca*; acetylenic alcohol; stereospecific reduction

Optically active acetylenic alcohols are useful for organic syntheses as chiral building blocks.[1-3] We have established the production of optically active 3-pentyn-2-ol (PYOH) through stereospecific hydrolysis of the corresponding esters[4,5] or stereoinversion of the racemate by microorganisms.[6,7] The production of (*R*)-PYOH from the racemate through stereoinversion by *Nocardia fusca* AKU 2123 seemed to involve the stereospecific oxidation of (*S*)-PYOH to 3-pentyn-2-one and the successive stereoselective reduction of 3-pentyn-2-one to (*R*)-PYOH.[7] To discover the mechanism of the stereoinversion catalyzed by *N. fusca* AKU 2123, and to obtain information for improving the practical process, purification and characterization of the enzyme involved in the stereoinversion were done. In this paper, we report the properties of the enzyme catalyzing (*S*)-PYOH oxidation. The purified enzyme showed NAD$^+$-dependent (*S*)-specific secondary alcohol dehydrogenase activity.

Materials and methods

Chemicals. (*R*)-, (*S*)- and (*RS*)-3-PYOH were kind gifts from Nippon Zeon Co., Ltd. (Japan). All other chemicals used in this work were of analytical grade, commercially available, and used without further purification.

Microorganism and cultivation. Nocardia fusca AKU 2123 preserved in our laboratory (AKU Culture Collection; Faculty of Agriculture, Kyoto University) was used as the enzyme source. The medium comprised 0.4% glucose, 1% malt extract, and 0.4% yeast extract (pH 7.3). *N. fusca* was inoculated into test tubes (16 × 165 mm) containing 5 ml of the medium and then incubated for 6 days at 28°C with shaking. The cultures were then transferred to 2-l shaking flasks containing 500 ml of medium. After cultivation for 6 days at 28°C with shaking, the cells were collected by centrifugation and washed twice with physiological saline, and then stored at −20°C.

Enzyme assay. The standard assay mixture for oxidation comprised, in 220 μl, 200 mM potassium phosphate buffer (pH 7.5), 0.9 mM NAD(P)$^+$, 10 mM (*RS*)-PYOH and an appropriate amount of the enzyme. For reduction, NAD(P)$^+$ and (*RS*)-PYOH were replaced by NAD(P)H (0.23 mM) and 2-hexanone (10 mM), respectively. The reaction mixture was incubated for 2 min without substrate at 30°C, and then the reaction was started by the addition of substrate. The increase or decrease in the amount of the coenzyme was measured spectrophotometrically at 340 nm. A molar extinction coefficient of 6,220 M^{-1} cm^{-1} for NAD(P)H was used for calculation of the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction/oxidation of 1 μmol of NAD$^+$/NADH per min under the assay conditions. Reaction conditions were investigated with (*RS*)-2-hexanol and 2-hexanone for oxidation and reduction, respectively, under the standard assay conditions except that the buffer (200 mM) was changed to CH_3COOH/CH_3COONa (pH 3.5–6.0), potassium phosphate (pH 5.5–8.0), Tris/HCl (pH 7.0–9.5), or glycine/NaOH (pH 8.5–12.5), and the temperature was changed in the range of 10 to 80°C.

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Enzyme purification. All purification procedures were done at 0–4°C, and 20 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM dithiothreitol was used as the buffer. Centrifugation was done at 14,000 × *g* for 30 min unless otherwise specified.

Step 1. Preparation of a cell-free extract. N. fusca cells (30 g wet cells) suspended in 30 ml of buffer were disrupted with 0.25 mm diameter glass beads (Dyno-Mill KDL, Switzerland) at 4°C for 60 min (270 ml). After centrifugation, the resulting supernatant was used as the cell-free extract.

Step 2. Solid ammonium sulfate fractionation. The cell-free extract was fractionated with solid ammonium sulfate. The precipitate obtained at 40–60% saturation was collected by centrifugation and dissolved in buffer (60 ml). The resultant enzyme solution was dialyzed against 20 liters of the buffer.

Step 3. DEAE-Sephacel chromatography. The dialysate was put on a DEAE-Sephacel column (5 × 15 cm) equilibrated with buffer. The column was washed with the buffer (200 ml), and then the enzyme was eluted with a linear gradient of 0–1 M NaCl in 600 ml of buffer. The active fractions were combined (128 ml) and concentrated to 9 ml by ultrafiltration with an Amicon YM-10 membrane.

Step 4. Alkyl-Superose HR10/10 column chromatography. After the ammonium sulfate concentration had been adjusted to 1.5 M with solid ammonium sulfate, the enzyme solution was put on an Alkyl-Superose HR10/10 column equilibrated with the buffer containing 1.5 M ammonium sulfate. The enzyme was eluted by lowering the ionic strength of ammonium sulfate linearly from 1.5 to 0 M (60 ml), and the active fractions were collected (6 ml).

Step 5. Sephacryl S-200 HR gel filtration chromatography. The enzyme solution was put on a Sephacryl S-200 HR column (1.25 × 80 cm), equilibrated with the buffer containing 0.2 M NaCl. The enzyme was eluted with 800 ml of the same buffer. The active fractions were collected (18 ml) and dialyzed against the buffer (20 l).

Step 6. MonoQ HR5/5 column chromatography. The dialyzed solution was put on a MonoQ HR5/5 column equilibrated with buffer. The enzyme was eluted with a linear gradient of NaCl, 0–1 M (30 ml). The active fractions were collected (1.5 ml).

Step 7. Phenyl-Superose HR5/5 chromatography. After the NaCl concentration had been adjusted to 4 M with NaCl, the enzyme solution was put on a Phenyl-Superose HR5/5 column equilibrated with the buffer containing 4 M NaCl. The enzyme was eluted by lowering the ionic strength of NaCl from 4 to 0 M. The active fractions were collected (1 ml), and used for characterization.

