Stereoselective Carveol Dehydrogenase from Rhodococcus erythropolis DCL14

A NOVEL NICOTINOPROTEIN BELONGING TO THE SHORT CHAIN DEHYDROGENASE/REDUCTASE SUPERFAMILY\(^*\)

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A novel nicotinoprotein, catalyzing the dichlorophenolindophenol-dependent oxidation of carveol to carvone, was purified to homogeneity from Rhodococcus erythropolis DCL14. The enzyme is specifically induced after growth on limonene and carveol. Dichlorophenolindophenol-dependent carveol dehydrogenase (CDH) is a homotetramer of 120 kDa with each subunit containing a tightly bound NAD(H) molecule. The enzyme is optimally active at pH 5.5 and 50 °C and displays a broad substrate specificity with a preference for substituted cyclohexanols. When incubated with a diastereomeric mixture of (\(4R\))- or (\(4S\))-carveol, CDH stereoselectively catalyzes the conversion of the (\(6S\))-carveol stereoisomers only. Kinetic studies with pure stereoisomers showed that this is due to large differences in \(V_{\text{max}}/K_m\) values and simultaneous product inhibition by (\(R\))- or (\(S\))-carvone. The \(R. \text{erythropolis}\) CDH gene (\(limC\)) was identified in an operon encoding the enzymes involved in limonene degradation. The CDH nucleotide sequence revealed an open reading frame of 831 base pairs encoding a 277-amino acid protein with a deduced mass of 29,531 Da. The CDH primary structure shares 10–30% sequence identity with members of the short chain dehydrogenase/reductase superfamily. Structure homology modeling with trihydroxynaphthalene reductase from \(M. \text{grisea}\) suggests that CDH from \(R. \text{erythropolis}\) DCL14 is an \(a\beta\)-one-domain protein with an extra loop insertion involved in NAD binding and a flexible C-terminal part involved in monoterpenoid binding.

Terpenes are the largest class of secondary plant metabolites (1). They are generally regarded as derivatives of isoprene and are classified based on the number of isoprene units linked. Monoterpenes are branched chain C10 hydrocarbons formed from two isoprene units. They are widely distributed in nature, and over 400 different naturally occurring monoterpenes have been identified (2). Volatile monoterpenoid emission from trees is estimated at \(127 \times 10^{14}\) g carbon/year (3).

Limonene (4-isopropenyl-1-methylcyclohexene), a monocyclic monoterpen, is the most widespread terpene in the world and is formed by over 300 plants (4). (\(4R\))-(\(+\))-Limonene is the most widespread form. (\(4R\))-Limonene is the major constituent of citrus peel essential oils, where it is usually found in concentrations between 90 and 96% (5). However, several plants form a mixture of both enantiomers of limonene, whereas others produce only the (\(4S\))-enantiomer (4).

Several pathways for the microbial degradation of limonene have been postulated so far (6–10), but most of these pathways have not been substantiated by biochemical studies. The best studied microbial degradation pathway for limonene involves the hydroxylation at the C7-methyl group resulting in the formation of perillyl alcohol (6, 7). Two enzymes of this pathway, perillyl alcohol dehydrogenase and perillyl aldehyde dehydrogenase, have been partially purified (11, 12). Also an enzyme from one of the other limonene transformation pathways, \(a\)-terpineol dehydratase, was partially purified from two different \(Pseudomonas\) species (8, 13).

We recently isolated \(R. \text{erythropolis}\) DCL14, a strain able to grow on limonene as sole carbon and energy source that contains a novel degradation pathway for limonene (14). Cells grown on limonene and carveol were found to contain a DCPIT\(3\)-dependent carvone dehydrogenase (CDH) that converts carveol into carvone (Fig. 1). We here report on the purification, characterization, gene cloning, sequence determination, and structure homology modeling of this novel enzyme.

