Canadian Journal of Chemistry
Revue canadienne de chimie

**Theoretical study on hydrolytic step in biotransformation of
 β -hexachlorocyclohexane degraded by haloalkane
dehalogenase LinB**

Journal:	<i>Canadian Journal of Chemistry</i>
Manuscript ID	cjc-2016-0653.R1
Manuscript Type:	Article
Date Submitted by the Author:	17-Mar-2017
Complete List of Authors:	Tang, Xiaowen; Shandong University Wang, Junjie; Shandong University Zhao, Nan; Shandong University Zhang, Qingzhu; Environment Research Institute Wang, Wenxing; Shandong University, Environment Research Institute
Is the invited manuscript for consideration in a Special Issue? Please select::	N/A
Keyword:	haloalkane dehalogenase LinB, β -hexachlorocyclohexane, hydrolytic mechanism, quantum mechanics/molecular mechanics, Boltzmann-weighted average

SCHOLARONE™
Manuscripts

**Theoretical study on hydrolytic step in
biotransformation of β -hexachlorocyclohexane
degraded by haloalkane dehalogenase LinB**

Xiaowen Tang, Junjie Wang, Nan Zhao, Qingzhu Zhang*, Wenxing Wang

Environment Research Institute, Shandong University,

Jinan 250100, P. R. China

Draft

Keywords

Haloalkane dehalogenase LinB, β -hexachlorocyclohexane, hydrolytic
mechanism, quantum mechanics/molecular mechanics,
Boltzmann-weighted average

*Corresponding authors. E-mail: zqz@sdu.edu.cn,

Fax: 86-531-8836 1990

Abstract

The hydrolytic process of LinB-catalyzed biotransformation of a notorious contaminant β -HCH was investigated in atomistic detail with a combined quantum mechanics/molecular mechanics approach. The Boltzmann-weighted averaging method amended by disproportionate effect analysis was showed to capture the fluctuation of a single molecule enzyme reaction. With the potential barriers of 18.7 and 2.6 kcal/mol, two elementary steps that refer to formation and decomposition of a tetrahedral intermediate are involved in the hydrolytic reaction, respectively. Polarized by Glu132, His272 serves as a proton carrier along the whole hydrolysis reaction. The electrostatic influence analysis highlighted residue Leu248 as a possible mutation target for rational design of LinB in enzyme modification. Further spatial location analysis provided explanation for the opposite effect of Asn38 toward the two elementary steps. Getting insight into the catalytic details as well as the structure and function of LinB can enrich the knowledge of it and promote its application in bioremediation of chlorinated hydrocarbon pollutants.

38 **Graphical abstract**

39

40

41

1. Introduction

Introduced in the 1940s, the technical hexachlorocyclohexane (t-HCH) are widely used as a broad-spectrum organochlorine insecticide to control a wide range of agricultural, horticultural, and public health pests.¹⁻³ It is comprised of various stable isomers and the β -isomer, accounting for about 5-12% in t-HCH, is the most notorious component due to its extreme recalcitrance in the environment.⁴ Although its non-target toxicity and environmental persistence have caused it to be prohibited in most countries, the t-HCH is still widely used as an effective and inexpensive insecticide in many regions.^{3,5} It is estimated that about four to six million tons of various HCH isomer have been released to the environment heretofore.^{4,6} Residues of the HCH isomers have now been detected in numerous samples from different areas, such as air, water, soil, milk, fish, mammals and even human blood and adipose tissue.⁴ Therefore, the HCH contamination, especially β -HCH contamination, is continuously a serious threat to the environment and the residue problem of β -HCH should not be ignored.