Characterization of the enzyme. The relative molecular mass was measured by HPLC on a G3000 SW column (7.5 × 600 mm, Tosoh), as described previously.[8] SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% polyacylamide gel and protein-concentration measurement were done as described previously.[9]

NH_2-Terminal amino acid sequence analysis. The NH_2-terminal amino acid sequence was analyzed with a model 476A pulsed-liquid-phase protein sequencer with an on-line phenylthiohydantoin analyzer. The NH_2-terminal amino acid sequence obtained was compared with those of proteins stored in the SWISS-PROT, PIR, and PRF protein databases.

Analytical methods. Measurements of PYOH, 3-pentyn-2-one, 2-hexanol, and 2-hexanone were done by gas-liquid chromatography (GLC). A GLC apparatus, Shimadzu GC-14B, equipped with a flame ionization detector and a glass column (3 mm × 2 m) packed with 10% polyethyleneglycol (PEG 20M) on 60/80 mesh Chromosorb W (Nishio Kogyo, Japan) was used: column temperature, 70°C for PYOH and 3-pentyn-2-one (retention times, t_r: 6.9 min and 2.7 min, respectively), 40°C for 2-hexanol and 2-hexanone (t_r: 12.8 min and 4.3 min, respectively); injection port temperature, 230°C; N_2 flow rate, 60 ml/min; air and H_2, 0.6 kg/cm^2. The optical purity of PYOH and 2-hexanol was analyzed by GLC. A GLC apparatus, Shimadzu GC-17A, with a flame ionization detector and a capillary column (Chiralsil-DEX CB, 0.25 mm × 25 m; Chrompack, USA) was used: column temperature, 70°C; injection port temperature; 230°C. t_r: (*R*)-PYOH, 8.6 min; (*S*)-PYOH, 9.4 min; (*R*)-2-hexanol, 11.4 min; (*S*)-2-hexanol, 11.7 min. The carrier gas was helium (2 ml/min), and N_2 (60 ml/min) was used as the make-up gas. The enantiomeric excess (*e.e.*) was calculated from the peak areas of the stereoisomers.

Measurements of ethyl 4-chloro-3-hydroxybutanoate (CHBE) and ethyl 4-chloro-3-oxobutanoate (COBE) were done by GLC on a glass column packed with 10% PEG 20M on 60/80 mesh Chromosorb W (Nishio Kogyo, Japan), as described previously.[10] The optical purity of CHBE was measured by HPLC on a Chiralcel OB packed column (4.6 × 250 mm; Daicel Chemical Industries, Japan), as described previously.[11]

Results

Cofactor requirement and stereospecificity

The cofactor requirement and stereospecificity of

Table 1. Purification of the NAD^+-Dependent (*S*)-Specific Alcohol Dehydrogenase from *N. fusca*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
1) Cell-free extract	2750	966	0.35	100
2) Ammonium sulfate	777	724	0.93	75
3) DEAE-Sephacel	124	531	4.28	55
4) Alkyl-Superose HR 10/10	37.5	338	9.02	35
5) Sephacryl S-200 HR	22.0	232	10.5	24
6) MonoQ HR 5/5	5.40	78.3	14.5	8.1
7) Phenyl-Superose HR 5/5	0.45	18.7	41.5	2.0

Enzyme activity was assayed under the standard reaction conditions for oxidation with (*RS*)-PYOH as a substrate.

PYOH oxidation catalyzed by the cell-free extract of *N. fusca* were investigated. In the presence of NAD^+ but not $NADP^+$, the cell-free extract catalyzed the oxidation, suggesting that the enzyme is an NAD^+-dependent dehydrogenase. With NAD^+ as a cofactor, both (*S*)-PYOH and (*R*)-PYOH were oxidized at the rates of 350×10^{-3} and 47×10^{-3} μmol/min/mg protein, respectively. (*S*)-PYOH was oxidized much faster than (*R*)-PYOH. These results suggest that the cell-free extract contained an (*S*)-specific alcohol dehydrogenase, and this enzyme was further purified.

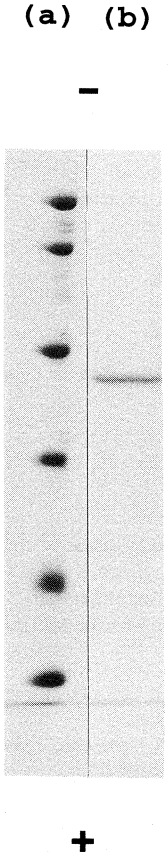

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis of the Enzyme.
(a) Standards (from top): phosphorylase *b* (M_r=97,400), bovine serum albumin (66,300), aldolase (42,400), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). (b) Purified enzyme. The gel was stained for protein with Coomassie Brilliant Blue R-250 and destained in ethanol/acetic acid/water (3:1:6, by vol.).

Purification of the enzyme

Typical results of enzyme purification are shown in Table 1. The enzyme was purified 118-fold, with a 2.0% yield, from the cell-free extract. The purified enzyme gave a single band on SDS-PAGE (Fig. 1).

Molecular mass and subunit structure

The relative molecular mass of the native enzyme was found to be 150,000 on high-performance gel-permeation liquid chromatography on a TSK G3000 SW column. The relative molecular mass of the subunit was estimated to be 39,000 on SDS-PAGE (Fig. 1). These results suggest that the enzyme is a homotetramer.

NH_2-Terminal amino acid sequence analysis

The NH_2-terminal amino acid sequence of the purified enzyme was analyzed as shown in Fig. 2. When this sequence was compared with those of proteins stored in the protein-sequence databases described under Materials and methods, it was found to show similarity with the carbonyl reductase from *Rhodococcus erythropolis* (RECR)[12] and phenylacetaldehyde reductase (PAR) from *Corynebacterium* strain ST-10 (Fig. 2).[13] There was 78 percent similarity for both of them.