EXPERIMENTAL PROCEDURES
Organism and Culture Conditions

\(R. \text{erythropolis}\) DCL14 was isolated with (\(4R\))-dihydrocarvone as the carbon and energy source as described previously (15). The strain was subcultured every month and grown at 30 °C on a yeast extract-glucose agar plates for 2 days, after which the plates were stored at room temperature. Cultures were grown in 5-liter Erlenmeyer flasks containing 1 liter of mineral salts medium (16) with 0.01% (v/v) carbon source and fitted with rubber stoppers. The flasks were incubated at 30 °C on a horizontal shaker oscillating at 1 Hz with an amplitude of 10 cm. After growth was observed, the concentration of the toxic substrates was increased with daily steps of 0.01% (v/v) until a total of 0.1% (v/v) carbon source had been added.

Cells for enzyme purification were grown fed-batch on limonene in a fermentor as described previously (15). Cells were collected by centrifugation (4 °C, 10 min at 16,000 \(\times\) g) and washed with 50 mM potassium phosphate buffer, pH 7.0. The pellet was resuspended in 7 ml of this buffer containing 0.1% Tween 80 and stored at –20 °C.

\(1\) The abbreviations used are: DCPIT, dichlorophenolindophenol; CDH, carveol dehydrogenase; GC, gas chromatography; MS, mass spectrometry; SDR, short chain dehydrogenase/reductase.

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Fig. 1. Reaction catalyzed by DCPIP-dependent CDH from R. erythropolis DCL 14. Numbers in carveol refer to the carbon atom numbering.

**Chemicals**

(4R)-Carveol (mixture of two diastereomers), (−)−(R)- and (+)−(S)-carvone were from Acros and DCPIP was from Merck. SDS was purchased from BDH. NAD⁺ (100%), NADP⁺ (98%), NADPH (98%), and NADPH (98%) were obtained from Roche Molecular Biochemicals. DEAE-Sepharose CL-6B, Octyl Sepharose, Sephacryl S300, Superdex 200 HR 10/30, and Resource-Q were obtained from Amersham Pharmacia Biotech. Hydroxyapatite and Bio-Gel P6DG were from Bio-Rad. (4S)-Carveol (mixture of two diastereomers) was prepared by reducing (S)-carvone with lithium aluminium hydride. (+)−(4S; 6S)-cis- (97.8%), (+)−(4S, 6R)-trans- (94.6%), (−)−(4R, 6R)-cis- (96.8%), and (−)−(4R, 6S)-trans-carpaveol (99.2%) were obtained after preparative GC separation.

**Enzyme Purification**

All purification steps were performed at 4 °C. Buffers contained 10% glycerol and 0.1% Tween 80, and the pH was 7.0. If necessary, pooled fractions were concentrated by ultrafiltration with an Amicon ultrafiltration unit using a YM-10 membrane.

**Hydroxyapatite Chromatography**—Cell extract (15 ml) was applied onto an Octyl Sepharose column (2.5 × 31 cm) equilibrated with 25 mm potassium phosphate buffer containing 1 mM NaCl and eluted with the same buffer (flow rate, 0.75 ml/min; collected fraction volume, 7.5 ml). Fractions containing DCPIP-dependent CDH activity were pooled and adjusted to 80% ammonium sulfate saturation. After 15 min at 0 °C, the precipitate was collected by centrifugation (15 min at 27,000 × g; final pellet was resuspended in 5 ml of 15 mM potassium phosphate buffer.

**Gel Filtration**—The solubilized precipitate was applied onto a Sephacryl S300 column (2.5 × 98 cm) equilibrated with 15 mM phosphate buffer and eluted with the same buffer (flow rate, 0.75 ml/min; collected fraction volume, 7.5 ml). Fractions containing CDH activity were pooled.

**Identification of Prosthetic Group of CDH**

The pooled fractions from the hydroxyapatite chromatography step were applied onto a DEAE-Sepharose CL-6B column (2.5 × 31 cm) equilibrated with 25 mM potassium phosphate buffer. The column was washed with 200 ml of the same buffer (flow rate, 0.75 ml/min; collected fraction volume, 7.5 ml), and subsequently the enzyme was eluted with a linear gradient of 0–1 M NaCl in the same buffer (total volume, 1 liter). CDH eluted at a concentration of 300 mM NaCl. Active fractions were pooled, concentrated by ultrafiltratation, and stored at 4 °C.