The degradation of β -HCH can hardly be carried out through dehydrochlorination reactions because of the lack of axial chlorine atoms.⁷ Instead, the hydrolytic dechlorination reactions seem to be more feasible.^{2,4} Haloalkane dehalogenases (EC 3.8.1.5) are a kind of microbial α/β -hydrolases⁸ that can catalyze the cleavage of the carbon-halogen bond in halogenated compounds through hydrolytic dechlorination mechanism. These enzymes have been generally employed for microbiological detection and removal of the halocarbon pollutants in the

64 environment.^{9,10} One such haloalkane dehalogenase, LinB, which was originally
65 isolated from the bacterial strain of *Sphingobium japonicum* UT26, can catalyze the
66 conversion of a broad range of halogenated alkanes, including β -HCH to their
67 corresponding alcohols.¹¹ The dehalogenation reaction catalyzed by LinB can be
68 accomplished in the absence of any other cofactor except water.^{12,13} Additionally,
69 crystallographic studies reveal that the active site of LinB is much larger than other
70 dehalogenases, which makes it have relatively broad substrate specificity.¹⁴
71 Consequently, LinB are receiving much more attention for bioremediation purposes
72 nowadays.

73 Haloalkane dehalogenase LinB metabolizes β -HCH to
74 2,3,4,5,6-pentachlorocyclohexanol (β -PCHL) via an alkyl-enzyme intermediate
75 (β -HCH-LinB), and a catalytic triad composed of Asp108, His272 and Glu132 plays a
76 crucial role in such a process.⁴ Two considerable steps, dechlorination of β -HCH and
77 hydrolysis of the β -HCH-LinB complex, are involved in this process. In the
78 dechlorination step, the carboxylate of Asp108 serves as a nucleophile to attack the
79 carbon atom of β -HCH with an S_N2 displacement mechanism, giving rise to the
80 cleavage of carbon-chlorine bond and formation of β -HCH-LinB complex. For the
81 subsequent hydrolysis step, the hydrolytic water molecule makes a nucleophilic attack
82 upon the β -HCH-LinB complex, bringing about the recovery of Asp108 and
83 formation of the product β -PCHL. His272 and Glu132 are indispensable to this step,
84 in which His272 acts as a general base to activate the hydrolytic water molecule
85 through a deprotonation process. Meanwhile the catalytic acid Glu132 is hydrogen

bonded to His272, it can assist His272 to keep its proper orientation, increase its basicity, and further stabilize its protonated imidazole ring.¹² For its broad range of potential applications, LinB has always been the subject of some crystallographic and dynamic studies,¹²⁻¹⁴ and the structural character and catalytic mechanism have been clarified distinctly. Even so, only a small minority of studies have been performed to elucidate the detailed reaction process for the LinB-catalyzed hydrolytic dechlorination of β -HCH. A previous computational study described the S_N2 dechlorination step between β -HCH and Asp108 of LinB in detail.¹⁵ However, the hydrolysis step involved in the formation of β -PCHL has never been discussed. The activation energy of the hydrolysis step is not provided and the effect of residues surrounding the active site on the reaction is still undefined.

In an attempt to provide a deep insight into the hydrolytic step of LinB-catalyzed degradation of β -HCH in atomic detail and give a supplement to the activation energy data of each elementary reaction along the whole catalytic cycle, we have investigated the hydrolysis step with the aid of a combined quantum mechanics/molecular mechanics (QM/MM) method. QM/MM computations of the enzyme-catalyzed reaction can provide the atomic-level details of the enzyme mechanism and is therefore becoming an increasingly important tool to supplement the experimental enzyme chemistry.¹⁶ Through our present work, a more comprehensive understanding toward the degradation mechanism of β -HCH metabolized by haloalkane dehalogenase LinB is established. The effect of residues surrounding the active site on the reaction is also obtained, which can present valuable

108 guidance for rational design of the mutants of haloalkane dehalogenase LinB with a
109 more efficient enzymatic activity for industrial and environmental applications.