Substrate specificity and catalytic properties

The substrate specificity of the enzyme is summarized in Table 2. For oxidation, aliphatic secondary alcohols, including alkyl, alkenyl, alkynyl, and aromatic ones, and β-hydroxyacid ester were accepted as substrates. Primary alcohols such as 1-hexanol, 2-butene-1-ol, 3-pentyn-1-ol, and benzyl alcohol were not accepted as substrates, suggesting that the enzyme is a secondary alcohol dehydrogenase. Medium-chain secondary alcohols such as (*RS*)-2-hexanol and (*RS*)-2-heptanol served as good substrates, and alkyl alcohols were preferably oxidized compared to alkenyl and alkynyl alcohols.

The enzyme catalyzed the reverse reaction, *i.e.*, NADH-dependent reduction of ketones, aldehydes, and keto esters. Alkyl, alkenyl, and aromatic ketones served as substrates, and medium-chain alkyl ketones were more favorable substrates for reduction. 2-Heptanone was the best substrate for reduction among the tested compounds.

Kinetic parameters were measured by double reciprocal Lineweaver-Burk plots with (*RS*)-, (*S*)- or (*R*)-PYOH, or (*RS*)-, (*S*)- or (*R*)-2-hexanol as the substrate for oxidation, and 2-hexanone as the substrate for reduction. For (*RS*)-, (*S*)- or (*R*)-PYOH, K_m and V_{max} were

NFADH	M	K	A	L	Q	Y	V	T	V	G	A	E	P	E	V	R	E	I	P	19
RECR	M	K	A	I	Q	Y	T	R	I	G	A	E	P	E	L	T	E	I	P	19
PAR	M	K	A	M	Q	Y	T	R	I	G	A	E	P	E	L	T	E	I	P	19

NFADH	T	P	E	P	G	P	G	E	V	L	L	R	V	T	A	A	G	V	37
RECR	K	P	E	P	G	P	G	E	V	L	L	E	V	T	A	A	G	V	37
PAR	K	P	E	P	G	P	G	E	V	L	L	E	V	T	A	A	G	V	37

Fig. 2. Comparison of the NH_2-Terminal Amino Acid Sequence of the Secondary Alcohol Dehydrogenase from *N. fusca* (NFADH), Carbonyl Reductase from *Rhodococcus erythropolis* DSM 743 (RECR), and Phenylacetaldehyde Reductase (PAR) from *Corynebacterium*.
Identical amino acid residues are enclosed in boxes.

Table 2. Substrate Specificity of the NAD^+-Dependent (*S*)-Specific Alcohol Dehydrogenase from *N. fusca*[a]

Substrate[b]	Relative[c] activity (%)	Substrate	Relative activity (%)
Alcohol		Ketone	
(*RS*)-2-propanol	8.1	acetone	19
(*RS*)-2-butanol	19	2-butanone	23
(*RS*)-2-pentanol	76	2-pentanone	59
(*RS*)-2-hexanol	100	2-hexanone	225
(*RS*)-2-heptanol	92	2-heptanone	334
(*RS*)-2-octanol	84	2-octanone	223
(*RS*)-2-nonanol	81	2-nonanone	259
(*RS*)-2-decanol	85	2-decanone	159
(*RS*)-2-undecanol	27	2-undecanone	58
(*RS*)-2-tetradecanol	8.9	2-dodecanone	25
(*RS*)-2-tridecanol	14	2-tridecanone	17
(*RS*)-3-buten-2-ol	5.6	2-tetradecanone	27
(*RS*)-3-penten-2-ol	26	3-penten-2-one	1.6
(*RS*)-3-butyn-2-ol	2.7	6-methyl-5-hepten-2-one	188
(*RS*)-3-pentyn-2-ol	23	chloroacetone	28
(*RS*)-4-pentyn-2-ol	1.4	acetophenone	14
(*RS*)-1-phenylethanol	9.5	benzyl acetone	136
(*RS*)-1-phenyl-2-propanol	12	ethyl 3-oxobutanone	166
(*RS*)-4-phenyl-2-butanol	46	1-phenyl-1,2-propanedione	142
(*RS*)-2-phenyl-2-propanol	0.3	2,3-pentanedione	29
Diol		Aldehyde	
(*RS*)-1,2-propanediol	0.8	propionaldehyde	7.4
(*RS*)-1,2-butanediol	0.3	*n*-butyraldehyde	24.2
(*RS*)-2,3-butanediol	1.1	isobutyraldehyde	15.9
(*RS*)-1,2-pentanediol	1.3	*n*-valeraldehyde	84
(*RS*)-2,4-pentanediol	2.6	isovaleraldehyde	9.7
(*RS*)-1,2-hexanediol	5.1	1-hexanal	254
(*RS*)-2,5-hexanediol	46.8	*n*-heptaldehyde	0.9
		1-octanal	3.5
Ester			
ethyl (*RS*)-3-hydroxybutanoate	10	Keto ester	
ethyl (*RS*)-4-chloro-3-hydroxybutanoate	3.3	pyruvic acid methyl ester	110
		pyruvic acid ethyl ester	225
		ethyl 4-chloro-3-oxobutanoate	38.1
		methyl 4-chloro-3-oxobutanoate	5.2

[a] The reaction pHs for the oxidation and reduction were 9.0 and 6.0, respectively.
[b] The 1-phenyl-1,2-propanedione substrate concentration was 5 mM, the others being 10 mM.
[c] To calculate the relative activity, the activity for (*RS*)-2-hexanol (92 μmol/min/mg protein) was taken as 100%.
[d] The following compounds did not serve as substrates: for oxidation, methanol, ethanol, 1-propanol, 1-butanol, 1-propanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol, 1-dodecanol, 2-butene-1-ol, 2-butyn-1-ol, 3-butyn-1-ol, 2-pentyn-1-ol, 3-pentyn-1-ol, and benzyl alcohol; for reduction, 3-methyl-2-butanone, 2,4-pentanedione, benzaldehyde, phenylacetaldehyde, and chloroacetaldehyde.

