

Mechanism of *cis-trans* Isomerization of Unsaturated Fatty Acids in *Pseudomonas putida*

Angelika von Wallbrunn,¹ Hans Hermann Richnow,² Grit Neumann,²
Friedhelm Meinhardt,¹ and Hermann J. Heipieper^{2*}

Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, 48149 Münster,¹ and Department of Remediation Research, Centre for Environmental Research (UFZ) Leipzig-Halle, 04318 Leipzig,² Germany

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We studied the pattern of the *cis-trans* isomerization of unsaturated fatty acids in cells of *Pseudomonas putida* S12 grown in a medium supplemented with oleic acid which was deuterated at both of the C atoms of its double bond. Direct evidence that isomerization does not include a transient saturation of the double bond was obtained. In addition, analysis of the amino acid sequences of the seven known Cti proteins identified them as heme-containing proteins of the cytochrome *c* type.

Notwithstanding the toxicity of aromatic solvents to most microorganisms, bacteria that are able to tolerate high concentrations of these compounds in their environment do exist, and most of them belong to the genus *Pseudomonas* (11). One of the solvent adaptation mechanisms enabling these *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds is the isomerization of *cis*- to *trans*-unsaturated fatty acids (for reviews, see references 1 and 13). The extent of the isomerization apparently correlates with the toxicity and the concentration of such organic compounds in the membrane (7, 8).

***cis-trans* isomerization of unsaturated fatty acids in the solvent-tolerant bacterium *Pseudomonas putida* S12.** The *cis-trans* isomerase activity is constitutively present in *Pseudomonas*, does not require ATP or other cofactors like NAD(P)H or glutathione, and works in the absence of de novo synthesis of lipids (2, 5, 7, 15). Its independence from ATP is consistent with the negative free energy of the *cis*-to-*trans* isomerization (19). The enzyme has been purified from the periplasmic fraction of *Pseudomonas oleovorans* (17) and *Pseudomonas* sp. strain E3 (16) and has been isolated as a His-tagged *P. putida* P8 protein heterologously expressed in *Escherichia coli* (9). The *cis-trans* isomerase gene cloned and sequenced from *P. putida* P8 (10, 21) and *P. putida* DOT-T1E (12) made evident that the isomerase has an N-terminal hydrophobic signal sequence, which is cleaved off after the enzyme has been targeted to the periplasmic space. Holtwick et al. (9) provided evidence that the enzyme is a cytochrome *c*-type protein, as they found CXXCH, a heme-binding site (14), in the predicted Cti polypeptide. For an enzyme preparation from *Pseudomonas* sp. strain E3, which is presumably homologous to the *cti* gene product of *P. putida* P8, it was suggested that iron (probably Fe³⁺) plays a crucial role in the catalytic reaction (16).

Despite the wealth of available information, the biochemical mechanism of the solvent-induced regulation of isomerase ac-

tivity, in relation to membrane homeostasis (18), still remains obscure.

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Cultivation of the strain and supplementation with deuterated oleic acid. *P. putida* S12 (4) was cultivated in a mineral medium (3) with 15 mM glucose as the sole carbon source. Cells were grown in 100-ml shake cultures in a horizontally shaking water bath at 30°C. Growth was monitored by measuring the turbidity at 560 nm. When indicated, mineral medium was supplemented with 0.01% (wt/vol) [9,10-²H₂]oleic acid and 0.01% (vol/vol) Triton X-100 as described by Diefenbach and Keweloh (2). After 4 h of exponential growth, cells were harvested by centrifugation and resuspended in the same volume of mineral medium without oleic acid and Triton X-100; prior to the addition of 1-octanol, the cells were incubated for 30 min. For activation of the *cis-trans* isomerase, 1-octanol at a concentration of 0.04% (vol/vol) was added to the cells. The cultures were incubated in the presence of octanol for 2 h in a shaking water bath at 30°C. The cells were then harvested, and the lipids were extracted and transferred to fatty acid methyl esters (FAME) (6). Analysis of FAME in hexane was performed with a quadrupole gas chromatography-mass spectrometry (GC-MS) system (models HP6890 and HP5973; Hewlett-Packard, Palo Alto, Calif.) equipped with a split/splitless injector. A CP-Sil 88 capillary column (inside diameter, 0.32 mm; length, 30 m; film, 0.25 µm; Chrompack, Middelburg, The Netherlands) was used for the separation of the FAME. GC conditions were as follows. The injector temperature was held at 250°C. The split flow was 1:10, and the carrier gas was He. The temperature program was performed as follows: 80°C for 1 min isotherm, an increase of 15°C per min to 140°C, and an increase of 4°C per min to 280°C. The MS conditions were electron ionization mode with an ionization energy of 70 eV. The relative amounts of the carboxylic acids were determined by using their peak areas in total ion chromatograms. The fatty acids were identified by GC-MS and

* Corresponding author. Mailing address: Department of Remediation Research, Centre for Environmental Research (UFZ) Leipzig-Halle, Permoserstr. 15, 04318 Leipzig, Germany. Phone: 49 341 235 2772. Fax: 49 341 235 2492. E-mail: heipiep@san.ufz.de.

