Kinetic and Sequence-Structure-Function Analysis of LinB Enzyme Variants with β- and δ-Hexachlorocyclohexane

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Abstract

Organochlorine insecticide hexachlorocyclohexane (HCH) has recently been classified as a ‘Persistent Organic pollutant’ by the Stockholm Convention. The LinB haloalkane dehalogenase is a key upstream enzyme in the recently evolved Lin pathway for the catabolism of HCH in bacteria. Here we report a sequence-structure-function analysis of ten naturally occurring and thirteen synthetic mutants of LinB. One of the synthetic mutants was found to have ~80 fold more activity for β- and δ-hexachlorocyclohexane. Based on detailed biophysical calculations, molecular dynamics and ensemble docking calculations, we propose that the latter variant is more active because of alterations to the shape of its active site and increased conformational plasticity.

Introduction

Isomers of hexachlorocyclohexane (HCH) have been widely used as insecticides in various agricultural systems around the world for most of the last 70 years [1,2]. Despite mounting concerns about their human and eco-toxicity, they still find heavy use in some developing countries in particular [2,3]. Only the concerns about their human and eco-toxicity, they still find heavy use in some developing countries in particular [2,3]. Only the γ isomer is insecticidal but either a mixture of the four major isomers (α, β, γ and δ), known as technical HCH, or purified γ-HCH, known as lindane, have been used commercially. The purification of lindane from technical HCH has led to massive dumps (> 50,000 tonnes) of the other isomers in several countries and the stability of all isomers (but particularly of β-HCH) has led to widespread contamination of the environment, originating both from the dump sites and broad scale agricultural uses [1–4].

Over 60 bacterial strains which can degrade HCH, about half of them Sphingomonads, have now been reported, and all those characterized biochemically and genetically have proven to use the well established Lin pathway (encoded by various lin genes) to degrade HCH [2,5,6]. There are two major forms of the pathway which differ in their initial reactions but subsequently converge. One form operates on α-, γ- and δ-HCH and is initiated by two rounds of dehydrochlorination, followed by two rounds of hydrolytic dechlorination. The other form of the pathway operates on β- and δ-HCH and is initiated by two rounds of hydrolytic dechlorination, albeit some of the subsequent steps remain to be elucidated. The linA/LinA gene/enzyme system catalyzes dehydrochlorinations in both forms of the pathway via an E2 reaction mechanism, and the linB/LinB gene/ enzyme system catalyzes the hydrolytic dechlorinations via S_N2 displacement reactions [2,5,6].

The LinB enzyme is a monomeric 32 kDa protein from the α/β hydrolase fold superfamily [7]. Its structure has been solved [8] and its catalytic mechanism is reasonably well understood, with an Asp-His-Glu catalytic triad mediating nucleophilic attack on the substrate and then on an acyl-enzyme intermediate, and an oxyanion hole stabilizing the intermediate [9–12]. The best characterized LinB, LinBUT26A from Sphingobium japonicum strain UT26, has a broad substrate specificity, mainly due to a large active site volume, which includes monochloroalkanes (C3–C10), dichloroalkanes, bromoalkanes and chlorinated aliphatic alcohols [7,10]. Notably, this variant yields a significantly lower specificity constant for β-HCH (0.02 mM⁻¹ s⁻¹) as compared to another relatively well characterized LinB, namely, LinB90A

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Supporting Information files.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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on the basis of these data and the known structure of the LinBut26 occurring LinB variants, plus another 13 synthetic variants derived from Sphingobium indicum strain B90A (0.20 mM HCH) [13]. Nonetheless the activity of LinBb90a for β-HCH is much lower than that of LinBt26 for some of the other halogenated aliphatic compounds mentioned above (see [7,13] for a comprehensive list of kinetic constants).

A total of ten naturally occurring LinB variants have now been described which differ by as many as 16 (5.4%) of their amino acid residues [2]. At least some of these variants differ qualitatively in their substrate specificities; in addition to the β- and δ-HCH difference above, LinBb90a will hydrolytically dechlorinate the metabolite tetrachlorocyclohexol (TCXL), whereas LinBt26 does not [14]. A molecular dynamics simulation study suggests that this is mainly due to a difference in the flexibility of the entrance of the substrate access tunnel mediated by six out of the seven amino acid differences between the two enzyme variants [13].

