 Reaction Mechanism and Stereochemistry of γ-Hexachlorocyclohexane Dehydrochlorinase LinA*

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γ-Hexachlorocyclohexane dehydrochlorinase (LinA) catalyzes the initial steps in the biotransformation of the important insecticide γ-hexachlorocyclohexane (γ-HCH) by the soil bacterium Sphingomonas paucimobilis UT26. Stereoechemical analysis of the reaction products formed during conversion of γ-HCH by LinA was investigated by GC-MS, NMR, CD, and molecular modeling. The NMR spectra of 1,3,4,5,6-pentachlorocyclohexene (PCCH) produced from γ-HCH using either enzymatic dehydrochlorination or alkaline dehydrochlorination were compared and found to be identical. Both enantiomers present in the racemate of synthetic γ-PCCH were converted by LinA, each at a different rate. 1,2,4-trichlorobenzene (1,2,4-TCB) was detected as the only product of the biotransformation of biosynthetic γ-PCCH. 1,2,4-TCB and 1,2,3-TCB were identified as the dehydrochlorination products of racemic γ-PCCH. δ-PCCH was detected as the only product of dehydrochlorination of δ-HCH. LinA requires the presence of a 1,2-biaxial HCl pair on a substrate molecule. LinA enantiotopologically differentiates two 1,2-biaxial HCl pairs present on γ-HCH and gives rise to a single PCCH enantiomer 1,3(R),4(S),5(S),6(R)-PCCH. Furthermore, LinA enantiomerically differentiates 1,3(S),4(R),5(R),6(S)-PCCH and 1,3(R),4(S),5(S),6(R)-PCCH. The proposed mechanism of enzymatic biotransformation of γ-HCH to 1,2,4-TCB by LinA consists of two 1,2-anti conformationally dependent dehydrochlorinations followed by 1,4-anti dehydrochlorination.

Dehydrochlorinases are enzymes that eliminate HCl from a substrate molecule leading to the formation of a double bond. Three different dehydrochlorinases have been reported to date. DDT dehydrochlorinase (1) isolated from Musca domestica catalyzes dehydrochlorination of 1,1-trichloro-2,2-bis(4-chlorophenyl)ethane to 1,1-dichloro-2,2-bis(4-chlorophenyl) ethene and requires glutathione for its activity (2). 3-Chloro-β-alanine dehydrochlorinase (3) from Pseudomonas putida employs the cofactor pyridoxal 5'-phosphate during catalysis. γ-Hexachlorocyclohexane dehydrochlorinase (LinA)3 (4) from the γ-hexachlorocyclohexane-degrading bacterium Sphingomonas paucimobilis UT26 catalyzes the conversion of γ-hexachlorocyclohexane (γ-HCH) to 1,2,4-trichlorobenzene (1,2,4-TCB) via γ-1,3,4,5,6-pentachlorocyclohexene (γ-PCCH). LinA does not require any cofactor for its activity and therefore represents a distinct type of enzyme from the former two dehydrochlorinases.

The linA gene encoding γ-hexachlorocyclohexane dehydrochlorinase was cloned by Imai et al. (5). The nucleotide sequence of the linA did not show sequence similarity to any sequence deposited in the databases. Recently, a gene identical to linA was cloned by Thomas et al. (6) from the newly isolated γ-HCH-degrading bacterium. The G+C content of linA (53%) is considerably lower than that of other genes and of the total DNA of Sp. paucimobilis strains, suggesting that linA originates from the genome of some other genus or organism. The linA gene was expressed in Escherichia coli, and the translation product (γ-HCH dehydrochlorinase LinA) was purified to homogeneity by Nagata et al. (4). Purified LinA showed activity with α-, γ-, and δ-HCH, but not with β-HCH. Because β-HCH does not contain a 1,2-biaxial HCl group, it was proposed that LinA dehydrochlorinates stereoelectronically at this pair of hydrogen and chlorine (7).

This paper presents stereoechemical analysis of the reaction products of enzymatic dehydrochlorination of γ-HCH by LinA. The absolute configuration and conformation of the reaction products is established, and the reaction mechanism of dehydrochlorination of LinA is proposed.