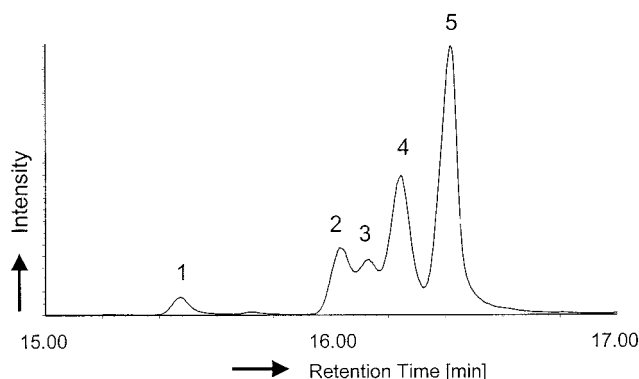


FIG. 1. Capillary GC and peak assignment of methylated total lipid extracts in hexane, prepared from $[9,10\text{-}^2\text{H}_2]$ oleic acid- and 1-octanol-treated cells of *P. putida* S12. The FAME were identified according to GC-MS analysis (see Fig. 2) and coinjection with the following authentic reference compounds (identified by number on the figure): 1, stearic acid ($\text{C}_{18:0}$); 2, $[9,10\text{-}^2\text{H}_2]$ elaidic acid ($\text{C}_{18:1}$ $\Delta 9\text{-trans}$; retention time, 16.037 min); 3, *trans*-vaccenic acid ($\text{C}_{18:1}$ $\Delta 11\text{-trans}$; retention time, 16.141 min); 4, $[9,10\text{-}^2\text{H}_2]$ oleic acid ($\text{C}_{18:1}$ $\Delta 9\text{-cis}$; retention time, 16.238 min); 5, *cis*-vaccenic acid ($\text{C}_{18:1}$ $\Delta 11\text{-cis}$; retention time, 16.420 min)

coinjection with authentic reference compounds obtained from Supelco (Bellefonte, Pa.).

Isomerization of deuterated oleic acid incorporated in the plasma membrane. *P. putida* uses the anaerobic pathway to synthesize unsaturated fatty acids (13) and its membranes therefore contain *cis*-vaccenic acid ($\Delta 11\text{-cis}$ -octadecenoic acid) as a C_{18} unsaturated fatty acid. Thus, in order to investigate the molecular mechanism of the *cis-trans* isomerase, cells of *P. putida* S12 were supplemented with oleic acid ($\Delta 9\text{-cis}$ -octadecenoic acid), which does not occur naturally in the cells. For monitoring possible changes at the double bond during isomerization from the *cis* to the *trans* configuration, oleic acid with a deuteration at the two C atoms of the double bond ($[9,10\text{-}^2\text{H}_2]$ oleic acid) was used in these experiments. Incorporation into the membrane lipids of the cells was found at levels of about 15% of total fatty acid; these findings are in accordance with observations for other bacteria (2, 6).

The addition of 1-octanol to cells previously grown exponentially in medium supplemented with $[9,10\text{-}^2\text{H}_2]$ oleic acid for 4 h induced the formation of *trans* isomers of the native and supplemented fatty acids. Figures 1 and 2 show a representative GC together with the corresponding mass spectra of the extracted C_{18} FAME obtained from the membranes after incubation for 2 h in the presence of 0.04% (vol/vol) 1-octanol. The corresponding mass spectra of the $\text{C}_{18:1}$ peaks are presented in

