Enantioselective Dehydrochlorination of δ -Hexachlorocyclohexane and δ -Pentachlorocyclohexene by LinA1 and LinA2 from *Sphingobium indicum* B90A

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δ -Hexachlorocyclohexane (δ -HCH), one of the prevalent isomers of technical HCH, was enantioselectively dehydrochlorinated by the dehydrochlorinases LinA1 and LinA2 from *Sphingobium indicum* B90A to the very same δ -pentachlorocyclohexene enantiomer. Racemic δ -pentachlorocyclohexene, however, was transformed with opposite enantioselectivities by the two enzymes. A transformation pathway based on an *anti*-1,2-elimination, followed by a *syn*-1,4-elimination and a subsequent *syn*-1,2-elimination is postulated.

Hexachlorocyclohexane (HCH) was extensively used as an insecticide during the last century (1). Indiscriminate agricultural application of technical HCH and inadequate disposal of isomeric waste from lindane (γ -HCH) production led to widespread pollution of agricultural soils and production dump sites, respectively, with insecticidally inactive ballast isomers (mainly α -, β -, δ -, and ϵ -HCH) (1–4).

The first two steps in the aerobic degradation pathway of γ -HCH are catalyzed by a dehydrochlorinase of the LinA type (3, 5, 6) and, recently, the crystal structure of the dehydrochlorinase LinA from *Sphingobium japonicum* UT26, which is identical to LinA2 from *Sphingobium indicum* B90A, was solved; those study authors discussed in detail the binding mode of γ -HCH to the active site (7). The other environmentally relevant HCH isomers, except for β -HCH, are also substrates of LinA-type dehydrochlorinases, and the dehydrochlorination of α -, γ -, and ϵ -HCH by LinA enzymes was shown to proceed via pentachlorocyclohexenes (PCCHs) and the putative intermediate tetrachlorocyclohexadiene (TCDN) to trichlorobenzenes (TCBs) (2, 8, 9).

It was postulated early on that only HCH and PCCH isomers with *trans*-diaxial HCl pairs are substrates for dehydrochlorinases (10). This observation also provided an explanation for the missing activity of these enzymes toward β -HCH, which does not have such arrangements. δ -HCH was reported to be a substrate for LinA, but different end products of this reaction were described (11, 12). In this study, we investigated the conversion of δ -HCH and δ -PCCH by two LinA variants from *S. indicum* B90A with respect to total mass balances, final products, and enantioselectivity of the reactions. Based on the data, we postulate a reaction mechanism that is composed of three HCl elimination steps: an *anti*-1,2-elimination, followed by a *syn*-1,4-elimination and a subsequent *syn*-1,2-elimination.

δ -HCH was incubated with purified, His-tagged LinA1 and LinA2 according to previously published procedures (2). To monitor substrate degradation and emerging metabolites, we performed chiral gas chromatography-mass spectrometry (GC-MS) using a Trace GC Ultra apparatus equipped with an ITQ 900 ion trap system (Thermo Scientific, Waltham, MA) under electron impact ionization (EI; 70 eV, 200°C) and full scan conditions. The

FIG 1 Reaction scheme used for modeling of δ -HCH and δ -PCCH degradation kinetics.

GC conditions were as follows: split/splitless injection (60 s splitless, 250°C) on a 25-m chiral BGB171 column (0.25-mm inner diameter by 0.12- μ m film thickness; BGB Analytik AG, Bockten, Switzerland). The temperature program started at 70°C for 2 min, then increased at 15°C/min to 110°C, at 2°C/min to 200°C, and at 25°C/min to 230°C, followed by a 2-min isothermal hold.

For both enzymes, the δ -HCH concentrations decreased with a concomitant increase of δ -PCCH. As the absolute configurations of the two δ -PCCH enantiomers were not available, we designated them δ -PCCH1 and δ -PCCH2, respectively, according to their elution order on the GC column. For parameter estimation by nonlinear regression, we used the modeling software AQUASIM (13). Degradation of parent compounds and formation of metabolites were fitted to experimental data, assuming first-order reac-

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TABLE 1 Modeled reaction rates, stoichiometric factors, and initial substrate concentrations for the formation of chlorinated benzenes from δ -HCH and δ -PCCH