**EXPERIMENTAL PROCEDURES**

Chemical Synthesis of γ-PCCH—γ-PCCH was synthesized by alkaline dehydrochlorination of γ-HCH (8). γ-HCH of more than 98% purity was purchased from Sigma-Aldrich. 50 mg of γ-HCH was dissolved in 5 ml of acetonitrile. The synthesis was started by addition of 2.5 ml of 0.1 M NaOH to the reaction mixture. The reaction mixture was heated for 20 min at 40 °C. The products of synthesis were extracted with hexane. γ-PCCH was purified by preparative liquid chromatography with a steel column (8 × 250 mm) packed by silica gel (7 μm). 20% dichloromethane in hexane was used as the mobile phase.

Biocatalytic Synthesis of γ-PCCH—Purified enzyme LinA was prepared as described previously (4). 10 mg of γ-HCH and 100 ml of phosphate buffer, pH 7.5, were equilibrated at 35 °C in a shaking water bath. The enzymatic reaction was initiated by adding 100 μl of LinA (protein concentration: 45 mg/l). The reaction was stopped after 5 min by extraction with hexane. The product γ-PCCH was purified using the same procedure as described above for chemically synthesized γ-PCCH.

Kinetics of LinA with γ-PCCH—Synthesized γ-PCCH was dissolved in phosphate buffer (10 ml, pH 7.5) and equilibrated at 35 °C in a water bath. The rate of dehydrochlorination was followed by gas chromatography-mass spectrometry; HCH, hexachlorocyclohexane; PCCH, pentachlorocyclohexene; NMR, nuclear magnetic resonance; TCB, trichlorobenzene; TCDN, tetrachlorodicyclohexa-1,4-diene; DFT, density functional theory; CD, circular dichroism.

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2 The abbreviations used are: LinA, γ-hexachlorocyclohexane dehydrochlorinase from S. paucimobilis UT26; BPS, N-[1-[4-bromophenyl]ethyl]-5-fluoro-salicilamide; GC-MS, gas chromatography-mass spectrometry; HCH, hexachlorocyclohexane; PCCH, pentachlorocyclohexene; NMR, nuclear magnetic resonance; TCB, trichlorobenzene; TCDN, tetrachlorodicyclohexa-1,4-diene; DFT, density functional theory; CD, circular dichroism.

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shaking water bath. The enzymatic reaction was initiated by adding 10 ml of LinA (protein concentration: 45 mg/l). The progress of the reaction was monitored in 1 ml of the reaction mixture at 5, 10, 20, 40 min, and 24 h. Samples from the reaction mixture were extracted with 0.3 ml of hexane and analyzed by GC-MS as described below.

**GC-MS Analysis**—Reaction products were identified and quantified on a GC-MS system (Hewlett Packard 6890) with helium as a carrier gas. The temperature of the DB-5MS capillary column (59.5 m × 0.25 mm × 0.25 μm, J&W Scientific) was kept at 50 °C for 2 min and then increased to 300 °C at a rate of 15 °C/min. The scan mode at 50–550 m/z was used for searching and for identification of products, whereas SIM mode was used for quantification.

**Testing of Enantiomeric Purity**—The enantiomeric purity of the reaction products was monitored on a GC system (Hewlett Packard 5890) equipped with ECD detector and CYCLODEX-B capillary column (30 m × 0.25 mm × 0.25 μm, J&W Scientific). The column temperature was increased from 80 to 220 °C at a rate 10 °C/min and then the temperature was kept for 10 min at 220 °C.

**NMR Spectroscopy**—NMR spectra were collected on a Bruker AVANCE 500 MHz spectrometer equipped with a z-gradient triple resonance1H/13C/BB probehead at 298.2 K. The NMR samples were prepared in total volumes of 260 μl in 99.99% CD3CN. Selective one-dimensional 1H TOCSY (9) with a mixing time of 50 ms and two-dimensional NOESY (10) with a mixing time of 900 ms were acquired for resonance assignment of γ-PCCH. The acquisition parameters used for selective one-dimensional 1H TOCSY were: spectral width 5000 Hz, 8192 complex points, 2.2 s recycle delay, mixing time 50 ms and 8 scans. The spectrum was zero-filled to 12288 real points, and resolution was enhanced by 82° shifted-square sine bell apodization function. The acquisition parameters used for two-dimensional NOESY were: spectral width 5000 Hz in the both dimensions (t1, t2), 2048 complex points in the t2 dimension, 8192 complex points in t1, 2.2 s recycle delay, mixing time 50 ms and 8 scans. The spectrum was collected with the States-TPPI quadrature detection in t1 (11) with mixing time 900 ms. The spectrum was zero-filled to 2048 real points in t2 and to 8192 real points in the t1 dimension, and resolution was enhanced by a 82° shifted-square sine bell apodization function. Three bond proton-proton scalar coupling constants were obtained from standard high resoluted one-dimensional 1H NMR spectrum. The acquisition parameters were: spectral width 5000 Hz, 12288 complex points, 2.2 s recycle delay, mixing time 50 ms and 8 scans. The spectrum was zero-filled to 32 120 real points, and resolution was enhanced using a 45° shifted-square sine bell apodization function.