1.9 mM and 43 μmol/min/mg, 1.6 mM and 53 μmol/min/mg, and 3.0 mM and 26 μmol/min/mg, at pH 9.0, respectively. For (*RS*)-, (*S*)- or (*R*)-2-hexanol, K_m and V_{max} were 0.45 mM and 93 μmol/min/mg, 0.33 mM and 130 μmol/min/mg, and 1.8 mM and 25 μmol/min/mg, at pH 9.0, respectively. For 2-hexanone, K_m and V_{max} were 2.5 mM and 260 μmol/min/mg, at pH 6.0, respectively. The apparent K_m for NAD^+ on (*RS*)-2-hexanol oxidation is 0.5 mM, and that of NADH on 2-hexanone reduction is 0.057 mM. The double reciprocal Lineweaver-Burk plots for (*RS*)-2-hexanol oxidation with various NAD^+ concentrations and a fixed (*RS*)-2-hexanol concentration showed an intersecting initial velocity pattern (Fig. 3(A)). The same plots of the reverse reaction, 2-hexanone reduction, showed a parallel pattern (Fig. 3(B)). These results suggest that the reaction mechanism is a sequentially ordered one, because two substrate reactions following sequentially ordered mechanism give an intersecting initial pattern for the slow reaction (oxidation at pH 9.0) and a nearly parallel pattern for the reverse direction (reduction at pH 6.0) on the condition that the forward and backward initial reaction rates are sufficiently different.[14,15]

Stereospecificity

The stereospecificity for oxidation, as for some secondary alcohols, was examined (Table 3). With all alcohols tested, the initial oxidation rates of (*S*)-isomers were much faster than those of (*R*)-isomers. These results were also supported by the results of K_m and V_{max} measurements for (*S*)- and (*R*)-isomers described above. After prolonged incubation with (*RS*)-PYOH or (*RS*)-2-hexanol as the substrate, only the (*R*)-isomers of the corresponding alcohols remained in the reaction mixtures though they underwent some oxidation (Fig. 4). With 2-hexanone or ethyl 4-chloro-3-oxobutanoate

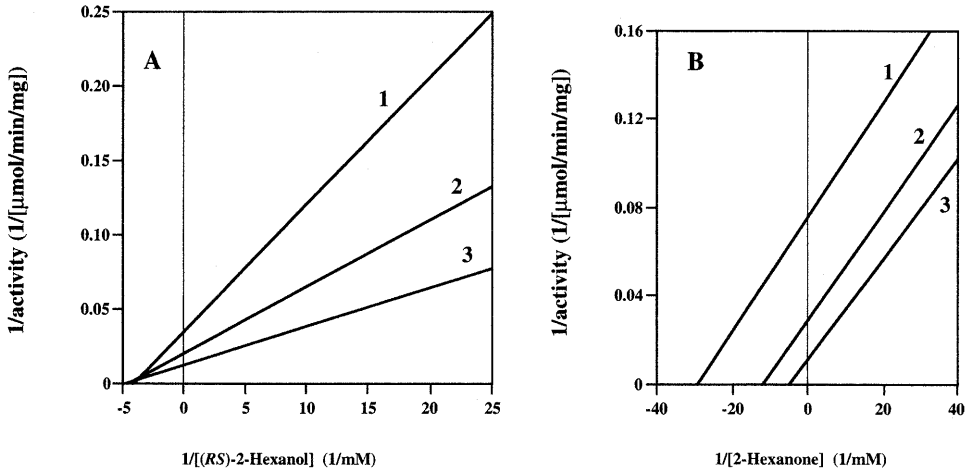

Fig. 3. The Initial Velocity Patterns of the Secondary Alcohol Dehydrogenase from *N. fusca* for the Oxidation (A) and Reduction (B) by Double Reciprocal Plots of Lineweaver-Burk.

The reactions were done under the standard assay conditions with varied concentrations of (*RS*)-2-hexanol and fixed concentrations of NAD^+ at 0.03 (1), 0.06 (2) and 0.18 (3) mM for oxidation, or with varied concentrations of 2-hexanone and fixed concentrations of NADH at 0.03 (1), 0.06 (2) and 0.18 (3) mM for reduction.

Table 3. Stereoselectivity of NAD^+-Dependent Secondary Alcohol Oxidation[a]

Substrate[b]	Relative activity[c] (%)
(*RS*)-2-Butanol	19
(*R*)-2-Butanol	6.0
(*S*)-2-Butanol	27
(*RS*)-2-Pentanol	76
(*R*)-2-Pentanol	15
(*S*)-2-Pentanol	91
(*RS*)-2-Hexanol	100
(*R*)-2-Hexanol	30
(*S*)-2-Hexanol	121
(*RS*)-3-Butyn-2-ol	2.7
(*R*)-3-Butyn-2-ol	0.5
(*S*)-3-Butyn-2-ol	6.9
(*RS*)-3-Pentyn-2-ol	23
(*R*)-3-Pentyn-2-ol	5.6
(*S*)-3-Pentyn-2-ol	33
(*RS*)-1-Phenylethanol	9.5
(*R*)-1-Phenylethanol	0.9
(*S*)-1-Phenylethanol	21

[a] The reactions were done under the standard assay conditions at pH 9.0 with the substates (10 mM) indicated in the table.

[b] To calculate the relative activity, the activity for (*RS*)-2-hexanol (92 μmol/min/mg protein) was taken as 100%.

