Linalool Dehydratase-Isomerase, a Bifunctional Enzyme in the Anaerobic Degradation of Monoterpenes

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Castellaniella (ex Alcaligenes) defragrans strain 65Phen mineralizes monoterpenes in the absence of oxygen. Soluble cell extracts anaerobically catalyzed the isomerization of geraniol to linalool and the dehydration of linalool to myrcene. The linalool dehydratase was present in cells grown on monoterpenes, but not if grown on acetate. We purified the novel enzyme ~1800-fold to complete homogeneity. The native enzyme had a molecular mass of 160 kDa. Denaturing gel electrophoresis revealed one single protein band with a molecular mass of 40 kDa, which indicated a homotetramer as native conformation. The aerobically purified enzyme was anaerobically activated in the presence of 2 mM DTT. The linalool dehydratase catalyzed in vitro two reactions in both directions depending on the thermodynamic driving forces: a water secession from the tertiary alcohol linalool to the corresponding acyclic monoterpane myrcene and an isomerization of the primary allylalcohol geraniol in its stereoisomer linalool. The specific activities (Vmax) were 140 nanokatals mg⁻¹ for the linalool dehydratase and 410 nanokatals mg⁻¹ for the geraniol isomerase, with apparent Kₘ values of 750 μM and 500 μM, respectively. The corresponding open reading frame was identified and revealed a precursor protein with a signal peptide for a periplasmatic location. The amino acid sequence did not affiliate with any described enzymes. We suggest naming the enzyme linalool dehydratase-isomerase according to its bifunctionality and placing it as a member of a new protein family within the hydrolyses (EC 4.2.1.X).

The monoterpenes are divided into acyclic compounds, such as myrcene (7-methyl-3-methylene-1,6-octadiene) and ocimene, monocyclic monoterpenes, e.g. limonene and phellandrene, and bicyclic monoterpenes, e.g. pine and sabine. These unsaturated hydrocarbons are classified as highly volatile organic compounds. Plants as major producers emit more than 100 million tons/year to the atmosphere (6) where they are photooxidized and contribute to aerosol formation (7, 8). An example of physiological function is as defense against herbivores: plants often induce the synthesis of monoterpenes as repellents upon insect damage (9).

The mineralization of monoterpenes by aerobic microorganisms has been studied in detail with Pseudomonas species (10, 11). The aerobic metabolism depends on oxygenases that catalyze hydrogenation reactions with molecular oxygen as co-substrate (12). In the absence of oxygen, alternative biochemical pathways have been identified for hydrocarbon-mineralizing bacteria. Alkanes, e.g. n-hexane, and aromatic hydrocarbons with alkyl substituents, e.g. toluene, are anaerobically activated by glycine radical enzymes, and the radical intermediates add to fumarate, yielding methylalkylsuccinate and benzylsuccinate, respectively (13–15). Molybdenum-containing enzymes anaerobically hydroxylate ethylbenzene (16) and cholesterol (17).

For monoterpenes, no pathway has been elucidated so far. The anaerobic mineralization of monoterpenes to carbon dioxide is frequently present in denitrifying bacteria (18). Cultivation approaches established the enrichment of monoterpenemineralizing microorganisms (19) and the isolation of strains of Alcaligenes defragrans (20) and Thauera terpenica (21). A. defragrans was recently placed in the newly defined genus Castellaniella, as C. defragrans (22). Initial studies on potential metabolites of the degradation pathway identified isoterpinolene as metabolite that was apparently not further metabolized (23) and geranic acid as ionic intermediate present in nitrate-respiring cells that were grown on acyclic or cyclic monoterpenes, e.g. myrcene or limonene (24).

A simple pathway hypothesis is a hydration of myrcene, leading to geraniol and further to geranic acid (Fig. 1). We initiated biotransformation studies with soluble extracts of C. defragrans. In this article we report on the detection of novel enzyme activities and the isolation and characterization of an anaerobic linalool dehydratase-isomerase, a bifunctional enzyme that catalyzes the reversible dehydration and isomerization of linalool (3,7-dimethyl-1,6-octadien-3-ol) (Fig. 1).