**CD Spectroscopy**—The CD spectra were measured in acetonitrile at 298.2 K using a Jasco J-720 spectropolarimeter using a 1-cm path length and a wavelength of 200–350 nm.

**Quantum Chemical Calculations**—Ab initio geometry optimizations were conducted with Gaussian 98 (Gaussian, Inc.) using density func-
tional theory (DFT) method. These optimizations employed the Becke3P86 hybrid functional and 6-31G** basis set. The scalar couplings were calculated using the program deMon-NMR (MASTERS-CS, Universite de Montreal, Canada). The PERDEW functional and the basis set IGLO-III of Kutzelnigg et al. (12) were used in the calculations.

The electric and magnetic transition moments, respectively, were calculated for the six energetically lowest transitions using the time-dependent adiabatic extension of DFT, Becke3P86 hybrid functional and 6-31G** basis set. Quantum-mechanical calculations were performed on a SGI R10000 (SGI).

Molecular Modeling—The homology model of LinA dehalogenase was constructed using the method of satisfaction of spatial restraints as described elsewhere.2 The crystal structure of scytalone dehydratase (13), nuclear transport factor-2 (14), 3-oxo-5-steroid isomerase (15), and naphthalene 1,2-dioxygenase (16) served as the template structures (PDB accession codes 1std, 1oun, 1opy, and 1indo). The substrate molecule γ-HCH was docked in the active site manually using the program O, version 6.2.1. (17).

RESULTS

Identification of the Reaction Products by GC-MS—The numbering of the atoms in γ-HCH and γ-PCCH molecules used in the article is given in Fig. 1. The nomenclature of Izumi and Tai (18) was used for classification of the stereochemical course of the reactions. The activity of LinA toward γ-PCCH, originating from the alkaline dehydrochlorination of γ-HCH, was tested, and the end products of the reaction were identified using GC-MS. These products were compared with the end products of the enzymatic transformation of γ-HCH (Fig. 2, A and B). The same compound, 1,2,4-trichlorobenzene (1,2,4-TCB), was identified as the product of dehydrochlorination of both synthetic and biosynthetic γ-PCCH. In addition to 1,2,4-TCB, 1,2,3-TCB was also found in the reaction mixture obtained from the dehydrochlorination of synthetic γ-PCCH by LinA. Chromatography of the γ-PCCH formed by enzymatic dehydrochlorination using chiral stationary phase confirmed the formation of a single enantiomer in the reaction mixture (Fig. 2 A, inset). Alkaline dehydrochlorination of γ-HCH is known to proceed primarily by an E2 (AxhDHDN according to IUPAC) anti eli-
nation mechanism (19, 20) resulting in formation of the racemate of 1,3(S),4(R),5(S),6(R)- and 1,3(R),4(S),5(S),6(R)-PCCH. Consequently, one of the stereoisomers present in the racemate of γ-PCH from alkaline dehydrochlorination is identical with the stereoisomer of γ-PCH formed during enzymatic dehydrochlorination, whereas 1,2,3-TCB is the product formed by dehydrochlorination of the remaining enantiomer present in the racemate (Fig. 2B, inset). The formation of the single enantiomer during enzymatic dehydrochlorination of γ-HCH suggests that LinA enzyme specifically differentiates the enantiotopical pairs of the vicinal HCl.