**Electrophoresis and Molecular Mass Determinations**

The relative subunit molecular mass of CDH was determined by SDS-polyacrylamide gel electrophoresis. A 12.5% (w/v) slab gel was prepared by the method of Laemmli (19). The Amersham Pharmacia Biotech low molecular mass calibration kit containing phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and a-lactalbumin (14.4 kDa) was used as a reference. Proteins were stained with Coomassie Brilliant Blue G. The relative molecular mass of native CDH was determined by gel filtration on Superdex 200. The column was calibrated with blue dextran 2000, ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), p-hydroxybenzoate hydroxylase (88 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).

**Identification of Prosthetic Group of CDH**

The prosthetic group of CDH was extracted by urea unfolding, essentially as described by Arfman et al. (20). Urea (final concentration, 6 M) was added to 1.85 mg of purified CDH in 1 ml of 25 mM Hepes/NaOH, pH 7.5, containing 1 mM dithiothreitol. After standing for 10 min at 50 °C, the enzyme sample (1.4 ml) was eluted over a Bio-Gel P6DG column (1 × 10 cm), eluted with 10 mM Tris/Cl/HCl, pH 8.0, contain-
**Analytical Methods**

Protein was determined by the method of Bradford (22) with bovine serum albumin as the standard. The N-terminal amino acid sequence of purified CDH was determined by Edman degradation at the Protein Sequencing Facility Leiden, Department of Medical Biochemistry, Sylvius Laboratory, Leiden, The Netherlands. The metal composition of CDH was determined by inductively coupled plasma-MS using a Perkin-Elmer Elan 6000.

Carveol and carvone were analyzed by chiral GC on fused silica cyclodextrin-DEX 120 capillary column (30 m × 0.25 mm, 0.25-μm film coating; Supelco, Zwijndrecht, the Netherlands). GC was performed on a Chrompack CP9000 GC equipped with a FID detector using a 1 ml min⁻¹ as the carrier gas. The detector and injector temperatures were 250 and 200 °C, respectively, and the split ratio was 1:50.

**Absorption Spectra**

Absorption spectra were recorded at 25 °C on a Hewlett Packard HP 8453 diode array spectrophotometer. Fluorescence spectra were recorded at 20 °C with a SLM-Amino SPF506C spectrofluorimeter. Measurements were performed in a quartz cuvette (10-mm path length) in a total volume of 1 ml. Fluorescence emission spectra were recorded between 360 and 560 nm (bandwidth, 4 nm) with the excitation wavelength fixed at 280, 295, or 340 nm (bandwidth, 4 nm). Fluorescence excitation spectra were scanned between 240 and 440 nm (bandwidth, 4 nm) at a fixed emission wavelength of 450 nm (bandwidth, 4 nm). All fluorescence spectra were corrected against the appropriate reference solution.

**Cloning and Sequencing of the CDH Gene (limC)**

The limA gene coding for limonene-1,2-epoxide hydrolase, an enzyme involved in limonene degradation in *R. erythropolis* DCL14, was cloned using a combination of polymerase chain reaction and colony filter hybridization (21). Using the N-terminal amino acid sequence of the purified enzyme (15), two degenerated primers were designed at the beginning and the end of the 50-amino acid-long stretch. These primers were used to synthesize a homologous probe with which the complete *limA* gene and the flanking regions were isolated from a cosmid library of *R. erythropolis* DCL14 (21). The nucleotide sequences of the flanking regions of *limA*, containing the *limC* gene, were determined by a combination of subcloning and primer walking. The nucleotide sequences of double stranded inserts in pGEM-T were determined by the DyeDeoxy Terminator Cycle Sequencing kit using AmiTag FC DNA polymerase (Perkin-Elmer).

**Sequence Alignment**

For sequence alignment studies a PSI-BLAST (23) search was performed at the National Center for Biotechnology Information for related sequences with known structure. A multiple sequence alignment was performed by superimposing the structures of the selected sequences with the program TOP (24), followed by the addition of the CDH gene to the alignment profile with MACAW (25), and manual optimization.