(COBE) as the substrate for reduction, the optical purity of the reduction product was analyzed. (*S*)-2-Hexanol with 100% *e.e.* or ethyl (*R*)-4-chloro-3-hydroxybutanoate (CHBE) with 98.2% *e.e.* was formed from the corresponding ketone (Fig. 5). (The stereostructure of (*R*)-CHBE is the same as those of (*S*)-PYOH and (*S*)-2-hexanol, but the notation is opposite because of the existence of chlorine at the C-4 position of CHBE). As reported for RECR[12] and PAR,[13,16] strict stereoselectivity for reduction and slightly relaxed stereospecificity for oxidation were observed at all pHs tested.

Table 4. Effects of Various Chemicals on the Enzyme Activity[a]

Compound	Concentration (mM)	Addition	Relative activity (%)
None			100
o-Phenanthroline	2		0.4
EDTA	2		56
EDTA	2	$ZnSO_4$(5 mM)	115
EDTA	2	$NiCl_2$ (5 mM)	101
Trypaflavine	1		7.7
Quinaerine	2		28
Iodoacetate	2		67
$HgCl_2$	2		0
$AgNO_3$	2		0
$CdCl_2$	2		6.6
$SnCl_2$	2		46
$ZnSO_4$	2		95
$CoCl_2$	2		88
$NiCl_2$	2		92
$CuSO_4$	2		89
$MgCl_2$	2		88
$MnCl_2$	2		83

[a] Enzyme activity was measured as described under Materials and methods, except for the addition of the indicated compounds. (*RS*)-2-Hexanol (10 mM) was used the substrate and the activity without an inhibitor (92 μmol/min/mg protein) was taken as 100%. The following compounds did not cause much inhibition (0–20% inhibition); diphenylhydantoin, phenylmethylsulfonyl fluoride, *N*-bromosuccinimide, 2-mercaptoethanol, dithiothreitol, $NaIO_4$, $AlCl_3$, $CeSO_4$, $CaCl_2$, $FeSO_4$, $FeCl_3$ and CsCl.

Effects of chemicals

The various compounds listed in Table 4 were added to the standard reaction mixture, and then the relative activity was measured with (*RS*)-2-hexanol as the substrate. The enzyme activity was significantly inhibited by metal ion chelators such as *o*-phenanthroline and EDTA, suggesting that the enzyme requires metal ions for its activity. The inhibition caused by EDTA was reversed by the addition of $ZnSO_4$ or $NiCl_2$, suggesting that such divalent metal ions are important in the en-

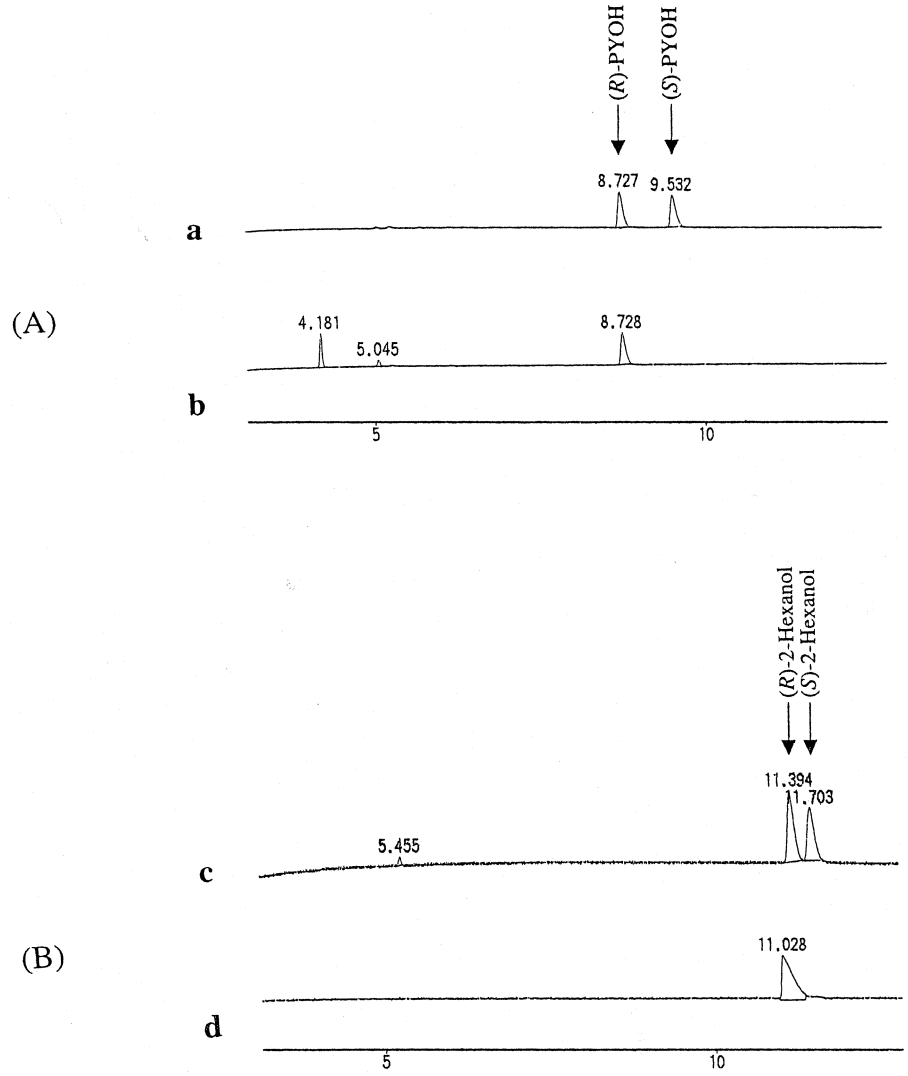

Fig. 4. Stereospecificity of the Secondary Alcohol Dehydrogenase from *N. fusca* in the Oxidation of (*RS*)-PYOH (A) and (*RS*)-2-Hexanol (B). **a** and **c**, before reactions. **b** and **d**, after reactions. The oxidations of (*RS*)-PYOH (10 mM) and (*RS*)-2-hexanol (10 mM) were done in 200 mM glycine/NaOH buffer (pH 9.0) with 25 mM NAD^+ for 24 hours at 30°C respectively, and then the products were analyzed as described under Materials and methods. PYOH, 3-pentyn-2-ol.

zyme activity. The enzyme activity was also inhibited by trypaflavine and quinacrine (nonspecific inhibitors of carbonyl reductase and aldehyde reductase), iodoacetate (sulfhydryl reagent), and heavy metal ions such as $HgCl_2$, $AgNO_3$, $CdCl_2$, and $SnCl_2$.