Different rates of formation of 1,2,4-TCB and 1,2,3-TCB were observed in kinetic measurements of dehydrochlorination of synthetic γ-PCH by LinA enzyme (Fig. 3). At the same time, different rates of consumption of the two enantiomers of synthetic γ-PCH were observed by gas chromatographic analysis employing the column for chiral separations. The enzyme eventually transformed all compounds present in the racemate. The formation of the single enantiomer during enzymatic dehydrochlorination of γ-HCH suggests that LinA enzyme specifically differentiates the enantiotopical pairs of the vicinal HCl.

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family of proteins (26) revealed that LinA shows distant relationships with scytalone dehydratase (13), nuclear transport factor-2 (14), and 3-oxo-D5-steroid isomerase (15). The proteins in this superfamily have diverged beyond notable sequence similarity and have evolved different function, but retain the general design of the active site cavity (26). This enabled us to construct a three-dimensional model of LinA by homology and dock the substrate molecule in its active site. A molecule of $\gamma$-HCH was manually docked into the LinA active site in a way that would allow efficient abstraction of a hydrogen from the 1,2-biaxial $\text{HCl}$ pair of the substrate molecule by the general base His-73 (Fig. 6B). The theoretical model of LinA complexed with the substrate is compared with the crystal structure of scytalone dehydratase complexed with its inhibitor (Fig. 6). The figure illustrates the common fold and conserved catalytic dyad His-73/Asp-25 of these enzymes. The essential role of the putative catalytic dyad for LinA activity was confirmed experimentally by site-directed mutagenesis.

**DISCUSSION**

**Reaction Mechanism of $\gamma$-HCH Dehydrochlorinase LinA**—The following experimental observations have been taken into account for the proposed reaction mechanism of LinA: (i) $\gamma$-PCCH formed by the enzymatic dehydrochlorination of $\gamma$-HCH is in the configuration corresponding to anti elimination. (ii) LinA exclusively dehydrochlorinates HCH substrates containing at least one 1,2-biaxial pair of hydrogen and chlorine. (iii) $1,3(R),4(S),5(S),6(R)$-PCCH is the exclusive product of enzymatic dehydrochlorination of $\gamma$-HCH, and (iv) His-73 and Asp-25 form the catalytic dyad of LinA.

The putative reaction mechanism for dehydrochlorination of $\gamma$-HCH by LinA enzyme is depicted in Fig. 7. Molecular modeling revealed that the most probable conformation of the $\gamma$-HCH in the active site is the chair conformation (see next paragraph). The HCl pair involved in the reaction is forced to adopt the 1,2-biaxial position in the enzyme active site. The requirement for the presence of a 1,2-biaxial HCl pair in the substrate molecules, the geometry of the active site as well as the kinetic measurements, indicate an $E_2$-like dehydrochlorination mechanism (27). We propose that His-73 acts as the base and attacks the hydrogen atom on C3, resulting in breaking of the C3–H bond based on the analogous role of catalytic histidine in the scytalone dehydratase (13). Asp-25 assists in the catalysis by keeping His-73 in the proper orientation and by stabilizing the positive charge that develops on the histidine imidazole ring during the reaction. There are probably other residues, which stabilize the transition state and the reaction products, e.g. via nonbonding interactions with hydrogen and chlorine atoms on the ring. Possible candidates are Lys-20 and Arg-129, which were shown to be important for the catalytic activity of LinA by site-directed mutagenesis experiments.

A dehydrochlorination of $\gamma$-PCCH is considered to proceed in two successive steps as shown in Reactions 1 and 2 (7, 19, 28).

$\begin{align*}
\text{Eq. 1: } & \text{C}_2\text{H}_4\text{Cl}_3 \rightarrow \text{C}_3\text{H}_4\text{Cl}_4 + \text{HCl} \\
\text{Eq. 2: } & \text{C}_2\text{H}_4\text{Cl}_4 \rightarrow \text{C}_3\text{H}_4\text{Cl}_3 + \text{HCl}
\end{align*}$