**Structure Homology Modeling**

Model building of CDH was performed with MODELLER4 (26) using the CHARMM forcefield (27). The stereochemical quality of the homology model was verified by PROCHECK (28), and the protein folding was assessed with PROFILE (29) and PROSAII (30), which evaluate the compatibility of each residue to its environment independently. After this initial verification, bad regions of the model were optimized with the simulated annealing procedure (molecular dynamics) of the XPLOR package (31), thereby fixing the other parts of the protein. A simulated
R. erythropolis DCL14 were present in cell extracts of revealed at least four different CDH activities: an NADP\(^{(4)}\) with (4\(^{-}\)) dependent CDH, an NAD\(^{(1)}\) was verified after several rounds of energy minimization. The model positions of NAD(P)H in SDR structures. NADH forcefield constants for ing was performed with the program O (32), based on highly similar positions of NAD\(\text{(P)H}\) in SDR structures. NADH forcefield constants for energy minimization were derived from CHARMM. Finally, the model was energy minimized with the conjugate gradient algorithm of step taking 0.5 femtosecond. Before and after molecular dynamics the annealing calculation was performed for 1000 steps at 900 K, with each step taking 0.5 femtosecond. Before and after molecular dynamics the model was energy minimized with the conjugate gradient algorithm of XPLOR (31); the minimization converged after 2000 cycles (gradient, 0.1 kcal/mol). Again, the model was checked and verified. NADH docking was performed with the program O (32), based on highly similar positions of NAD\(\text{(P)H}\) in SDR structures. NADH forcefield constants for energy minimization were derived from CHARMM. Finally, the model was verified after several rounds of energy minimization.

**RESULTS**

**Induction of CDH Activities in R. erythropolis—**R. erythropolis DCL14 is able to grow on a variety of monoterpenes. During growth on these substrates, several CDH activities are induced (Table I). Both NAD\(^{-}\)- and DCPIP-dependent CDH activities were present in cell extracts of R. erythropolis DCL14 grown on (4S)-carveol. Separation by anion exchange chromatography revealed at least four different CDH activities: an NAD\(^{\text{P}}\)-dependent CDH, an NAD\(^{\text{P}}\)-dependent CDH with a high activity with (4S)-carveol, an NAD\(^{\text{S}}\)-dependent CDH with a high activity with (4R)-carveol, and a DCPIP-dependent CDH (results not shown). Only the latter activity was markedly induced after growth on limonene and carveol (Table I).

**Purification of DCPIP-dependent CDH—**During our initial efforts to purify the inducible CDH, the enzyme appeared to be rather unstable, especially at high ionic strength. A mixture of 0.1\(\%\) (v/v) Tween 80 and 10\(\%\) (v/v) glycerol stabilized CDH. The purification scheme for CDH is presented in Table II. The enzyme was purified 13-fold with an overall yield of 50\%. The ratio of the activity of CDH with (4S)- and (4R)-carveol remained constant during purification. SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed one distinct band, corresponding to a protein with a subunit mass of about 40 kDa (Fig. 2). Analytical gel filtration revealed one symmetric protein peak corresponding to a relative molecular mass of about 120 kDa. Together with the sequence data (see below), this suggests that the native enzyme is a homotetramer. Inducibly coupled plasma MS analysis revealed no metal ions as cofactors (<5\% mol metal/mol enzyme).

**Spectral Properties—**The absorption spectrum of CDH displayed a typical maximum at 275 nm and a broad band of relatively low intensity around 330 nm (Fig. 3A). Addition of dithionite, carveol, or carvone did not affect the absorption spectrum. Upon excitation at 340 nm, the holoenzyme exhibited a fluorescence emission maximum near 440 nm (Fig. 3B), whereas upon excitation at 280 nm, the native enzyme showed fluorescence emission maxima at 340 and 440 nm (Fig. 4A). This indicates that CDH contains protein-bound NAD(P)H.