Given the low but quantitatively different activities of the best characterized LinBt26 and LinBb90a variants for β- and δ-HCH, and their quantitative differences in respect to TCXL, our aim in the work described herein has been to quantitatively assess the difference in activities across the three sequence-based groups of LinB variants, plus another 13 synthetic variants derived from the LinBt26 protein. We find one synthetic variant with nearly 80-fold higher activity than LinBb90a for β-HCH and we suggest an explanation for its performance on the basis of increased mobility of its cap domain and increased affinity for the substrate.

Materials and Methods

Gene synthesis, expression vectors and chaperones

Codon optimized linB genes for expression in E. coli were synthesized by Genent AG, Regensburg Germany (Table S1). These genes were PCR amplified with respective attB1, attB2 and attB2-R2 primers (Table S2) and the amplicons were then cloned into pDONR201 and transferred to pDEST17, following the manufacturer’s instructions (Invitrogen, CA). The host E. coli BL21-AI (Invitrogen) cells for some clones co-expressed chaperones from the plasmid pGro7 (Takara, Japan).

Gene expression, enzyme purification and enzyme assays

Gene expression, enzyme purification and enzyme assays were performed as described earlier [15]. Briefly, the expression clones were grown in LB at 28°C until the OD600 had reached 0.5. At this point L-(+) arabinose was added at a final concentration of 2 g/L. Cultures were grown overnight, cells were harvested by centrifugation and cell free extract was prepared in 10 mM imidazole buffer containing 1X Bugbuster solution (Novagen, Darmstadt). The cell free extract was centrifuged at 16,000 g for 20 min at 4°C and the supernatant was subject to the N-terminal affinity chromatography to purify the His-tagged enzyme using standard procedures. The purified enzymes were quantified using Nanodrop (Thermo Scientific, DE) and stored in storage buffer (pH 7.5) containing 10% glycerol and 1 mM 2-mercaptoethanol at 4°C.

Results and Discussion

Eight of the ten natural LinB variants showed clearly measurable activities with both β- and δ-HCH (Table 1). As expected from previous reports, LinBt26 yielded lower activities with either isomer [7,13,14], as did the closely related LinBsp4. The other eight variants all showed 10–20 fold greater activities with β- than δ-HCH.

There was about a three-fold range of activities for each isomer across the latter eight variants. However, there was no obvious difference in activities across the three sequence-based groups of variants recognized by Lal et al [2] [again excluding LinBt26 and LinBsp4]. Instead, there was considerable variation within their...
Table 1. Activities towards \( \beta \)- and \( \delta \)-HCH of the ten known naturally occurring LinB variants (organised according to the three sequence-based groups recognised by Lal et al [2], the thirteen synthetic mutants made herein, and the eight variants analysed by Ito et al [14]).