EXPERIMENTAL PROCEDURES

Reagents—R-Limonene (95%), myrcene (90%), linalool (99%), and geraniol (98%) were purchased from Sigma-Aldrich. All
other chemicals used were of the highest available purity and were purchased from Aldrich, Boehringer, Fluka (Neu-Ulm, Germany), Merck, Sigma, and Bio-Rad Laboratories. Gases (CO₂ grade 4.8, N₂ grade 5.0, and O₂ grade 2.0) were supplied by Air Liquide (Düsseldorf, Germany). Chromatography media and instruments were from GE Healthcare.

Cell Growth and Preparation of Soluble Extracts—C. defrags strain 65Phen was maintained as described (20). For biomass production, the strain was cultivated on 30 mM limonene and 100 mM nitrate (24). A 1-liter preculture was inoculated in a 10-liter vessel of carbonate-buffered mineral salt medium at pH 7.0. Filter-sterilized limonene and vitamins (25) were added after cooling, and the culture was incubated for 6–7 days with a CO₂/N₂ (10/90 (v/v)) gas stream of 24 ml h⁻¹ at 28 °C. The stirrer frequency was initially 150 rpm and was increased during exponential growth phase of C. defragrans up to 250 rpm to ensure optimal substrate availability.

Cell harvest began after the addition of reducing agents, 50 μM Fe(II)Cl₃ and 2 mM DTT. Cells in the late exponential growth phase (A₆₀₀ ≈ 3) were transferred by gas pressure to centrifuge tubes and then collected by centrifugation for 15 min at 9000 × g at 4 °C. For the preparation of the soluble proteins, 40 g of wet or frozen cells were suspended in 60 ml of 25 mM sodium phosphate buffer, pH 8.0, containing 2 mM DTT and disintegrated in two passages through a French pressure cell press (Amincon, Rochester, NY) at 10.3 MPa. The soluble fraction was obtained by ultracentrifugation for 90 min at 150,000 × g at 4 °C to remove cell debris, unbroken cells, and membrane proteins.

Assays for Geraniol Isomerization and Linalool Dehydration—Salt or urea containing linalool dehydratase fractions were dialyzed three times against a 1000-fold volume of 80 mM Tris-HCl buffer, pH 9.0, for 20 min at 4 °C and under magnetic stirring. Purified and dialyzed linalool dehydratase fractions were stored under an anoxic gas phase at 4 °C.

Geraniol isomerization and linalool dehydrogenation were assayed routinely in a two-phase system. Vials (17 × 38 mm; Zinsser Analytic, Frankfurt, Germany) were prewarmed at 35 °C. Anoxic protein solution was transferred into the vials, and DTT was added to 2 mM. The tests were sealed with a butyl septum, and the headspace was flushed with CO₂/N₂ (10/90 (v/v)). The reaction was started by adding a distinct linalool or geraniol concentration to investigate the reaction to myrcene. 10–100 mM organic substrate was dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN).² The organic phase was added in a 1:1 ratio to the aqueous protein solution. Kinetic parameters of the enzyme activity were determined in a one-phase system with 10% (v/v) DMSO. The reaction was started by adding monoterpenes (0.1–10 mM) that was dissolved in anoxic 80 mM Tris-HCl buffer, pH 9.0, with 10% DMSO. In a third assay system, a pure myrcene phase (1/2 (v/v) myrcene/Tris-HCl buffer) was applied for measuring linalool and subsequently geraniol formation. The tubes were immediately transferred into a 35 °C shaking incubator. For kinetic analyses and the myrcene turnover, aqueous samples were taken at different time points and directly injected into the GC. In the two-phase system, 1 μl of the organic HMN carrier phase was injected to determine the substrate and product concentration.

To estimate the effect of temperature on linalool dehydratase activity, the two-phase assay was performed at temperatures between 4 °C and 45 °C. The pH optimum was tested by varying the buffer systems with pH values near the specific pKₐ values at 35 °C. The two-phase assay was also used to determine the influence of different effectors on enzyme activities.