110 **2. Calculation Methods**

111 **2.1 Model Preparation and Molecular Dynamics**

112 The initial enzyme model for the present simulation was built on the
113 basis of the X-ray crystal structure of haloalkane dehalogenase LinB from
114 *Sphingomonas paucimobilis* UT26 (PDB code: 1MJ5, resolution: 0.95 Å) obtained
115 from the Protein Data Bank (www.rcsb.org)¹⁴ and the substrate model of β-HCH was
116 created by Materials Studio 7.0 program (Accelrys Software Inc.). When creating the
117 β-HCH-LinB binary complex, Asp108 in LinB was alkylated by β-HCH and the
118 configurational coordinate was set according to the configuration of the product in
119 dechlorination of β-HCH catalyzed by the haloalkane dehalogenase LinB.¹⁵ Since the
120 enzyme crystal structure was determined with a high resolution of 0.95 Å, the location
121 of some small atoms and even hydrogen atom were provided accurately. Therefore, it
122 is reasonable to decide the protonation state of ionizable residues consulting from the
123 crystal structure directly. MolProbity software was used to check the flipped
124 Asn/Gln/His residues.¹⁷ The boundary condition applied in the present work was
125 nonperiodic. During the preparation of computational model, a cubic water box
126 (TIP3P model¹⁸) with a size of 23×23×23 was employed to dissolve the β-HCH-LinB
127 binary complex. The box was then altered to a sphere with a radius of 34.80 Å and
128 water molecules overlapping within 2.5 Å of the binary complex were deleted. As a

129 result, a total of 8751 water molecules were included in the system. Subsequently, the
130 whole system was relaxed through energy minimizations after adding eleven sodium
131 ions at random positions to keep it neutral. After that, the system was heated
132 progressively from 0 K to 298.15 K within 50 ps and then forced to the thermal
133 equilibration state in a trajectory of 500 ps (1 fs/step) by using the stochastic boundary
134 molecular dynamic (SBMD) simulation. At last, a 15 ns SBMD simulation with
135 canonical ensemble (NVT, 298.15 K) was performed for conformational sampling.¹⁹
136 The leap-frog algorithm and Langevin dynamics attached in the CHARMM package
137 were applied during the simulation.

138 2.2 QM/MM Calculations

139 In our current QM/MM calculations, the QM region was analyzed by
140 Turbomole quantum mechanics package²⁰ with the DFT method²¹ and the MM region
141 was treated by DL_POLY²² program with the CHARMM36 forced field²³. The
142 ChemShell platform²⁴ was employed for integrating the two programs together. For
143 keeping the reasonability of the present study, some essential criteria were considered
144 when partitioning the QM region. Atoms involved in the bond formation or cleavage
145 must be incorporated into the QM region deservedly, moreover, residues strongly
146 interacting with the reactive center should also be classified in it. In the QM region,
147 the fractured covalent bonds across the boundary of the QM and MM regions were
148 complemented through adding hydrogen-link atoms. Electrostatic embedding
149 method²⁵ and charge shift model²⁶ were used to avoid over-polarization of the QM

density in the QM region. As a consequence, with a charge of -1 and a spin multiplicity of 1, the QM region of the LinB-catalyzed hydrolysis reaction in the current study contains a nucleophilic water molecule, the side chains of residues Glu132, His272 and the β -HCH-alkylated Asp108 as well as three hydrogen-link atoms. Including water molecules, all the atoms within 18 Å of C $^{\alpha}$ atom (Scheme 1) from Asp108 were set to be the active region (about 2700 movable atoms) and those located beyond 18 Å of C $^{\alpha}$ were fixed during the QM/MM calculation. Geometries were optimized by adopting the hybrid delocalized internal coordinate (HDLC) optimizer²⁷ at the B3LYP/6-31G(d,p)//CHARMM36 level. A larger basis set, B3LYP/6-311++G(d,p), was accepted for receiving a more accurate result of the single point energy calculation. The transition state structure was determined by scanning the potential energy profile from reactant to product, reaction coordinates were defined as “d(O $^{\omega}$ -H 1)-d(O $^{\omega}$ -C $^{\alpha}$)” for formation process and “d(C $^{\alpha}$ -O 1)-d(O 1 -H 1)” for decomposition process (superscript can be consulted in Scheme 1). The corresponding structure with the highest energy in the potential energy profile was selected and further optimized by microiterative TS optimizer which combines the partitioned rational function optimizer (P-RFO) algorithm²⁸ and the low-memory Broyden-Fletcher-Goldfarb-Shanno (L-BFGS) algorithm²⁹. Additional harmonic vibrational frequency calculation was performed to verify the one imaginary frequency character of transition state structure. Further details of the QM/MM setup can be found in Supporting Information.