**Identification of CDH-bound Cofactor—**The cofactor of CDH was firmly bound as indicated by gel filtration and dialysis experiments. Fluorescence spectral analysis revealed that the cofactor could be conveniently released from the CDH apoprotein by treatment with 0.5\% SDS, 6\(\times\) urea, or 6\(\times\) guanidinium hydrochloride. Unfolding of CDH in 0.5\% SDS was rather slow as indicated by the time-dependent disappearance of the fluorescence emission maximum at 440 nm and increase of protein fluorescence at 340 nm (Fig. 4A). In contrast, unfolding in 6\(\times\) urea (or 6\(\times\) guanidinium hydrochloride) was a rather rapid process, taking less than 10 min to complete (not shown).

The urea-released cofactor was separated from the apoprotein by gel filtration. Spectral analysis revealed that the apoprotein fraction exhibited an absorption maximum near 275 nm and no residual absorbance at 330 nm (Fig. 4B, inset). In line with this, no fluorescence emission was observed at 440 nm when the apoprotein sample was excited at either 280 or 340 nm. The absorption spectrum of the dissociated cofactor exhibited maxima at 260 and 340 nm, characteristic of NAD(P)H (Fig. 4B). Furthermore, the rather high A\(_{290}\)/A\(_{340}\) ratio and relatively low fluorescence emission at 440 nm suggested that the CDH-bound cofactor is a mixture of oxidized and reduced pyridine nucleotide forms. The identity of the isolated cofactor was further established by anion-exchange chromatography, using conditions resulting in clear separation of NAD(H) and NAD(P)(H) (20). The CDH-derived cofactor preparation showed two peaks of nearly equal intensity at 260 nm, corresponding to...
the positions of NAD$^+$ and NADH, respectively. In line with this, only the second elution peak of the CDH-derived cofactor preparation displayed absorption at 340 nm. From this it is concluded that CDH contains NAD(H) as the tightly bound cofactor. Moreover, assuming a molar absorption coefficient $\varepsilon_{340} = 6.22$ m$^{-1}$ cm$^{-1}$, the amount of NAD(H) per CDH subunit was estimated. Different enzyme preparations yielded values ranging from 0.55–0.98 mol NAD(H)/subunit, indicating that each CDH monomer contains one NAD(H) molecule as tightly bound cofactor.

Catalytic Properties—Under the conditions of the standard assay, CDH was optimally active at pH 5.5 and showed 25% of the optimal activity at pH 4.6 and 7.0. The temperature optimum of CDH activity was near 50 °C. At pH 5.5, the specific activity gradually increased from 550 nmol min$^{-1}$ mg$^{-1}$ at 6 °C to 2825 nmol min$^{-1}$ mg$^{-1}$ at 50 °C. At temperatures above 35 °C, enzyme inactivation was observed during the time span of the activity assay, and at 60 °C activity was no longer detected. CDH used DCPIP as the electron acceptor. Over the pH range 6–9, no activity was observed with 9,10-anthraquinone, nitroblue tetrazolium, oxygen, phenazine methosulfate, quinalizarin, tetramethyl-p-benzoquinone, triphenyltetrazolium and ubiquinone. The presence of phenazine methosulfate or NAD$^+$ did not affect the DCPIP-dependent CDH activity at any of the tested pH values.

Substrate Specificity and Stereochemistry—CDH showed a broad substrate specificity (Table III). The best substrates were substituted cyclohexanols such as carveol, 3-methylcyclohexanol, and 3,5,5-trimethyl-2-cyclohexen-1-ol. Primary alcohols such as ethanol and perillyl alcohol and short chain secondary alcohols like 2-propanol and 1-butanol were no substrates for CDH (Table III). Furthermore, no reverse reaction was detectable when CDH was assayed with (R)- or (S)-carvone or (4R)-dihydrocarvone in the presence of reduced DCPIP or NADH.