The concentrations of the monoterpenes were analyzed by GC (Auto System XL; PerkinElmer Life Sciences) equipped with an Optima®-5 (0.25-μm film thickness, 50 m × 0.32-mm inner diameter; Macherey-Nagel, Düren, Germany) column and flame ionization detector. The following temperature program was applied: injection port temperature, 250 °C; column start temperature, 85 °C for 1 min, increasing to 120 °C at a rate of 5 °C min⁻¹, 120 °C for 0.1 min, increasing to 290 °C at a rate of 45 °C min⁻¹, 290 °C for 1 min; detection temperature, 350 °C. The split ratio was set to 1:25.

Purification of Linalool Dehydratase—The purification was performed with an Äkta system (GE Healthcare). All purification procedures were carried out at 4 °C with filtered (0.2 μm) and degassed buffers. 100 ml of soluble extract obtained from cells grown on limonene was applied to a Source 30Q column (5 × 30 cm) equilibrated in 50 mM sodium phosphate, pH 8.0 (AIE-Q1). The enzyme eluted at 200 mM NaCl in the aforementioned buffer during a stepwise gradient performed with 3.5 ml min⁻¹. Fractions containing linalool dehydratase were pooled, and saturated ammonium sulfate solution was added to a final concentration of 15% (v/v). The protein solution was applied to a Butyl-Sepharose FF column (4.7 ml) preequilibrated with 15% (v/v) saturated ammonium sulfate in 80 mM Tris-HCl, pH 8.0. After a first elution with 80 mM Tris-HCl, pH 8.0, the target enzyme was eluted with 6 M urea. The urea fraction, typically 20 ml, was mixed with saturated ammonium sulfate solution to a final concentration of 40% (v/v). After centrifugation for 10 min at 20,000 × g and 20 °C, the supernatant was withdrawn, and the pellet was solved in 2 ml of 100 mM Tris-HCl, pH 8.0. The concentrated solution was passed through a Superdex™ 200-pg column (120 ml) equilibrated with 10 mM Tris-HCl, pH 9.0. The active fractions from the gel filtration were applied to a second anion exchange chromatography with a different column material (ResourceQ, 1 ml) that was preequilibrated with 10 mM Tris-HCl, pH 7.0. The enzyme eluted at 120 mM NaCl in a step gradient performed with 2 ml min⁻¹ (AIE-Q2).

² The abbreviations used are: HMN, 2,2,4,4,6,8,8-heptamethylnonane; DMSO, dimethyl sulfoxide.
Determination of Relative Molecular Mass—The apparent relative molecular mass of the native enzyme was determined by gel filtration on a Superdex™ 200-pg column (120 ml) in 80 mM Tris-HCl buffer, pH 9.0. The standard proteins were: catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). The molecular mass of the monomeric enzyme was determined using a 12% SDS-polyacrylamide gel stained with Coomassie Blue R-250 (26). The protein ladder (Fermentas, St. Leon-Rot, Germany) covered a range molecular masses from 10 to 170 kDa.

UV-Visible Spectroscopy—Standards and purified linalool dehydratase fractions were dissolved in 80 mM Tris-HCl, pH 9.0, in the range between 10 and 100 μM Tris-HCl. UV absorption spectra were obtained by using a DU 600 UV-visible spectrophotometer (Beckman Coulter, Krefeld, Germany).

Protein Determination—The protein content was measured by Coomassie Blue R-250 protein assay (27) and by using bovine serum albumin as the standard.

N-terminal Amino Acid Sequence Analysis—Purified linalool dehydratase was separated by 12% SDS-PAGE and electroblotted on a PVDF membrane (Sequi-Blot; Bio-Rad Laboratories) according to the method of Towbin et al. (28). The membrane was washed for 1 min with distilled water and stained with Coomassie Blue R-250 (0.025% v/v) in 40% (v/v) methanol for 30 s before destaining with a water/methanol/acetic acid mixture (50/45/5, v/v/v). The PVDF membrane was washed with distilled water and dried for 6 h. The protein was excised from the membrane, and Edman degradation of the N-terminal amino acid residues was performed by Toplab GmbH (Martinsried, Germany). The gene and the protein sequence were deposited at GenBank under accession no. FR669447.