3. Results and Discussion

172 3.1 Reliability Test

173 The β -HCH-LinB binary complex was extracted per picosecond during
174 the SBMD simulation. The corresponding root-mean-square deviation (RMSD) for
175 the backbone of the enzymatic reaction model was checked and displayed in Figure
176 S1 of the Supporting Information. For convenience of description, several key atoms
177 in the QM region were numbered, as presented in Scheme 1. Three distance variations,
178 $O^{\omega}-C^{\alpha}$, $N^{\epsilon}-O^{\omega}$ and $O^{\theta}-H^{\gamma}$, along the 15 nanoseconds trajectory were depicted in Figure
179 S2. All the three selected distances were stable with small variation during our SBMD
180 simulation, indicating that the system has been stabilized. The average distances of
181 $O^{\omega}-C^{\alpha}$ (3.50 Å) and $N^{\epsilon}-O^{\omega}$ (3.30 Å) give the water molecule a suitable position to
182 initiate the hydrolysis reaction. Furthermore, the catalytic residues of LinB
183 (Glu132-His272) interact with each other through a stable and strong hydrogen bond
184 ($O^{\alpha}-H^{\beta}$, 1.80 Å) according to the SBMD simulation.

185 For more details to identify the reliability of the model used in our
186 present work, three dimensional models for the docked structure, MD snapshot, and
187 QM/MM-optimised structure were exhibited in Supporting Information. His272,
188 β -HCH-alkylated Asp108 and the nucleophilic water molecule were selected for the
189 comparison of the three models, as displayed in Figure S3. They overlapped in the
190 active site with a relatively stable position. Separate assessment for the three models
191 was also provided. The nucleophilic water molecule was restrained by His272 with
192 hydrogen bond. Its spatial position is also measured through distance of $O^{\omega}-C^{\alpha}$, which
193 is 3.31 Å in docked structure, 3.27 Å in MD snapshot, and 3.22 Å in

194 QM/MM-optimised structure. Maintaining its chair conformation, β -HCH links to
195 Asp108 with covalent bond. The bond angle of $C^1-O^1-C^\alpha$ is 117.2° , 116.9° and 122.3°
196 for the three models, respectively. Hence, it might be inferred that the model used in
197 our present work could be credible for the present study.

198 It has been well known that the accuracy of theoretical calculations for
199 organic reaction systems is highly dependent of the levels of theory and basis set
200 selection³⁰⁻³². So, it is necessary to illustrate the reliability of the theoretical
201 calculations in this study. Due to the lack of experimental structure of β -HCH-LinB
202 binary complex, it is difficult to compare the theoretical results with the
203 corresponding experimental values directly. For the purpose of verifying the
204 reliability of theoretical levels in this study, we optimized the X-ray crystal structure
205 of LinB (PDB code: 1MJ5) at the B3LYP/6-31G(d)//CHARMM36 level. The
206 calculated results agreed well with the available experimental data. For a detailed
207 comparison, the catalytic residues (Glu132 and His272) involved in the hydrolysis
208 reaction were observed and some bonds were selected and displayed in Figure S4 of
209 Supporting Information. All the eight distances were in accordance with the X-ray
210 values. So, it might be concluded that the same accuracy could be expected for the
211 geometrical parameters of the β -HCH-LinB binary complex optimized at the
212 B3LYP/6-31G(d)//CHARMM36 level.