Effects of pH and temperature

The optimum pH for the oxidation with (*RS*)-2-hexanol as the substrate was 8.5–9.5 (glycine/NaOH), and that for the reduction with 2-hexanone as the substrate was 5.5–6.5 (potassium phosphate). The optimum temperature for the oxidation with (*RS*)-2-hexanol as the substrate was 60°C, and that for the reduction with 2-hexanone as the substrate was 65°C. The enzyme was stable between pH 5.5–9.5 (more than 90% activity remaining) for 30 min of incubation at 30°C. It was also stable up to 30°C (more than 90% activity remaining) for 30 min of incubation under pH 7.0. At 40°C, about 85% activity remained, but only 50% at 50°C.

Discussion

The enzyme catalyzing the stereospecific oxidation of (*S*)-PYOH, which is the first reaction in the stereoinversion for the production of (*R*)-PYOH from the racemate (Fig. 6), was purified to homogeneity from *N. fusca*. Based on its cofactor requirement, stereoselectivity, and substrate specificity, the enzyme was identified as an NAD^+-dependent (*S*)-specific secondary alcohol dehydrogenase.

In the (*R*)-PYOH production from the racemate of PYOH through stereoinversion with whole cells of *N. fusca*, the addition of NADPH accelerates the (*R*)-PYOH production, while the addition of NADH inhibits the reaction at the stage of (*S*)-PYOH oxidation.[7] This indicated that the oxidation of (*S*)-PYOH by *N. fusca* is a reversible reaction catalyzed by an NAD(H)-dependent dehydrogenase. The characteristics of the purified enzyme well explain this, *i.e.*, the enzyme also

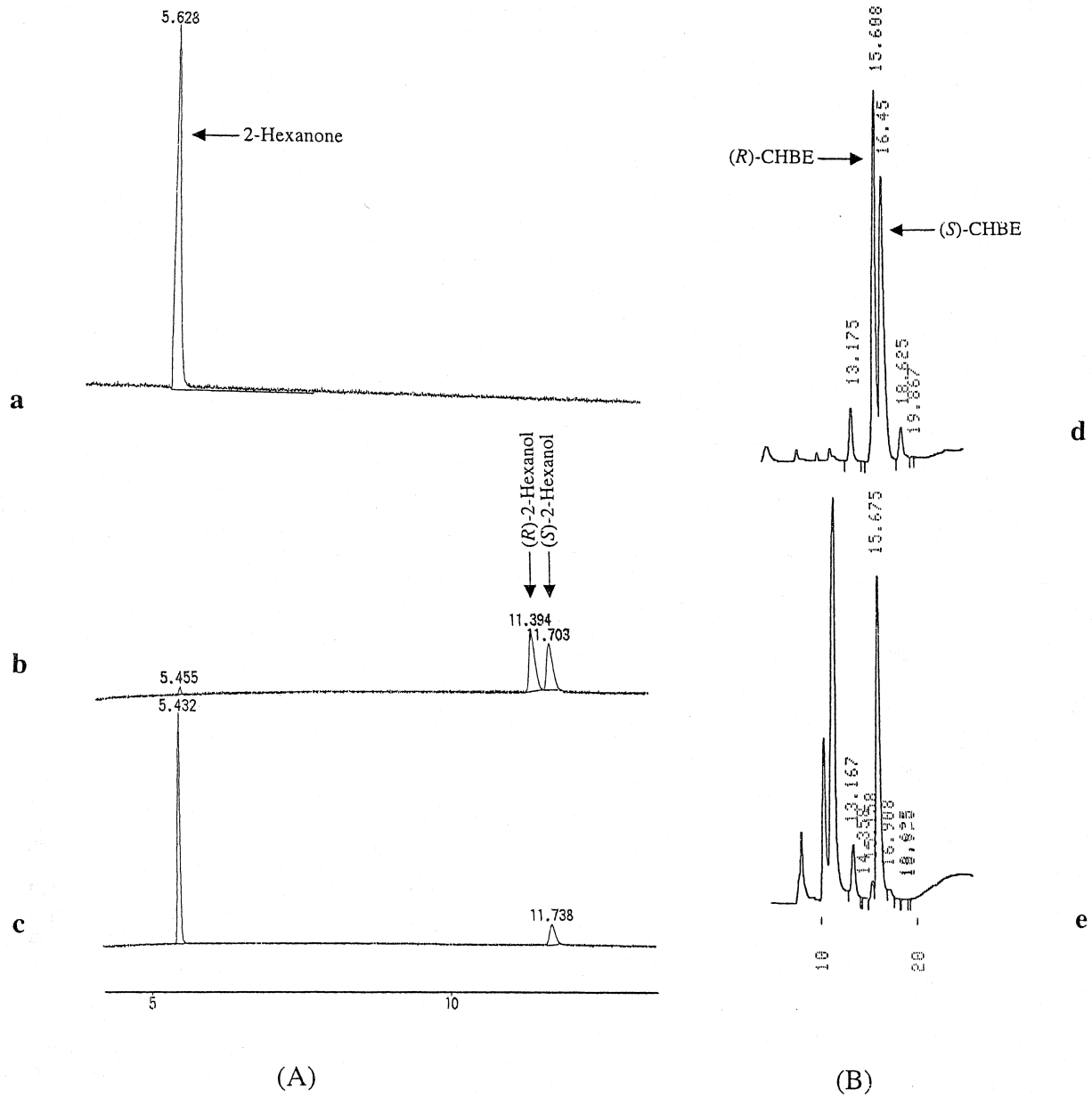

Fig. 5. Stereospecificity of the Secondary Alcohol Dehydrogenase from *N. fusca* in the Reduction of 2-Hexanone (A) and COBE (B).
a, **b** and **d**, authentic samples. **c** and **e**, enzyme reaction products. The reductions of 2-hexanone (10 mM) and COBE (10 mM) were done in 200 mM potassium phosphate buffer (pH 6.0) with 25 mM NADH for 24 hours at 30°C, and then the products were analyzed as described under Materials and methods. CHBE, ethyl 4-chloro-3-hydroxybutanoate; COBE, ethyl 4-chloro-3-oxobutanoate.