Overexpression in Escherichia coli—Standard molecular biology methods were applied. In short, the _ldi_ gene was amplified with the primers _ldi_Ndel_fw_ (TGCGACATATGATGCGGT-TCACATTGTGCGGT-TCACATTG) and _ldi_BglII_rw_ (CGCGAGATCTTTATTTC-CCTGCAGGCTTGCGGT-TCACATTG) from genomic _C. defragrans_ DNA and ligated into pCR4-TOPO (Invitrogen). The Ndel-BglII-flanked gene was transferred into pET-42a(+) (Novagen, Merck KGaA), and the gene was expressed in _E. coli_ BL21 Star™ (DE3) (Invitrogen). The construct correctness was confirmed by sequencing. Cultures were induced with isopropyl 1-thio-β-D-galactopyranoside. Soluble extracts were assayed in the anaerobic two-phase system.

RESULTS

Soluble extracts of _C. defragrans_ catalyzed the transformation of geranion in two directions (Fig. 1). A NAD⁺-reducing activity showed the presence of a geraniol dehydrogenase.³ In the absence of an electron acceptor, the dialyzed soluble extract initially formed linalool, and then, after a certain linalool concentration was reached, myrcene appeared. Both compounds, linalool and myrcene, were detected and identified by GC and GC-MS (data not shown). In separate experiments, the dialyzed soluble extract transformed linalool to myrcene.

Biomass yields in a pH-controlled fermenter were lower on myrcene than on limonene. Hence, we grew _C. defragrans_ on limonene. The crude extracts showed comparable specific linalool dehydratase activities. In contrast, the enzyme activity was not detected in cells grown on acetate. Addition of limonene (10 mM) to the culture growing on acetate resulted in induction of the enzyme activity after 10 h (data not shown).

Purification of Linalool Dehydratase from _C. defragrans_ Strain 65Phen—The purification of the linalool dehydratase initially yielded a preparation with several proteins (data not shown). The purification procedure was significantly improved by including a Butyl-Sepharose column. The protein eluted from this column with 6 M urea. In a five-step protocol, the enzyme activity was purified to a single protein band (Fig. 2). The linalool dehydratase protein yield was 0.02% of the initial protein, accompanied by a 1846-fold increase in the specific activity (Table 1). Gel filtration chromatography on a Superdex™ 200-pg column gave a single peak of active protein after a retention volume of 62 ml. Based on a calibration with standard proteins, linalool dehydratase exhibited a native molecular mass of 160 kDa. SDS-PAGE of the purified enzyme showed a single band with a molecular mass of 40 kDa (Fig. 2). These observations suggested that the native form of linalool dehydratase from _C. defragrans_ 65Phen is likely a homotetramer (α₄). UV-visible absorbance spectra revealed the absence of chromophors between 300 and 850 nm, suggesting that there was no prosthetic group present.

Catalytic Properties of Purified Linalool Dehydratase—The purified protein catalyzed the dehydration of linalool to myrcene in the absence of molecular oxygen but required 2 mM DTT. Geraniol was isomerized initially to linalool, and subsequently myrcene appeared (Fig. 3A). Both activities occurred concurrently in all purification steps; e.g. Fig. 4 shows the final anion exchange purification. The purification of the geraniol isomerase activity was 1740-fold similar to the 1846-fold purification of the linalool dehydratase activity.

³ J. Harder, unpublished results.
FIGURE 3. Time course of monoterpene transformation by the purified linalool dehydratase-isomerase in a two-phase-system with HMN as organic carrier phase (A) and in the presence of a myrcene phase (B). 

The enzyme activity measurements were performed in a two-phase system with HMN as organic phase. Like other monoterpenes, myrcene is 100-fold less soluble in water than monoterpenoids, e.g. geraniol or linalool: myrcene has a solubility of 43 μM and an octanol/water partition coefficient of logP = 4.5 (29). The organic phase served also as reservoir for the monoterpenoids. This dilution influences the actual concentrations of geraniol and linalool in aqueous solution. In equilibrium with the organic phase, calculation revealed micromolar concentrations for geraniol and linalool in the aqueous phase. Observed rates under these conditions were low, 14.5 picokatals mg⁻¹ for linalool dehydratase and 8.8 picokatals mg⁻¹ for geraniol isomerase.