213 3.2 Reaction Mechanism and Potential Barriers

214 The rate constant of a single enzyme reaction is generally not invariable

but exhibits a wide range of fluctuations according to the room-temperature single molecule experiments.^{33,34} The experimentally determined overall k_{cat} of an enzymatic reaction is supposed to be a statistic value by considering all the fluctuant results. It is assumed that each snapshot extracted from the trajectory corresponds to a local rate constant. Studying only one snapshot in estimating the rate constant of an enzymatic reaction is not reliable. In order to obtain a calculated potential barrier that agrees well with the overall k_{cat} , the Boltzmann-weighted averaging method is introduced.³² It can be described by the following equation:^{36,37}

$$\Delta E_{\text{ea}} = -RT \ln \left\{ \frac{1}{n} \sum_{i=1}^n \exp \left(\frac{-\Delta E_i}{RT} \right) \right\}$$

where, ΔE_{ea} is the Boltzmann-weighted average potential barrier, R is gas constant, T is the temperature, n is the number of snapshots, and ΔE_i is the potential barrier of pathway i . In the present study, ten different snapshots extracted every 0.5 nanoseconds from 10 to 15 ns in the SBMD simulation were served as the starting configurations in the following geometry optimization and transition-state search. It can interfere with the final Boltzmann-weighted average potential barrier if the set of starting geometries include one with an anomalously low energy barrier. So, the disproportionate effect is employed to assess the reliability of calculated barriers. The disproportionate effect can be evaluated by the following equation:³⁸

$$E_{\text{de}} = \frac{\Delta E^{a-1} - \Delta E^a}{\Delta E^a} \times 100\%$$

where E_{de} is the disproportionate effect, ΔE^{a-1} is the Boltzmann-weighted average potential barrier calculated by neglecting the evaluated snapshot and ΔE^a is the

236 Boltzmann-weighted average barrier with all the snapshots considered.

237 As a general ester hydrolysis reaction which has been studied
238 sufficiently,³⁶ the hydrolysis of the β -HCH-LinB binary complex refers to the
239 formation and decomposition of a tetrahedral intermediate as indicated in Scheme 1.
240 The catalytic water molecule acts as a nucleophile to initiate the reaction. Polarized by
241 Glu132, His272 acts as a general base to accept a proton in the catalytic water
242 molecule and further transfers it to the alkoxide ion. The calculated potential barriers
243 along the hydrolysis reaction in different snapshots were displayed in Table 1. A
244 substantial potential barrier spread from 13.6 to 29.9 kcal/mol has been found in the
245 formation of the tetrahedral intermediate among different snapshots, similar potential
246 barrier spread from 1.9 to 6.6 kcal/mol were also found in the decomposition process.
247 These observed potential barrier fluctuations may be helpful in comprehending the
248 room-temperature single molecule experimental evidence that the reaction rate of a
249 single enzyme molecule exhibits large fluctuations.^{33,34} The potential barrier for
250 decomposition step is extremely low that makes the disproportionate effect analysis
251 meaningless and no anomalously low energy barrier was found among the
252 decomposition step. So the following disproportionate effect analysis will mainly
253 focus on formation step rather than decomposition step. The calculated potential
254 barrier for the snapshot in 12.0 ns is anomalously low compared with other barriers.
255 Disproportionate effect of it is as high as 24.8%, which can be considered as a severe
256 interference to the final Boltzmann-weighted average potential barrier. While the
257 highest disproportionate effect changes to only 3.3% by ignoring the snapshot in 12.0

ns. It can be identified as an acceptable value when evaluating the final Boltzmann-weighted average potential barrier. Based on the analysis above, the snapshot with a barrier of 13.6 kcal/mol was neglected and the final Boltzmann-weighted average potential barrier for the formation step is 18.7 kcal/mol. In addition, the average potential barrier for decomposition step is 2.6 kcal/mol. It is much lower than that of the formation step, which reveals the overall potential barrier of the hydrolytic step is 18.7 kcal/mol. This value is consistent with the experimental value of hydrolysis of some common alkyl-enzyme complexes.^{39,40} As a consequence, the Boltzmann-weighted averaging method revised by disproportionate effect analysis is qualified to estimate the fluctuant values in the present study.

3.3 Catalytic Itinerary and Structural Details

Table 2 shows some crucial internuclear distances obtained at the B3LYP/6-31G(d,p)//CHARMM36 level. All the selected distances in optimized configurations are approximately the same among the ten snapshots. Since a majority of the catalytic reactions occur through the pathway with the highest reaction rates, the following investigation towards the hydrolysis of the β -HCH-LinB binary complex will mainly focus on the pathway with the lowest potential barrier. For a more intuitive observation, the three dimensional structures of R, TS1, IM, TS2 and P involved in the hydrolysis of the β -HCH-LinB binary complex are displayed in Figure 1.