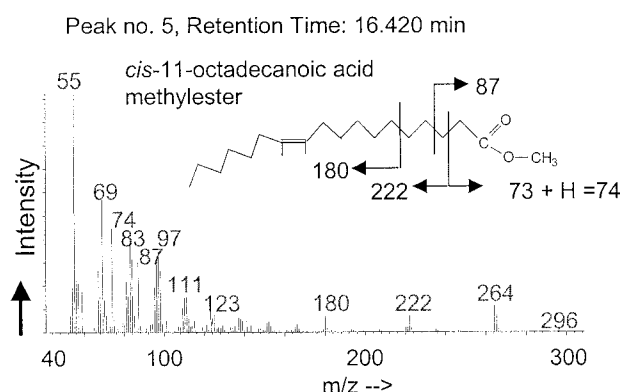
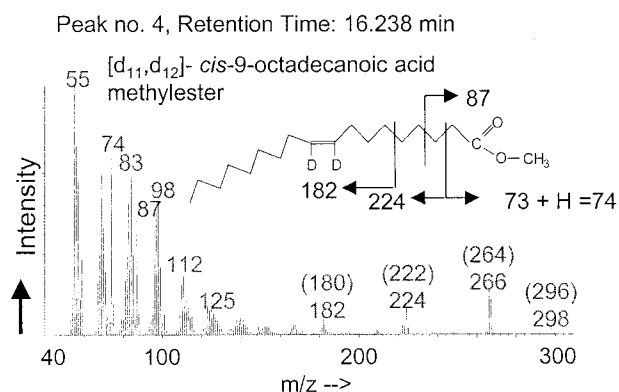
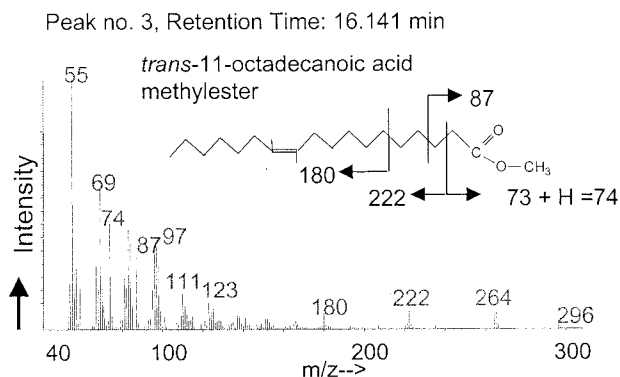
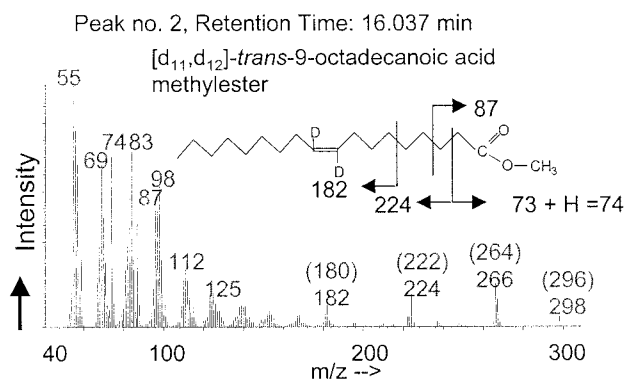


FIG. 2. Fragmentation patterns of unsaturated fatty acids, whereby mass numbers in brackets show the mass fragments of nondeuterated species (peak no. 3 and 5). The shift of 2 atomic mass units indicates that no loss of deuterium was observed during the isomerization reaction. Peak numbers and retention times for the GC are indicated (see Fig. 1).

Fig. 2. Next to the native C_{18} fatty acids of this bacterium, *trans*-vaccenic acid ($\Delta 11$ -*trans*-octadecenoic acid methylester, peak no. 3, retention time 16.141 min, m/z 296, nondeuterated) and *cis*-vaccenic acid ($\Delta 11$ -*cis*-octadecenoic acid methylester, peak no. 5, retention time 16.420 min, m/z 296, nondeuterated), the two nonnative fatty acids oleic acid ($\Delta 9$ -*cis*-octadecenoic acid methylester, peak no. 4, retention time 16.238 min, m/z 298, doubly deuterated) and elaidic acid ($\Delta 9$ -*trans*-octadecenoic acid methylester, peak no. 2, retention time 16.037 min, m/z 298, doubly deuterated) were detected. The occurrence of only one peak of elaidic acid with the molecular mass m/z 298 is most interesting, as it proves that the oleic acid was exclusively converted into the doubly deuterated derivative of elaidic acid. The mass fragmentogram of elaidic acid displays a completely labeled compound with two deuterium atoms and thus confirms that no deuterium was lost during the isomerization reaction (Fig. 2).

Alignment of the amino acid sequences of seven known Cti proteins. The alignment of the amino acid sequences of seven known Cti proteins was carried out with the ClustalW program (20) and the Malign (Heidelberg Unix Sequence Analysis Resources [HUSAR] from DKFZ Heidelberg, Heidelberg, Germany) and Bio-Edit (version 5.09) programs.

Irrespective of the taxon, a heme group of the cytochrome *c* type is present as a highly conserved motif and as a functional domain in all the enzymes compared (9). The covalent binding site of the heme in cytochrome *c* proteins is located between the heme-vinyl groups and the two cysteines of the conserved heme-binding motif CXXCH (reference 14 and data not shown).