The enzyme activities were not inhibited by 10% (v/v) DMSO. Thus, we performed the kinetic characterization in a single-phase system with HMN as organic phase as well as with a myrcene phase (Fig. 4A). Like other monoterpenes α- and β-ocimene nor the monoterpenoids citronellol and nerol were transformed. A 3-methylene group is absent in the oicmenes that have a 3-methyl-1,3-diene structure. Of the cis-3-methyl-2-en-1-ol motif present in geraniol, citronellol lacks the double bond at the C2-carbon atom, and nerol is the trans-isomer to geraniol. This suggests a highly specific binding site for the substrates.

Effects of Various Compounds—The purified linalool dehydratase required only DTT as a reducing agent and an oxygen free microenvironment (<1% (v/v)) for the dehydration of linalool. The activity was not detectable in the presence of 1 mM Ti(III)citrate. Other inhibitors were molecular oxygen (Table 2) and high salt concentrations. NaCl, KCl, or MgCl₂ at a concentration of 220 mM inhibited the enzyme activity completely. The metal-chelating agent EDTA (5 mM) did not affect the enzyme activity, suggesting that either the protein does not require metal ions for activity or the chelating molecule was not able to remove the metal ions under the assay conditions. Potassium nitrite or nitrate (20 mM) did not influence the enzyme activity. Coenzyme A was ineffective as a cofactor. However, phosphate as buffer or pyridoxal phosphate as well as S-adenosylmethionine modulated the enzyme activity. Cozyme A was ineffective as a cofactor. However, phosphate as buffer or pyridoxal phosphate as well as S-adenosylmethionine modulated the enzyme activity. Cozyme A was ineffective as a cofactor. However, phosphate as buffer or pyridoxal phosphate as well as S-adenosylmethionine modulated the enzyme activity. The enzyme is inhibited by urea: 20% activity remained at 3 M urea, and no activity was detected in 6 M urea.

Optimal pH and Thermophilicity—The linalool dehydratase activity had an optimal temperature at 35 °C. The enzyme activity had a pH maximum at low alkaline conditions (supplemental Fig. S1), but there was a sharp decrease in linalool dehydratase activity beyond pH 9.0. The optimal pH was 9.0 with Tris-HCl buffer. The temperature dependence of the reaction showed a linear Arrhenius plot in the range from 22 °C to 35 °C (supplemental Fig. S2), with an activation energy of Eₐ = 68.6 kJ/mol.

Identification of the Open Reading Frame—The N-terminal protein sequence was determined, and the corresponding open reading frame was found within a fosmid sequence obtained from C. defragrans 65Phen. The gene coded for a preprotein carrier nor in a DMSO-containing aqueous system. Myrcene was the only product detected in these experiments. We tested other acyclic monoterpenes as substrate for the enzyme. Neither the monoterpenes α- and β-ocimene nor the monoterpenoids citronellol and nerol were transformed. A 3-methylene group is absent in the oicmenes that have a 3-methyl-1,3-diene structure. Of the cis-3-methyl-2-en-1-ol motif present in geraniol, citronellol lacks the double bond at the C2-carbon atom, and nerol is the trans-isomer to geraniol. This suggests a highly specific binding site for the substrates.

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Identification of the Open Reading Frame—The N-terminal protein sequence was determined, and the corresponding open reading frame was found within a fosmid sequence obtained from C. defragrans 65Phen. The gene coded for a preprotein...
with 397 amino acids, including an N-terminal signal peptide sequence (MRFTLKTTAIVSAAALLAGFGPPPRAA) for transport into the periplasmatic space (supplemental Fig. S3). The alanine pair residues represent the cleavage motif. The experimentally determined N terminus of the purified protein started with the second alanine of the predicted cleavage motif (AELPPGRLATTE). Analyses with SignalP 3.0 (30) based on studies of signal-sequence cleavage sites (31) suggested a Sec-dependent membrane translocation mechanism for the preprotein into the periplasmatic space. Thus, the purified protein represents a mature protein. According to in silico mass calculation the precursor exhibits a molecular mass of 43 kDa.