In the reactant, the catalytic water molecule is stabilized by His

residue through hydrogen bond with a length of 1.90 Å. It makes it feasible for His272 to grab the proton (H^1) of the catalytic water molecule and further facilitate the nucleophilic reaction. The distance between O^ω and C^α is 3.22 Å, indicating that the catalytic water molecule is situated in an appropriate position to attack the C^α atom of Asp108. Therefore, the catalytic water molecule is fully competent to initiate the hydrolysis reaction. For the formation of the tetrahedral intermediate ($R \rightarrow TS1 \rightarrow IM$), a covalent bond $O^\omega-C^\alpha$ is formed with a bond length of 1.55 Å. The distance of $O^\omega-H^1$ is stretched to 1.85 Å, which implies the deprotonation of the catalytic water molecule. The leaving proton is transferred to His272 residue, forming a positively charged imidazole ring. Obviously, the nucleophilic attack, deprotonation of water molecule and proton transfer occur synchronously. The dihedral angle of $O^1-C^\alpha C^\beta-O^2$ changes from 178.8° (R) to 134.1° (IM) via 153.1° (TS1) during the formation process. It suggests that the sp^2 -hybridized C^α atom turns into sp^3 -hybridized and the tetrahedral intermediate is generated. Correspondingly, the dihedral angle of $O^2-C^\alpha C^\beta-O^\omega$ varies from 126.2° (IM) to 141.5° (TS2) via 168.7° (P) during the decomposition process. It reveals that the hybridization state of C^α atom transforms from sp^3 to sp^2 and the carboxyl of Asp108 is reconstituted. In the product, the distance of $C^\alpha-O^1$ (2.79 Å) suggests the decomposition of the tetrahedral intermediate. The proton (H^1) has transferred from the imidazole ring of His272 to the alkoxide ion with a bond length of 1.74 Å for $N^\epsilon-H^1$ and 1.01 Å for H^1-O^1 . The hydrolysis product β -PCHL is generated and the alkylated enzyme is recovered as presented in Figure 2. It is obvious that the alkoxide ion grabs proton from His272 instead of Asp108. Along the

whole hydrolysis reaction, His272 serves as a proton carrier to hold the proton of the catalytic water molecule at first and transfer it to the hydrolysis product at last. Also displayed in Figure 2, it is compelling to note that the hydrogen bond between $O^{\ominus}$ and H^{γ} becomes stronger when the imidazole ring of His272 is protonated. Hence, the function of Glu132 is proved that it can distribute the positive charge in protonated imidazole ring of His272 and further stabilize its protonated imidazole ring.

For a more detailed description, the internuclear distance and Mulliken population analysis charge variations are introduced. Figure 2A shows the variations of five crucial internuclear distances along the hydrolysis of β -HCH-LinB binary complex. Obviously, the most significant changes occur on the distance of $O^{\ominus}-C^{\alpha}$ and $C^{\alpha}-O^1$ during the hydrolysis reaction. However, the variations of them are asynchronous. The distance of $O^{\ominus}-C^{\alpha}$ is drastically reduced while $C^{\alpha}-O^1$ is almost unchanged from reactant to intermediate, which suggests the formation of the tetrahedral intermediate. For the process from intermediate to product, the distance of $O^{\ominus}-C^{\alpha}$ is nearly invariable whereas $C^{\alpha}-O^1$ is increased dramatically, which indicates the decomposition of the tetrahedral intermediate. Besides, the distance of $N^{\epsilon}-H^1$ decreases first and then increases showing a U-shaped curve. It is in accordance with the fact that His272 is protonated at the beginning of the reaction ($R \rightarrow IM$) and deprotonated at last ($IM \rightarrow P$). The atomic charge analysis of several key atoms is displayed in Figure 2B. Deprotonation of the catalytic water molecule is conformed again by an enhanced negative charge of $O^{\ominus}$ in TS1 (-0.74 e). A covalent bond $O^{\ominus}-C^{\alpha}$ is formed in the subsequent step and the negative charge of $O^{\ominus}$ weakens gradually as