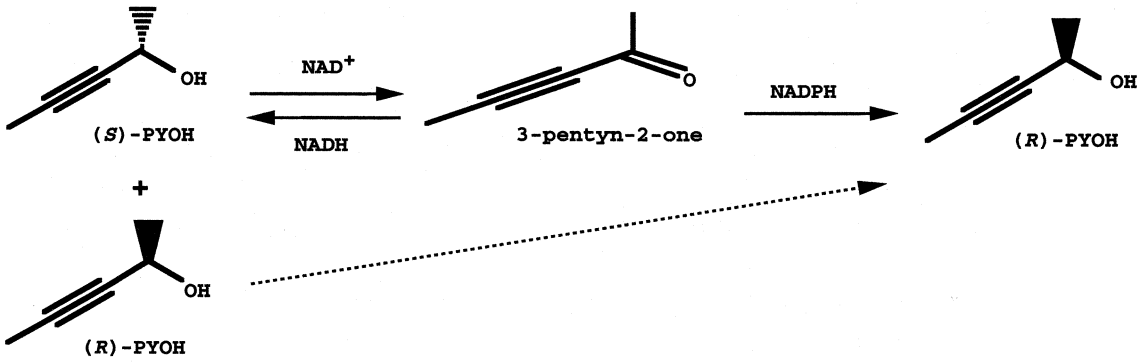

Fig. 6. Stereoinversion of (*RS*)-PYOH to (*R*)-PYOH Catalyzed by *N. fusca*.
The first reaction, *i.e.*, NAD^+-dependent oxidation of (*S*)-PYOH to 3-pentyn-2-one, is catalyzed by the secondary alcohol dehydrogenase from *N. fusca*.

Table 5. Comparison of the Characteristics of Purified NAD(H)-dependent (*S*)-Specific Enzymes from Various Microorganisms

Source	Enzyme	M_r (kDa)		Oxidation		Reduction	
		Native	Subunit	Optimum pH	Optimum Temp (°C)	Optimum pH	Optimum Temp (°C)
Nocardia fusca[a]	ADH[b]	150	39	8.5–9.5	60	5.5–6.5	65
Rhodococcus erythropolis DSM 743[12]	CR[c]	161	40	9.5	42	5.5	40
Rhodococcus erythropolis ATCC 4277[21]	ADH	110	48	9	74	—	—
Rhodococcus erythropolis[22]	ADH	95	48	—	—	6	40
Candida parapsilosis[23]	CR	135	67.5	9–10	52–56	7.5–8.5	36–40
Corynebacterium sp. strain, ST-10[13]	PAR[d]	155	42	—	—	6.0–6.5	—

[a] This study, —: not determined.
[b] ADH: Alcohol dehydrogenase.
[c] CR: Carbonyl reductase.
[d] PAR: Phenylacetaldehyde reductase.

catalyzes the NADH-dependent reduction of ketones (*S*)-specifically. Considering the above, the key enzyme, which decides the direction of the stereoinversion reaction, is an NADP(H)-dependent (*R*)-specific dehydrogenase (or reductase). The purification of this enzyme is now underway.

The NAD^+-dependent (*S*)-specific secondary alcohol dehydrogenase from *N. fusca* (NFADH) is different from the general NAD^+-dependent (*S*)-specific alcohol dehydrogenase (EC 1.1.1.1),[17] $NAD^+/NADP^+$-dependent aryl-alcohol dehydrogenase (EC 1.1.1.90 and 91),[18,19] and $NADP^+$-dependent (*R*)-1-phenylethanol dehydrogenase from *Lactobacillus kefir*[20] in substrate specificity, but shows some similarities with other known NAD(H)-dependent (*S*)-specific enzymes (Table 5),[12,13,21-23] especially with the carbonyl reductase from *R. erythropolis* (RECR)[12] and phenylacetaldehyde reductase from *Corynebacterium* sp. (PAR)[13,16] in molecular size, NH_2-terminal amino acid sequence (Fig. 2), and stereoselectivity. RECR is classified as a medium-chain, zinc-containing alcohol dehydrogenase.[24] NFADH also seems to belong to this group because it has the same molecular size as RECR and requires Zn^{2+} for its complete activity. RECR and PAR were reported to show broad substrate specificities,[12,13,16] although their activities toward alkynyl alcohols and ketones were not investigated. The application of NFADH to a wider range of substrates other than PYOH is also of further interest. For example, NFADH is able to produce (*R*)-CHBE, which is a useful chiral building block for the chemical synthesis of pharmaceuticals, with high stereoselectivity in the presence of NADH and its regeneration system.