| LinB variants | Haplotypes | Turnover number (min \(^{-1}\)) | \( \beta \)-HCH | \( \delta \)-HCH |
|---------------|------------|-------------------------------|----------------|----------------|
| **Group 3**   |            |                               |                |                |
| W1.10 LinB\(_{\text{SS04-1}}\) |             | 74±7                          | 4±1            |
| W1.9 LinB\(_{\text{SS04-2}}\) |             | 177±9                         | 3±1            |
| W1.8 LinB\(_{\text{SS04-5}}\) |             | 214±13                        | 6±1            |
| W1.7 LinB\(_{\text{SWB}}\) |             | 0*                            | 0*             |
| W1.6 LinB\(_{\text{UT26}}\) |             | 0*                            | 0*             |
| **Group 2**   |            |                               |                |                |
| W1.5 LinB\(_{\text{RM05}}\) | I N A A . . . . . . . . Y M V H . A M | 124±13          | 6±1            |
| **Group 1**   |            |                               |                |                |
| W1.4 LinB\(_{\text{SS04-3}}\) |             | 170±15                        | 8±1            |
| W1.3 LinB\(_{\text{NC-5-A}}\) |             | 154±31                        | 8±1            |
| W1.2 LinB\(_{\text{NC-5-B}}\) |             | 65±9                          | 5±0            |
| W1.1 LinB\(_{\text{RM06}}\) | S K P T A V V T L D L A I A H I | 90±1            | 4±1            |
| **Synthetic variants** | | | | |
| G1.1 A83P | . . . . . . . . . . . . . . . S | 182±17 | 6±1 |
| G1.2 V134L | . . . . . . . . . . . . . . L | 138±10 | 9±2 |
| G1.3 T135L | . . . . . . . . . . . . . . L | 194±3 | 10±2 |
| G1.4 L138I | . . . . . . . . . . . . . . I | 9±1 | 11±2 |
| G1.5 H247S | . . . . . . . . . . . . . . S | 1±1 | 0* |
| G1.6 I253M | . . . . . . . . . . . . . . M | 20±1 | 0* |
| G1.7 A83P | . . . . . . . . . . . . . . S | 17±2 | 0* |
| G1.8 A247H | I N A A . . . . . . . . Y M V H . H M C | 24±4 | 5±1 |
| G2.1 T81A/A83P | . . . . . . . . . . . . . . . | 44±6 | 4±1 |
| G2.2 V134L/T135L | . . . . . . . . . . . . . . L | 6920±272 | 241±6 |
| G2.3 V134L/T135L/T81A | . . . . . . . . . . . . . . L | 422±12 | 31±6 |
| G2.4 L138I/H247S/I253M | . . . . . . . . . . . . . . I | 10±2 | 3±1 |
| G2.5 A81T/A83P | . . . . . . . . . . . . . . P | 4±1 | 2±1 |

| Activity (mM \(^{-1}\) min \(^{-1}\)) |ND |
|--------------------------------------|---|
| LinB\(_{\text{UT26}}\) | . . . . . . . . . . . . . . L | 1.626 | ND |
| **Mutations from Ito et al. [13]** | | | | |
| I134V | . . . . . . . . . . . . . . A | 5.814 | ND |
| A247H | . . . . . . . . . . . . . . L | 0.462 | 1.626 |
| I134V/A247H | . . . . . . . . . . . . . . A | 1.458 | ND |
| LinB\(_{\text{RM08-1060A}}\) | . . . . . . . . . . . . . . I | 12.30 | ND |
| V134I | . . . . . . . . . . . . . . I | 7.44 | ND |
| H247A | . . . . . . . . . . . . . . A | 12.6 | 1.23 |
two major groups (1 and 3 in Table 1), including some large differences between otherwise very closely related variants. The sequence differences between these variants frequently involved residues (eg 112, 134, 135, 138 and 253) constituting the enzyme’s active site.

Based on the above results, six generation one synthetic variants (denoted G1.1–G1.6) on the LinB B90A background and one synthetic variant each on the LinB SS04-3 (G1.7) and LinB NM05 (G1.8) background were made. One of the former (G1.3; T135L) was generated by mistake (physio-chemically similar T135A was intended) during gene synthesis. These eight generation one mutants were analyzed for β- and δ-HCH activities in order to determine the contributions of individual amino acid differences to the activity variation. Three LinB B90A variants (G1.1–G1.3) showed ~2 fold higher activity (Table 1), and these mutations (A83P, V134L and T135L), along with certain others, were used to make five second generation double and triple mutants on the LinB B90A background. These mutants were also tested on β- and δ-HCH.

Overall eleven of the synthetic variants from both generations one and two yielded activity values within the range seen for the natural variants (0–214 and 0–8 min⁻¹ for β- and δ-HCH respectively; Table 1). However, LinB G2.1 and LinB G2.2 yielded activities 2–4 and 30 fold, respectively, higher than any previously seen, and 4–8 and about 80 fold higher than those of the LinB B90A reference variant (Table 1). β-HCH activities were again much higher than δ-HCH for all the synthetic variants and there was a strong correlation between β- and δ-HCH activities across the total of 23 natural and synthetic variants (r² = 0.99; P <0.03).