the reaction proceeds. The atomic charge of O¹ varies rapidly from IM (0.10 e) to TS2 (-0.39 e), which reveals the cleavage of C^α-O¹ and emergence of alkoxide ion. In the product, the negative charge of O¹ is reduced to -0.25 e rather than increased continuously, showing a situation that the proton has been transferred to alkoxide ion. A wave-shaped change is presented for Mulliken population analysis charge variation of H¹. Its positive charge in transition states (TS1 and TS2) is higher than that in other stationary points (R, IM and P), which corresponds to the characteristic of proton transfer.

3.4 Individual Residue Influence

The reactive center of LinB is largely surrounded by thirteen residues, according to previous crystal structure study¹⁴ and further manually selected through visual inspection. Even though residues around the reactive center do not participate in the reaction directly, they can still make an electrostatic influence on the enzyme reaction. In order to elucidate the electrostatic influence of the residues surrounding the reactive center, electrostatic interaction energies of the thirteen residues towards the hydrolysis of the β-HCH-LinB complex were evaluated. The electrostatic influence of residue *i* can be described as:

$$\Delta E^{i-0} = \Delta E^i - \Delta E^0$$

Where, ΔE^{i-0} is the energy changes of the barrier, ΔE^i is the potential barrier with charges on each atom of residue *i* set to 0 and ΔE^0 is the original values of the potential barrier. The geometry structures of the stationary points were kept

unchanged during all these energy calculations. A positive ΔE^{i-0} value means that neglecting the influence of the i^{th} residue will increase the potential barrier. Namely, the i^{th} residue can decrease the potential barrier and promote the enzyme reaction. Contrarily, a negative ΔE^{i-0} value indicates that the i^{th} residue can increase the potential barrier and suppress the enzyme reaction.⁴¹

The ΔE^{i-0} values of the thirteen residues investigated in the current work were schematically represented in Figure 3. Results show that Gly37, Asn38, His107 and Phe273 are accelerants towards the formation of the tetrahedral intermediate ($\Delta E^{i-0} > 1$ kcal/mol), whereas Leu248 is a suppressant for it ($\Delta E^{i-0} < -1$ kcal/mol). For the decomposition process, only Asn38 is identified to make a significant impact and all other residues are found to perform an inappreciable influence (-1 kcal/mol $< \Delta E^{i-0} < 1$ kcal/mol). Among all the thirteen residues investigated in our electrostatic influence analysis, Asn38 is particularly intriguing because it can significantly facilitate the formation process by decreasing the potential barrier of 3.0 kcal/mol and strongly suppress the decomposition process by increasing the potential barrier of 3.9 kcal/mol ($\Delta E^{i-0} = -3.9$ kcal/mol). Spatial location analysis (Figure S5) reveals that a hydrogen bond between the hydrogen in peptide bond of Asn38 and O² of Asp108 is formed along with the reaction. It can promote the formation process through dispersing the negative charge and stabilizing the tetrahedral intermediate. However, a more stable intermediate will be more difficult to decompose.

4. Conclusions

365 With the aid of a combined QM/MM approach, we investigated the
366 hydrolytic process of LinB-catalyzed degradation of β -HCH in atomistic detail. It
367 provided new methods for gaining insight into the structure and function of enzymes.
368 In the present system, two elementary steps were involved in this process and the
369 formation step with a potential barrier of 18.7 kcal/mol costs a higher barrier. The
370 reaction barrier of a single enzyme molecule exhibited large fluctuations and
371 Boltzmann-weighted averaging method amended by disproportionate effect analysis
372 can process the fluctuations competently. Electrostatic influence of Asn38 was found
373 to be dramatic, for it can influence the two elementary steps with opposite effect.
374 Furthermore, the electrostatic influence analysis also revealed the facilitation of
375 Gly37, His107 and Phe273 as well as the inhibition of Leu248 for the formation step.
376 It can be a valuable base for rational design of mutant enzyme with a more efficient
377 activity towards the degradation of β -HCH and other chlorinated hydrocarbon
378 pollutants, which can enrich the knowledge of LinB and promote its application in
379 environment.