Parameter	Unit	LinA1 ^b	LinA2 ^b
δ -HCH ^a			
$c_{\delta\text{-HCH},0}$	μM	326 ± 2	309 ± 10
$k_{-(\delta\text{-HCH})}$	h^{-1}	0.039 ± 0.001	0.78 ± 0.07
$\delta\text{-HCH} \rightarrow \delta\text{-PCCH1}$	Stoichiometric factor χ	0.39 ± 0.03	0.006 ± 0.001
$\delta\text{-HCH} \rightarrow \delta\text{-PCCH2}$	Stoichiometric factor $1 - \chi$	0.61 ± 0.04	0.99 ± 0.02
$k_{\text{cat}}/K_m (\delta\text{-HCH})^c$	$\text{M}^{-1} \text{s}^{-1}$	0.77 ± 0.04	77.4 ± 7.9
δ -PCCH			
$c_{\delta\text{-PCCH1},0}$	μM	188 ± 3	188 ± 2
$c_{\delta\text{-PCCH2},0}$	μM	188 ± 3	188 ± 2
$k_{-(\delta\text{-PCCH1})}$	h^{-1}	0.57 ± 0.03	0.14 ± 0.01
$k_{-(\delta\text{-PCCH2})}$	h^{-1}	0.067 ± 0.005	0.30 ± 0.02
$\delta\text{-PCCH} \rightarrow 1,2,3\text{-TCB}$	Stoichiometric factor ϕ	0.064 ± 0.021	0.13 ± 0.01
$\delta\text{-PCCH} \rightarrow 1,2,4\text{-TCB}$	Stoichiometric factor $1 - \phi$	0.94 ± 0.02	0.87 ± 0.03
$k_{\text{cat}}/K_m (\delta\text{-PCCH1})^c$	$\text{M}^{-1} \text{s}^{-1}$	11.1 ± 0.9	13.4 ± 1.0
$k_{\text{cat}}/K_m (\delta\text{-PCCH2})^c$	$\text{M}^{-1} \text{s}^{-1}$	1.31 ± 0.11	30.2 ± 2.3

^a The modeled parameters (rate constants and stoichiometric factors) from the respective δ -PCCH degradation experiment were used to fit the kinetics of δ -HCH degradation.

^b Values are means $\pm$ standard deviations, as modeled by using AQUASIM, and proper error propagation was performed for derived quantities.

^c The k_{cat}/K_m values were calculated according to the following equation: $k_{\text{cat}}/K_m = k/[E_0]$, where $[E_0]$ is the stoichiometric concentration of active centers and k is the rate constant for the degradation of the respective substrate. The coefficient of variation for the determination of $[E_0]$ was estimated to be 5%.

tion kinetics, as shown in Fig. 1. In the experiment with LinA2, a certain amount of δ -HCH was not available for the enzymes. Therefore, we allowed for a residual substrate concentration as an additional fitting parameter ($85 \mu\text{M}$) in the model. LinA2 degraded δ -HCH with a 100-fold-higher specificity constant (k_{cat}/K_m) than LinA1 (Table 1). The maximum concentrations of δ -PCCH that were reached also strongly depended on the enzyme variant. δ -PCCH accumulated up to 60 and $150 \mu\text{M}$ when LinA1 and LinA2, respectively, were used as the catalysts (Fig. 2A and B). Wu et al. showed that δ -PCCH was further converted to 1,2,4-TCB by LinA from *Sphingobium* sp. BHC-A (12). We confirmed this observation but identified 1,2,3-TCB as an additional but minor product. The third possible TCB isomer, 1,3,5-TCB, was never observed. We found complete mass balances. Since δ -PCCH is a chiral molecule, we were interested in whether LinA1 and LinA2 showed enantioselectivity with respect to δ -PCCH formation. LinA1 and LinA2 both converted δ -HCH mainly to one δ -PCCH isomer (δ -PCCH2), although traces of δ -PCCH1 were also detected. Enantiomeric excesses of $>87\%$ and $>98\%$ were reached for δ -PCCH2 in reactions catalyzed by LinA1 and LinA2, respectively.

As the enantiomeric excess of transient PCCHs observed during incubation of δ -HCH is the result of enantioselective formation as well as degradation, we subsequently also studied the transformation of racemic δ -PCCHs by LinA1 and LinA2. Racemic δ -PCCH was prepared from δ -HCH according to the methods described by Trantírek et al. (11) and incubated with LinA1 and LinA2. Control reactions without enzyme did not show any degradation. We found opposite enantioselectivities of the two en-

FIG 2 Degradation of δ -HCH and racemic δ -PCCH as monitored by GC-MS. (A and C) LinA1 (0.4 mg ml^{-1}); (B and D) LinA2 (0.08 mg ml^{-1}). The substrates and metabolites are symbolized as follows: δ -HCH ($\blacklozenge$), δ -PCCH1 ($\bullet$), δ -PCCH2 ($\circ$), 1,2,3-TCB ($\blacktriangle$), 1,2,4-TCB ($\triangle$), sum of all reactants ($\diamond$). Continuous lines represent the model fits of the data; corresponding fitted parameters are summarized in Table 1.

zymes with respect to δ -PCCH degradation (Fig. 2C and D). LinA2 converted δ -PCCH2 2.4-fold faster than δ -PCCH1 (Table 1) and, in contrast, LinA1 degraded δ -PCCH1 8.5-fold faster than δ -PCCH2. Suar et al. (9) reported opposite enantioselectivities of LinA1 and LinA2 with respect to the formation as well as the dissipation of chiral β -PCCHs from chiral α -HCH. Here, we observed the same phenomenon with respect to dissipation but not formation of δ -PCCH enantiomers from achiral δ -HCH.