We confirmed that LinA is specific toward 1,2-biaxial hydrogen...
and the chlorine pair in Reaction 1. Enzymatic dehydrochlorination of \(\gamma\)-PCCH proceeds by a 1,2-anti dehydrochlorination reaction (Reaction 1), followed by 1,4-anti dehydrochlorination (Reaction 2). Because the enzymatic transformation of 1,3\((R)\),4\((S)\),5\((S)\),6\((R)\)-PCCH results exclusively in the formation of 1,2,4-TCB, the reaction must proceed through 1,3\((R)\),4,6\((R)\)-tetrachlorocyclohexa-1,4-diene (TCDN) as an intermediate (Fig. 8). Biotransformation of 1,3\((R)\),4\((S)\),5\((R)\),6\((S)\)-PCCH to 1,2,3-TCB then proceeds through 1,3,5,6-TCDN. The dehydrochlorination of the 1,3\((R)\),4\((S)\),5\((S)\),6\((R)\)-PCCH by LinA starts on the H4Cl5 pair and proceeds analogously from a stereochemical and mechanistic point of view to \(\gamma\)-HCH dehydrochlorination (Fig. 8).

Transformation of 1,3\((R)\),4,6\((R)\)-TCDN to 1,2,4-TCB has been proposed to proceed by a spontaneous nonenzymatic rearrangement, based on the assumption of an unstable diene-type structure (7). 1,3\((R)\),4,6\((R)\)-TCDN has never been directly detected in the reaction mixture, suggesting that 1,4 elimination of HCl from TCDN proceeds by the same or higher rate than enzymatic 1,2 elimination of HCl from \(\gamma\)-PCCH. 1,2,3-TCB is the exclusive product of a 1,4 elimination reaction of 1,3,5,6-TCDN. The specific formation of 1,2,3-TCB seems to support the enzymatic nature of Reaction 2. LinA could specifically differentiate between 1,4 H6Cl3 and H3Cl6 groups of 1,3,5,6-TCDN, when only elimination of H6Cl3 results in formation of 1,2,3-TCB. In case of nonenzymatic elimination, the preference for 1,4 elimination of H6Cl3 over H3Cl6, and lack of 1,3,5-TCB product (28) could be caused by the higher activation barrier or unfavorable thermodynamics of H3Cl6 elimination. More research is needed to elucidate the mechanism of Reaction 2.

**Active Conformations of \(\gamma\)-HCH Dehydrochlorinase Substrates**—The chair conformation of \(\gamma\)-HCH is expected to be the active conformation during its dehydrochlorination by LinA (Fig. 6B) for two reasons. One is that \(\gamma\)-PCCH is known to be both a LinA substrate and a competitive inhibitor of \(\gamma\)-HCH dehydrochlorinization (7), and the chair conformation allows similar binding modes for both \(\gamma\)-HCH and \(\gamma\)-PCCH substrates. The other reason is that in a chair conformation there is at least one axial chlorine atom laying in the same plane as the abstracted proton. The fact that both \(\gamma\)-PCCH enantiomers are LinA substrates strongly suggests that there may be more than one substrate-binding mode. Based on the shape of the active site and conformational analogy with the active conformation.
of γ-HCH, the twist conformation of α is the expected active conformation for γ-PCH.

**Stereodifferentiation of the Substrates Dehydrochlorinated by LinA**—The production of 1,3(R),4(S),5(S),6(R)-PCCH during the enzymatic transformation of γ-HCH proves differentiation of the enantiotropic H2Cl3/H5Cl6 and H3Cl2/H6Cl5 groups. The different rates of the consumption of the two enantiomers of the γ-PCH as well as different rates of the creation of the 1,2,3- and 1,2,4-TCB confirm the stereodifferentiation of the γ-PCH enantiomers by LinA. The production of the 1,2,4-TCB from 1,3(R),4(S),5(S),6(R)-PCCH by enzymatic dehydrochlorination proves differentiation of the diastereotopic H4Cl5 and H5Cl4 groups. Both topological differentiations are consequences of sharing a common conformation on one side of the ring facing the catalytic residues, the enantiomeric differentiation of γ-PCH enantiomers arises as a consequence of the opposite orientation of the hydrogen and chlorine atoms on the double bond at the active site. Whereas catalytic residues cause the topological differentiation, the enantiomeric differentiation is driven by the noncovalent interaction of the double bond substituents with noncatalytic residues in the active site of the LinA. The expected active conformations of 1,3(R),4(S),5(S),6(R)-PCCH and 1,3(S),4(R),5(R),6(S)-PCCH are depicted in Fig. 8.

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