Because carveol exists in four stereoisomeric forms the kinetic constants of CDH with the pure stereoisomers were determined (Table IV). The highest $V_{\text{max}}/K_m$ and lowest $K_m$ values were obtained for (4S)-carvone and (4S)-carveol. Also a strong competitive product inhibition was observed and a $K_i$ of 0.11 and 0.23 mM were determined for (R)- and (S)-carvone, respectively. Using a diastereomeric mixture of either (4R)- or (4S)-carveol as the substrate, the stereoselectivity of CDH was studied by chiral GC (Fig. 5). CDH converted only the (6S)-stereoisomers of carveol.

N-terminal Sequence—The N-terminal amino acid sequence determination of purified CDH gave a tandem sequence (a mixture of two identical peptide sequences, of which one lacked the first residue). The N-terminal sequence was determined to be Ala-Arg-Val-Glu-Gly-Gln-Val-Leu-Ile-Thr-Gly-Ala-Ala-Arg-Gly-Gln-Gly-Arg-Ser-His-Ala-Ile-Lys-(Lys/Leu)-(Ala/Leu)-Glu-Gly.

Nucleotide Sequence—Recently, we cloned and sequenced the limA gene coding for limonene-1,2-epoxide hydrolase from *R. erythropolis* DCL14 (21). Sequencing the flanking regions of limA suggested that limA is the first open reading frame of an operon encoding genes involved in the limonene degradation
pathway of this microorganism.\(^2\) The third open reading frame of this operon, *limC*, encodes a protein of 277 amino acids with a deduced molecular mass of 29.531 Da (Fig. 6). Residues 2–29 of this operon, *limC*, encodes CDH. A potential ribosomal-binding site (Shine-Dalgarno sequence), GGAGGGA, preceded the ATG translational initiation site by 2 base pairs (Fig. 6).

**Amino Acid Sequence Comparison**—The derived primary structure of CDH showed up to 33% sequence identity and 52% sequence homology with members of the SDR superfamily. The highest degree of sequence identity with enzymes of known structure was found for trihydroxynaphthalene reductase (Protein Data Bank code 1YBV) from *Magnaporthe grisea* (30%), human 17\(\beta\)-hydroxysteroid dehydrogenase (1FM) (29%), 3a,20\(\beta\)-hydroxysteroid dehydrogenase (2HSD) from *Streptomyces hydrogenans* (28%), and *cis*-biphenyl-2,3-dihydriodiol-2,3-dehydrogenase (1BDB) from *Burkholderia cepacia* (28%). Fig. 7 shows a sequence alignment of CDH and SDR enzymes of known three-dimensional structure. From the alignment it is obvious that CDH shares many structural features with the other members of the family. Conserved regions in the primary structure of CDH are present throughout the sequence indicating that the enzyme has an \(\alpha/\beta\) one-domain fold. Conserved features near the N terminus include the GXXGXG finger-print characteristic for the \(\beta\)-\(\alpha\)-\(\beta\) dinucleotide binding fold in dehydrogenases (42, 43) and Asp37, involved in determining the coenzyme specificity (43). Other conserved features include the Ser\(^{126}\)-Try\(^{169}\)-Lys\(^{172}\) catalytic triad with the YXXX fingerprint, involved in substrate oxidation (44) and the NAG binding site, which is new in relation to the other SDR members (Fig. 7).

**Table IV**

| Substrate | \(V_{\text{max}}\) mg min\(^{-1}\) | \(K_m\) mg \(l^{-1}\) min\(^{-1}\) | \(V_{\text{max}}/K_m\) |
|-----------|-----------------|-----------------|-----------------|
| (4S,SR)-Carveol | 1320 | 2.0 | 660 |
| (4S,SS)-Carveol | 3870 | 0.39 | 10 |
| (4R,SR)-Carveol | 1450 | 0.35 | 4.2 |
| (4R,SS)-Carveol | 4200 | 0.041 | 100 |

**Fig. 6**. Nucleotide sequence of the *limC* region of *R. erythropolis* DCL14 and the deduced amino acid sequence of CDH. Nucleotide sequence of the 834-base pair DNA fragment containing the *limC* gene. The open reading frame starts at base 37 and terminates at base 870. The deduced amino acid sequence is indicated below the DNA sequence. Underlined amino acid residues are identical to those determined by Edman degradation of the purified enzyme. A potential ribosome binding site (marked SD) upstream of the open reading frame is indicated.