Table 2 rearranges a subset of the data from Table 1 to show the effects of single amino acid differences at eight positions spread across the enzyme (T81A, A83P, V112A, V134L, T135L, L138I, H247S and I253M). In six of these cases the effects of each substitution could be examined in more than one haplotype background. All eight substitutions had significant effects in at least one, and generally, most of the backgrounds tested. For six of the substitutions the effect was 10 fold or greater. Notably however, all six substitutions tested in more than one background also showed significant, and often also large, effects of the background haplotype. The table shows clear cases of positive (V134L/ T135L in LinB G2.2) and negative epistasis (A81 and P83 producing two-fold increases in β- and δ-HCH activities alone but a two-fold decrease together). Also a comparison of the A83P mutation in LinB B90A and LinB SS04-3 illustrates the effect of the genetic background; this mutation doubled activity in the LinB B90A background but decreased it by 10 fold in the LinB SS04-3 background.

The fact that the use of chaperones was necessary for expression of a few variants with higher activity suggests that some of the mutations nevertheless were deleterious for protein solubility or stability. We are now exploring whether the chaperone dependency of these variants could be ameliorated by stabilizing mutations.

Molecular dynamics simulations were used to probe the conformational dynamics of the synthetic LinB G2.2 variant relative to LinB B90A (our reference variant). These simulations revealed large differences in conformational flexibility within the cap domain (Figure 1). This domain occludes the active site and has previously been shown to be more flexible in LinB relative to the related dehalogenases DhaA and DhIA [28]. Here we see that LinB G2.2 shows greatly enhanced flexibility in this region when compared to LinB B90A. Unlike some of the other variants of this enzyme, LinB B90A has a histidine at position 247. This residue has been hypothesized to enhance activity by reducing solvent access
Table 2. Pairwise comparisons of β- and δ-HCH data for variants characterized herein which differ by single amino acid changes.

| Residues | Mutants | Haplotype | Turnover number (min⁻¹) |
|----------|---------|-----------|-------------------------|
|          |         |           | β-HCH                   | δ-HCH                   |
|          |         |           | 2 | 6 | 7 | 81 | 83 | 112 | 134 | 135 | 138 | 147 | 150 | 222 | 223 | 224 | 247 | 253 |           | |
| T81A     | W1.1/W1.3 | - | - | - | T/A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/154 ± 31 | 4 ± 1/5 ± 1 |
| T81A     | G2.2/G2.3 | - | - | - | T/A | - | - | L | L | - | - | - | - | - | - | - | - | - | - | - | 6920 ± 272/422 ± 12 | 241 ± 6/31 ± 6 |
| T81A     | G2.5/G2.1 | - | - | - | T/A | P | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 4 ± 1/44 ± 6 | 2 ± 1/4 ± 1 |
| A83P     | W1.1/G1.1 | - | - | - | A/P | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/182 ± 17 | 4 ± 1/6 ± 1 |
| A83P     | W1.3/G2.1 | - | - | A | A/P | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 154 ± 31/44 ± 6 | 5 ± 1/4 ± 1 |
| A83P     | W1.7/W1.8 | - | - | - | A/P | A | L | A | I | - | - | - | S | M | - | - | - | - | - | - | 0/214 ± 13 | 0/6 ± 1 |
| A83P     | W1.4/G1.7 | - | - | A | A/P | - | - | - | - | - | - | - | S | - | - | - | - | - | - | - | 170 ± 15/7 ± 2 | 8 ± 1/0 |
| V112A    | W1.10/W1.9 | - | - | A | V/A | L | A | I | - | - | - | - | S | - | - | - | - | - | - | - | 74 ± 7/177 ± 9 | 4 ± 1/3 ± 1 |
| V134L    | W1.1/G1.2 | - | - | - | V/L | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/138 ± 10 | 4 ± 1/9 ± 2 |
| V134L    | G1.3/G2.2 | - | - | - | V/L | L | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 194 ± 3/6920 ± 272 | 10 ± 2/241 ± 6 |
| T135L    | W1.1/G1.3 | - | - | - | - | - | - | T/L | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/194 ± 3 | 4 ± 1/10 ± 2 |
| T135L    | G1.2/G2.2 | - | - | - | - | - | - | - | T/L | - | - | - | - | - | - | - | - | - | - | - | 138 ± 10/6920 ± 272 | 9 ± 2/241 ± 6 |
| L138I    | W1.1/G1.4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/9 ± 1 | 4 ± 1/11 ± 2 |
| H247S    | W1.1/G1.5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 90 ± 1/1 ± 1 | 4 ± 1/0 |
| H247S    | W1.3/W1.4 | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 154 ± 31/70 ± 15 | 5 ± 1/8 ± 1 |
| H247S    | G2.1/G1.7 | - | - | A | P | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 44 ± 6/17 ± 2 | 4 ± 1/0 |
| I253M    | W1.1/G1.6 | - | - | - | - | - | - | - | - | - | - | - | - | I/M | - | - | - | - | - | - | 90 ± 1/20 ± 1 | 4 ± 1/0 |