Comparisons of the protein and of the gene sequences with nucleotide, microbial genome and environmental metagenomic datasets did not reveal significant relationships to known proteins and genes. TblastN (32) identified the closest relative as a hypothetical partial mRNA protein from the eukaryotic ascomycota Aspergillus oryzae RIB40 with an E value of 2E-08. The biotransformation potential of Aspergillus species on myrcene has been elucidated previously (33), although linalool was not detected as a transformation product. TblastP identified a hypothetical protein from another eukaryotic ascomycota, Nectria hematococca mpV1, as closest related protein, with an E value of 2E-12.

A ClustalW alignment of the linalool dehydratase-isomerase showed no relevant scores with characterized alkene hydratases, namely a γ-carotene 1,2-hydratase (CruF) from Deinococcus radiodurans R1 (34), a hydroxyneurosporene synthase from Rhodospirillum rubrum ATCC 11170, and a γ-carotene 1,2-hydroxylase from Synechococcus sp. PCC 7002. This was a further indication of the novel character of this enzyme and its catalytic activity.

Expression of Linalool Dehydratase-Isomerase in E. coli—The identified open reading frame was used to construct the expression vector pET-42a(-H11001)-LDI. Isopropyl 1-thio-D-galactopyranoside-induced 3-ml cultures showed a linalool dehydratase activity of 380 nanokatals and a geraniol isomerase activity of 310 nanokatals in a 6-h assay. Control cultures with the vector lacking the ldi gene had no enzyme activity. Soluble extracts of the induced cells had a specific linalool dehydratase activity of 435 picokatals mg-1 protein and a geraniol isomerase activity of 116 picokatals mg-1 protein in the two-phase assay.

DISCUSSION

Myrcene is an acyclic C₁₀-hydrocarbon and represents a large fraction (74%) of monoterpenes extracted from the essential oils of the hop plant Humulus lupulus (35). The transformation of this unsaturated hydrocarbon at the enzymatic level has never investigated under anaerobic conditions. Here, we describe a new initial reaction in the anaerobic degradation of hydrocarbons: the hydration of myrcene. First, a water molecule is added to the methylene double bond. Mechanistically, it may be equivalent to a chemical water addition catalyzed by acids, leading to linalool, a tertiary allylalcohol (3,7-dimethyl-1,6-octadien-3-ol). A subsequent isomerization yielded the primary allylalcohol geraniol (3,7-dimethylocta-2,6-dien-1-ol). These two reactions are catalyzed by a single bifunctional enzyme, the linalool dehydratase-isomerase.

The thermodynamic equilibrium favors the formation of myrcene from geraniol. Linalool is thermodynamically more stable than geraniol: according to experimental observations in a two-phase system with Thauera linaloolentis (36), linalool is 5.9-kJ mol-1 more stable than geraniol. Our observations con-
The complete assay contained 150 μl of 100 mM monoterpenone dissolved in HMN and 150 μl of protein solution (0.5 mg/ml) including 2 mM DTT.

| Assay                  | Linalool dehydratase | Geraniol isomerase |
|------------------------|----------------------|--------------------|
| %                      | %                    |
| Complete assay         | 100                  | 100                |
| −2 mM DTT              | 10                   | 10                 |
| +1 mM Ti(III)citrate   | 0                    | 0                  |
| +0.1% (v/v) O₂         | 100                  | 100                |
| +0.5% (v/v) O₂         | 110                  | 110                |
| +1% (v/v) O₂           | 90                   | 90                 |
| +20% (v/v) O₂          | 5                    | 5                  |
| +99.9% (v/v) O₂        | 0                    | 0                  |
| +1 mM pyridoxal phosphate | 65               | 100                |
| +40 mM S-adenosylmethionine | 20               | 200                |

Table 2: Effectors on enzyme activity

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