\(^2\) C. J. B. van der Vlugt-Bergmans, F. Barbirato, J. A. M. de Bont, and M. J. van der Werf, unpublished results.
enzymes of the SDR family (33–36, 40). For the tetrameric contacts, probably the αE and αF interact along the Q-axis, the αG and βG along the P-axis, and the weakest interaction occurs with the short C-terminal regions along the R-axis.

Mode of Coenzyme Binding—The NADH cofactor was modeled in an extended conformation, highly similar to related SDR members. The NADH molecule is buried in the protein and probably shielded from solvent by the extra loop, comprising residues 40–50 (Fig. 8). The nicotinamide ring was modeled in the syn conformation, consistent with the proposed stereochemistry of hydride transfer in the SDR family (35). Asp 37 interacts in the model with the 2-OH ribose of NADH (Fig. 9), and the pyrophosphate moiety is located near the GXXXG sequence of the dinucleotide binding fold. The substrate binding cavity is enclosed on one side by the nicotinamide ring of NADH and the active site residue Tyr169 and on the other side by the C-terminal substrate binding loop.

**DISCUSSION**

This paper describes the purification and characterization of a DCPIP-dependent CDH, which is induced when *R. erythropolis* DCL14 is grown on monoterpenes. During the last decade it has become apparent that monoterpenes play an important role in chemical ecology where they act as attractants, repellents, sex pheromones, or alerting pheromones or are a part of defense secretion systems against predators (46). Remarkably, little is known about the metabolism of monoterpenes. Information regarding the enzymes involved in the monoterpene degradation pathways is especially scarce (6, 47, 48). Although CDH activity has been detected in crude extracts of spearmint (*Mentha spicata*) (49) and caraway (*Carum carvi*) (50), this is the first report of a CDH purified to homogeneity.

**CDH from *R. erythropolis* DCL14 is a homotetramer, and each 30-kDa CDH subunit contains a tightly bound cofactor.**

**FIG. 7.** Multiple sequence alignment of CDH from *R. erythropolis* DCL14 and short chain dehydrogenases/reductases of known three-dimensional structure. The deduced amino acid sequence of CDH is aligned with the amino acid sequences of 1,3,8-trihydroxynaphthalene reductase (1YBV) from *M. grisea* (33), 7α-hydroxysteroid dehydrogenase (1FMC) from *Escherichia coli* (34), 3α,20β-hydroxysteroid dehydrogenase (2HSD) from *S. hydrogenans* (35), cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (1BDB) from *B. cepacia* (36), enoyl acyl carrier protein reductase (1ENO) from *E. coli* (37), human 17β-hydroxysteroid dehydrogenase (1DHR) (38), liver dihydropteridine reductase (1DHR) (39), mouse lung carbonyl reductase (1CYD) (40), and mouse sepiapterin reductase (1SER) (41). Secondary structure elements are underlined, and residues discussed in the text are depicted in bold type.