References

1) Glänzer, B. I., Faber, K., and Griengl, H., Enantioselective hydrolysis by baker's yeast-III. Microbial resolution of alkynyl esters using lyophilized yeast. *Tetrahedron*, **43**, 5791–5796 (1987).
2) Burgess, K. and Jennings, L. D., Enantioselective esterifications of unsaturated alcohols mediated by a lipase prepared from *Pseudomonas* sp. *J. Am. Chem. Soc.*, **113**, 6129–6139 (1991).
3) Shimizu, M., Kawanami, H., and Fujisawa, T., A lipase mediated asymmetric hydrolysis of 3-acyloxy-1-octyns and 3-(*E*)-acyloxy-1-octens. *Chem. Lett.*, 107–110 (1992).
4) Ogawa, J., Xie, S.-X., and Shimizu, S., Production of (*R*)-3-pentyn-2-ol through stereospecific hydrolysis of racemic 3-pentyn-2-ol esters with microbial enzymes. *Appl. Microbiol. Biotechnol.*, **51**, 53–57 (1999).
5) Xie, S.-X., Ogawa, J., and Shimizu, S., (*S*)-3-Pentyn-2-ol production through microbial enzyme-catalyzed, highly enantioselective hydrolysis of racemic 3-pentyn-2-ol esters. *Biotechnol. Lett.*, **20**, 935–938 (1998).
6) Ogawa, J., Xie, S.-X., and Shimizu, S., Stereoinversion of optically active 3-pentyn-2-ol by *Nocardia* species. *Biotechnol. Lett.*, **21**, 331–335 (1999).
7) Xie, S.-X., Ogawa, J., and Shimizu, S., Production of (*R*)-3-pentyn-2-ol through stereoinversion of racemic 3-pentyn-2-ol by *Nocardia fusca* AKU 2123. *Appl. Microbiol. Biotechnol.* in press (1999).
8) Ogawa, J., Soong, C.-L., Honda, M., and Shimizu, S., Imidase, a dihydropyrimidinase-like enzyme involved in the metabolism of cyclic imides. *Eur. J. Biochem.*, **243**, 322–327 (1997).
9) Shimizu, S., Kataoka, M., Chung, M. C.-M., and Yamada, H., Ketopantoic acid reductase of *Pseudomonas maltophilia* 845: purification and characterization, and role in pantothenate biosynthesis. *J. Biol. Chem.*, **263**, 12077–12084 (1988).
10) Shimizu, S., Kataoka, M., Katoh, M., Morikawa, T., Miyoshi, T., and Yamada, H., Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reductase in an organic solvent-water diphasic system. *Appl. Environ. Microbiol.*, **56**, 2374–2377 (1990).
11) Kataoka, M., Rohani, L. P. S., Yamamoto, K., Wada, M., Kawabata, H., Kita, K., Yanase, H., and Shimizu, S., Enzymatic production of ethyl (*R*)-4-chloro-3-hydroxybutanoate: asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by an *Escherichia coli* transformant expressing the aldehyde reductase gene from yeast. *Appl. Microbiol. Biotechnol.*, **48**, 699–703 (1997).
12) Zelinski, T., Peters, J., and Kula, M.-R., Purification and characterization of a novel carbonyl reductase isolated from *Rhodococcus erythropolis*. *J. Biotechnol.*, **33**, 283–292 (1994).
13) Itoh, N., Morihama, R., Wang, J.-C., Okada, K., and Mizuguchi, N., Purification and characterization of phenylacetaldehyde reductase from a styrene-assimilating *Corynebacterium* strain, ST-10. *Appl. Environ. Microbiol.*, **63**, 3783–3788 (1997).
14) Peters, J., Minuth, T., and Kula, M.-R., Kinetic and mechanistic studies of a novel carbonyl reductase isolated from *Candida parapsilosis*. *Biocatalysis*, **8**, 31–46 (1993).
15) Zelinski, T. and Kula, M.-R., A kinetic study and application of a novel carbonyl reductase isolated from *Rhodococcus erythropolis*. *Bioorg. Med. Chem.*, **2**, 421–428 (1994).
16) Itoh, N., Mizuguchi, N., and Mabuchi, M., Production of chiral alcohols by enantioselective reduction with NADH-dependent phenylacetaldehyde reductase from *Corynebacterium* strain ST-10. *J. Molec. Catal. B: Enzymatic*, **6**, 41–50 (1999).
17) Brändén, C.-I., Jörnvall, H., Eklund, H., and Furugren, B., Alcohol dehydrogenases. In "The Enzymes, 3rd edn., Vol. 11", ed. by Boyer, P. D., Academic Press, New York, pp. 103–190 (1975).

18) Muheim, A., Waldner, R., Sanglard, D., Reiser, J., Schoemaker, H. E., and Leisola, M. S. A., Purification and properties of an aryl-alcohol dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*. *Eur. J. Biochem.*, **195**, 369–375 (1991).
19) Shaw, J. P., Rekik, M., Schwager, F., and Harayama, S., Kinetic studies on benzyl alcohol dehydrogenase encoded by TOL plasmid pWWO. *J. Biol. Chem.*, **268**, 10842–10850 (1993).
20) Hummel, W., Reduction of acetophenone to *R*(+)-phenylethanol by a new alcohol dehydrogenase from *Lactobacillus kefir*. *Appl. Microbiol. Biotechnol.*, **34**, 15–19 (1990).
21) Ludwig, B., Akundi, A., and Kendall, K., A long-chain secondary alcohol dehydrogenase from *Rhodococcus erythropolis* ATCC 4277. *Appl. Environ. Microbiol.*, **61**, 3729–3733 (1995).
22) Hummel, W. and Riebel, B., Chiral alcohols by enantioselective enzymatic oxidation. *Ann. N.Y. Acad. Sci.*, Vol. 799 (Enzyme Engineering 13) 713–716 (1996).
23) Peters, J., Mimuth, T., and Kula, M.-R., A novel NADH-dependent carbonyl reductase with an extremely broad substrate range from *Candida parapsilosis*: purification and characterization. *Enzyme. Microb. Technol.*, **15**, 950–958 (1993).
24) Hummel, W., New alcohol dehydrogenases for the synthesis of chiral compounds. *Adv. Biochem. Eng. Biotechnol.*, **58**, 144–184 (1997).