The activity data are taken from Table 1 and abbreviated variant names are also as in Table 1. Haplotypes are also shown as per Table 1 (with LinB90A again used as the reference), except that residues that did not differ among the subset of variants included in these comparisons have been omitted. Blue and red colours are used to show the residues and activities corresponding to each variant in the pairwise comparisons.

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to the active site [13]. Although this mechanism may be feasible for some of the smaller haloalkane dehalogenase substrates (such as dichloroethane and trichloropropane), HCH cannot easily diffuse into the partially occluded active site in LinB, nor can it easily enter through the side tunnels previously characterized for this enzyme [13]. As such, it seems reasonable to assume that increased flexibility in the cap domain plays an important role in the enhancement of catalysis as observed in LinBG2.2 relative to LinBB90A by removing kinetic barriers to substrate binding (in spite of the fact that this potentially allows greater solvent penetration into the active site).

Additionally, our simulations indicate that LinBG2.2 is able to bind β-HCH more tightly than LinBB90A. The average binding energy of β-HCH to LinBG2.2 was calculated to be $-4.25 \pm 0.13$
REU (Rosetta energy units) compared to that of LinB B90A, which was $2.54 \pm 0.22$. This modest increase in affinity arises in part from more favourable hydrophobic interactions between L134 and $\beta$-HCH (Figure 2). This is also supported by results from molecular dynamics simulations with $\beta$-HCH bound, which show that LinBG2.2 is able to keep the ligand much closer to the catastrophic conformation than LinB B90A (Figure 3). Both the distance between the nucleophilic oxygen and the ligand centre of mass as well as the RMSD from the catalytic conformation are much lower for LinBG2.2 relative to LinB B90A (Figure 3).

Collectively, these results imply that the V134L/T135L mutations of LinB G2.2 enhance catalysis by affecting the conformational plasticity of the cap domain and by holding the substrate in a suitable conformation for catalysis. More extensive simula-

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**Figure 2.** Approximate affinity of LinB B90A and LinB G2.2 for $\beta$-HCH using Rosetta Ligand Dock program. Highest scoring docked pose for LinB B90A (A) and LinB G2.2 (B). The ligand $\beta$-HCH is shown as Van der Waals spheres, catalytic residues (as well as residue 134 which differs between LinB B90A and LinB G2.2) are rendered as thick sticks. Other residues within 5 Å of $\beta$-HCH are rendered as thin sticks. The empty space in the binding pocket of LinB B90A that is filled by the V134L mutation of LinB G2.2 is outlined with red dashes. doi:10.1371/journal.pone.0103632.g002

**Figure 3.** Molecular dynamics simulations of LinB with $\beta$-HCH bound in the active site. The distance between the nucleophilic oxygen of Asp108 and the center of mass of $\beta$-HCH is shown as a function of time (LinB B90A in blue and LinB G2.2 in red) in panel A. Panel B shows the RMSD from the near-attack conformation for $\beta$-HCH as a function of time (LinB B90A in blue and LinB G2.2 in red) in panel B. doi:10.1371/journal.pone.0103632.g003
tions as well as additional biophysical and biochemical analysis are now needed to provide a more complete picture of the relevant conformational motions of this enzyme.

Supporting Information

Figure S1 Superposition of the ground state β-HCH structure and the β-HCH transition state as calculated by ab initio electronic structure theory.

Table S1 Enzyme variants and primers.

Table S2 Codon optimized gene sequences.

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Author Contributions

Conceived and designed the experiments: RP DL RL JGO GP. Performed the experiments: RP DL KK PS. Analyzed the data: RP DL KK PS JGO GP. Contributed reagents/materials/analysis tools: DL JGO GP. Contributed to the writing of the manuscript: RP DL JGO GP.