**FIG. 8.** Ribbon diagram of the modeled three-dimensional structure of CDH from *R. erythropolis* DCL14. The three-dimensional structure of CDH was predicted by structure homology model building (26). The crystal structure of trihydroxynaphthalene reductase (1YBV) from *M. grisea* (33), 7α-hydroxysteroid dehydrogenase (1FMC) from *Escherichia coli* (34), 3α,20β-hydroxysteroid dehydrogenase (2HSD) from *S. hydrogenans* (35), cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (1BDB) from *B. cepacia* (36), enoyl acyl carrier protein reductase (1ENO) from *E. coli* (37), human 17β-hydroxysteroid dehydrogenase (1DHR) (38), liver dihydropteridine reductase (1DHR) (39), mouse lung carbonyl reductase (1CYD) (40), and mouse sepiapterin reductase (1SER) (41). Secondary structural elements are annotated together with the N and C termini.
that is only released after treatment with unfolding agents. The cofactor was identified as NAD(H), indicating that CDH is a nicotinoprotein. Nicotinoproteins catalyze a large spread of reactions including transhydrogenation, dehydrogenation, dismutation, epimerization, and oxidation of alcohols and aldehydes (51–57). Nicotinoprotein dehydrogenases can be divided in two groups. The first group includes dehydrogenases that catalyze oxidations dependent on exogenous NAD(P). However, due to an unusually high affinity, the (reduced) cofactor remains (partly) bound to the enzyme, even after lengthy purification procedures or repeated crystallization. Examples are glyceraldehyde-3-phosphate dehydrogenase (58) and mammalian acetaldehyde dehydrogenase (56). The second group includes nicotinoprotein dehydrogenases that are, in vitro, only active when assayed in the presence of an artificial electron acceptor like N,N-dimethyl-4-nitrosoaniline or DCPIP (57, 59, 60). In vivo, these enzymes probably regenerate the cofactor by delivering the reduction equivalents directly to a component of an electron transport chain (61). Examples are methanol dehydrogenase from Amycolatopsis methanolica (57), short chain primary alcohol dehydrogenase from A. methanolica (60), and medium chain primary alcohol dehydrogenase from Methanosaerina barheri (59). Being exclusively active with DCPIP, it is evident that CDH belongs to this second group of nicotinoprotein dehydrogenases. However, the physiological electron acceptor of the enzyme remains to be elucidated.

Gene cloning confirmed that CDH is involved in the biodegradation of limonene and sequence analysis classified CDH as a member of the SDR superfamily. These enzymes are built up by subunits of about 30 kDa, do not contain metal cofactors, have a single α/β domain with a βα-α-β dinucleotide binding fold near the N termini, and contain a highly conserved Ser-Tyr-Lys triad at their active site (44, 62). In CDH, the catalytic machinery includes Ser156, Tyr169, and Lys173. Ser156 and Tyr169 are likely involved in substrate activation, whereas Lys173 presumably is involved in the orientation of the nicotinamide ring of the cofactor (33). Moreover, the presence of an apatic residue, 18 residues downstream of the GXXGXG fingerprint, confirmed the biochemical identification of the coenzyme specificity of CDH (40).

Structure prediction suggests that CDH contains a 10-amino acid-long loop (residues 40–50) located near the entrance of the NADH binding cleft. This loop, which is absent in other SDR enzymes, might be involved in shielding the NADH cofactor from the bulk solvent. Another intriguing feature concerns the functional role of the flexible loop comprising residues 204–236. As proposed for 7α-hydroxysteroid dehydrogenase (34) and confirmed by the structure of trihydroxynaphthalene reductase complexed with tricyclazole (33), this part of the sequence is likely involved in determining the substrate specificity. Moreover, from the conservation of several critical residues, we propose that in CDH a hinge bending motion of the substrate binding loop occurs. Thr204 (N-terminal hinge) and Asn236 (C-terminal hinge) might act as the hinges, whereas Pro199 and Pro239 probably stabilize the region close to the hinges (34).

CDH from R. erythropolis has a unique substrate specificity in comparison with previously reported (secondary) alcohol dehydrogenases (63–65). The enzyme has a preference for substituted cyclohexanols and does not catalyze the oxidation of primary or short chain aliphatic secondary alcohols. CDH shows an absolute stereoselectivity with carveol, converting only the (S)-stereoisomers when incubated with a diastereomic mixture. This stereoselectivity is explained by the kinetic constants of CDH, showing a much higher catalytic efficiency for the (S)-stereoisomers than for the (6R)-stereoisomers and the simultaneous strong competitive product inhibition. Absolute stereoselectiveness is a unique property among secondary alcohol dehydrogenases (66–68), potentially making the enzyme of interest for the biocatalytic production of (natural) enantiopure compounds.

In conclusion, DCPIP-dependent CDH from R. erythropolis DCL14 is a novel nicotinoprotein with a SDR α/β one-domain fold. The enzyme catalyzes the stereoselective conversion of monoterpenes, which further extends the functional diversity of the SDR superfamily.

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