In general, LinA2 exhibited higher k_{cat}/K_m values for both PCCH isomers than LinA1 did, which is in accordance with recently published results (2, 14) describing LinA2 as the more active enzyme. Although the enzymes did not degrade the δ -PCCH enantiomers in the same order, they both formed 1,2,4-TCB as major and 1,2,3-TCB as minor reaction products.

The fitted stoichiometric factors for the formation of δ -PCCH1 and δ -PCCH2 (χ and $1 - \chi$, respectively [Table 1]) indicated that both enzymes primarily form δ -PCCH2, but with different preferences. While LinA1 accumulated only a small excess of δ -PCCH2 (61% versus 39% δ -PCCH1), LinA2 almost exclusively formed δ -PCCH2.

Based on our results, we propose a pathway for the transformation of δ -HCH by LinA (Fig. 3). δ -HCH can adopt two different conformations, but one is strongly favored due to the presence of five equatorial chlorine atoms. δ -HCH carries two adjacent *trans*-diaxial HCl pairs that both involve the chlorine at position 1 (Fig. 3, bold bonds), and LinA can eliminate HCl at one of these two sites, leading either to δ -PCCH1 or to δ -PCCH2. Abstraction of H^+ at C-2 will lead to one enantiomer, and abstraction of H^+ at C-6 will lead to the other. Our experiments showed that mainly δ -PCCH2 was formed by LinA1 and LinA2, but we could not identify its absolute configuration. Thus, we arbitrarily chose one of the two possible δ -PCCH enantiomers for the illustration of the transformation pathway (Fig. 3). It must be noted that both PCCH enantiomers are degradable via the same TCDN isomer to 1,2,4- and 1,2,3-TCB. This was supported by the observation that racemic δ -PCCH was transformed to similar ratios of 1,2,4- and 1,2,3-TCB as found for δ -HCH (Fig. 2). Although δ -PCCH does not carry a *trans*-diaxial HCl pair, it obviously served as the substrate for both LinA enzymes. We postulate that δ -PCCH is dehydrochlorinated to 1,2,5,6-tetrachlorocyclohexa-1,3-diene by a *syn*-1,4-elimination (H^+ abstraction at C-6), as recently proposed for the transformation of a heptachlorocyclohexane isomer (2). We suggest that the second possible *syn*-1,4-elimination (H^+ abstraction at C-3) of δ -PCCH to 1,3,5,6-tetrachlorocyclohexa-1,3-diene did not occur, because we never observed the formation of 1,3,5-TCB, which would have been a likely product of the subsequent dehydrochlorination steps. Analogous to a mechanism that was published for chorismate lyase (15), 1,2,5,6-tetrachlorocyclohexa-1,3-diene could be further dehydrochlorinated by *syn*-1,2-elimination reactions, leading to 1,2,4- or 1,2,3-TCB.

Our data clearly support enantioselectivity of LinA enzymes with respect to dehydrochlorination of PCCHs, and they show that, besides 1,2-elimination reactions, these dehydrochlorinases also catalyze 1,4-eliminations of HCl.

LinA1 differs from LinA2 (which is identical to LinA from *Sphingobium japonicum* UT26) by 11 single amino acids and the insertion of a five-residue motif from the IS6100 sequence (3). Three of these changes (L96C, F113Y, and T133M) are located in the substrate-binding site pertaining to amino acid residues that have been suggested to be directly involved in the binding of

FIG 3 Postulated degradation pathway of δ -HCH by the dehydrochlorinases LinA1 and LinA2 from *S. indicum* B90A. The *syn*-1,2-elimination reactions of TCDN proceed by abstracting the proton at C-5 or -C6, leading to 1,2,4-TCB and 1,2,3-TCB, respectively. Please note that in the first reaction, abstraction of the proton at C-6 will lead to the formation of the opposite enantiomer. As the absolute conformations of the δ -PCCH enantiomers are not known, we arbitrarily show one configuration here.

γ -HCH and γ -PCCH. This provides some evidence that the change of these three amino acids is responsible for the opposite enantioselectivities of LinA1 and LinA2 with regard to α -HCH (9) and the PCCHs. However, additional work is required to fully understand how exactly these changes affect enantioselectivities of the enzymes.

It is likely that the findings described here analogously apply to the transformation of other halogenated cyclic compounds catalyzed by LinA-like enzymes.

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