In all Cti sequences of the six *Pseudomonas* strains, an N-terminal signal sequence is present, indicative of the periplasmic localization of the *cis-trans* isomerase. Such localization was already proven for *P. oleovorans* and *P. putida* DOT-T1E (12, 17). However, a signal peptide characterization for *sec*-dependent secretion is not present in the Cti protein of *Vibrio cholerae*.

A multiple sequence alignment of the seven known Cti proteins revealed that proteins from *Pseudomonas* and *Vibrio* strains form a phylogenetic tree composed of three main branches (Fig. 3), suggesting a common ancestor of the enzyme. Interestingly, the predicted polypeptide from *V. cholerae* obviously does not constitute a separate group but rather emanates from the diverse groups of proteins from *P. aeruginosa* and *Pseudomonas* sp. strain E3.

Though considerable research efforts have been carried out on the physiological, molecular biological, and biochemical mechanisms of the *cis-trans* isomerase of unsaturated fatty acids in *Pseudomonas* (and several *Vibrio*) strains, there are still at least two unanswered questions. One concerns the regulation of the activation of the constitutive protein which seems to be permanently present and potentially active in the periplasm. The regulation of enzyme activity may be brought about by giving the active center of the enzyme the ability to reach its substrate, the double bond, which in turn could depend on the fluidity of the membrane. Accordingly, the observed regiospecificity reflects penetration of the active site of the isomerase to a specific depth in the membrane (6). A periplasmic location of Cti supports the assumption that the enzyme is not an integral membrane protein; thus, only double

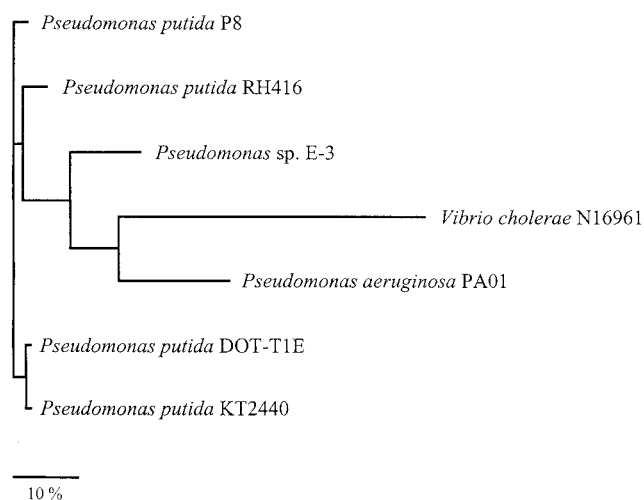


FIG. 3. Phylogenetic tree based on amino acid sequences of known fatty acid *cis-trans*-isomerases. Bar, 10 substitutions per 100 amino acids.

bonds at a certain depth of the membrane would be within reach of the active site of the enzyme (6).

The second unanswered question concerns the molecular mechanism of the isomerization reaction. In principle, two mechanisms are conceivable. From a mechanistic point of view, *cis-trans* isomerization of carbon-carbon double bonds may be a coupled hydration-dehydration reaction, as was demonstrated for β -oxidation enzymes, which show 2-*cis*-enoyl- and 3-*trans*-enoyl-coenzyme A isomerase activities (9).

Since double-deuterated elaidic acid appears exclusively after *cis-trans* isomerization in supplementation experiments with the double-deuterated oleic acid, an intermediate saturation of the double bond, e.g., by a coupled hydration-dehydration reaction, is clearly excluded as this would cause the loss of the deuteration. Thus, our results strongly indicate a mechanism in which an enzyme-substrate complex is formed whereupon the electrophilic iron (probably Fe^{3+}), provided by the heme domain present in the enzyme, removes an electron from the *cis* double bond, transferring the sp^2 linking into an sp^3 . The double bond is then reconstituted after rotation to the *trans* configuration has occurred. *cis-trans* isomerization of unsaturated fatty acids can also be catalyzed nonenzymatically by iron, which is attached to the double bond during the reaction, suggesting a transformation of the double bond via sp^2 to sp^3 and a reformation after rotation has taken place (19). These observations are in good accord with results of site-directed mutagenesis experiments carried out to destroy the heme-binding motif in Cti of *P. putida* P8 (9) that led to a loss of the enzyme activity and, therefore, indicated the presence of cytochrome *c* and heme, respectively, in the catalytic center of the enzyme. Cti activity is independent of additional factors such as ATP, NADPH, and O_2 (2, 16); Cti differs in this respect from all other known heme-containing enzymes acting on fatty acids as substrates. There is, however, no need of a cofactor because no net electron power is consumed.

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