380 Acknowledgments

381 The work was financially supported by NSFC (National Natural Science Foundation
382 of China, project Nos. 21337001 and 21577082).

383 Supplementary data

384 Root-mean-square deviations (RMSD) of backbone for the molecular

dynamic simulation (Figure S1); key distance variations along the molecular dynamic
simulation (Figure S2); three dimensional structures of the docked structure, the MD
snapshot, and the QM/MM-optimised structure (Figure S3); structure of catalytic
residues Glu132 and His272 as well as the comparison of theoretical results and
experimental values (Figure S4); spatial location of Asn38 in the active site of LinB
(Figure S5); and additional details on the methods.

Draft

References

- (1) Raina, V.; Hauser, A.; Buser, H. R.; Rentsch, D.; Sharma, P.; Lal, R.; Holliger, C.; Poiger, T.; Muller, M. D.; Kohler, H. P. E. *Environ. Sci. Technol.* **2007**, *41* (12), 4292-4298.
- (2) Okai, M.; Ohtsuka, J.; Imai, L. F.; Mase, T.; Moriuchi, R.; Tsuda, M.; Nagata, K.; Nagata, Y.; Tanokura, M. *J. Bacteriol.* **2013**, *195* (11), 2642-2651.
- (3) Li, Y. F. *Sci. Total Environ.* **1999**, *232* (3), 121-158.
- (4) Lal, R.; Pandey, G.; Sharma, P.; Kumari, K.; Malhotra, S.; Pandey, R.; Raina, V.; Kohler, H. P. E.; Holliger, C.; Jackson, C.; Oakeshott, J. G. *Microbiol. Mol. Biol. Rev.* **2010**, *74* (1), 58-80.
- (5) Willett, K. L.; Ulrich, E. M.; Hites, R. A. *Environ. Sci. Technol.* **1998**, *32* (15), 2197-2207.
- (6) Bala, K.; Geueke, B.; Miska, M.; Rentsch, D.; Poiger, T.; Dadhwal, M.; Lal, R.; Holliger, C.; Kohler, H.-P. *Environ. Sci. Technol.* **2012**, *46* (7), 4051-4058.
- (7) Deo, P. G.; Karanth, N. G.; Karanth, N. G. *Crit. Rev. Microbiol.* **1994**, *20* (1), 57-78.
- (8) de Jong, R. M.; Dijkstra, B. W. *Curr. Opin. Struct. Biol.* **2003**, *13* (6), 722-730.
- (9) Stucki, G.; Thuer, M. *Environ. Sci. Technol.* **1995**, *29* (9), 2339-2345.
- (10) Campbell, D. W.; Muller, C. M.; Reardon, K. F. *Biotechnol. Lett.* **2006**, *28* (12), 1-5.
- (11) Nagata, Y.; Miyauchi, K.; Damborsky, J.; Manova, K.; Ansorgova, A.; Takagi, M. *Appl. Environ. Microbiol.* **1997**, *63* (9), 3707-3710.

- 429 (12) Negri, A.; Marco, E.; Damborsky, J.; Gago, F. *J. Mol. Graph. Model.* **2007**, *26*
430 (3), 643-651.
- 431 (13) Marek, J.; Vevodova, J.; Smatanova, I. K.; Nagata, Y.; Svensson, L. A.; Newman,
432 J.; Takagi, M.; Damborsky, J. *Biochemistry* **2000**, *39* (46), 14802-14086.
- 433 (14) Oakley, A. J.; Klvana, M.; Otyepka, M.; Nagata, Y.; Wilce, M. C. J.; Damborsky,
434 J. *Biochemistry* **2004**, *43* (4), 870-878.
- 435 (15) Manna, R. N.; Dybala-Defratyka, A. *Arch. Biochem. Biophys.* **2014**, *562*, 43-50.
- 436 (16) Thiel, W.; Hummer, G. *Nature* **2013**, *504* (7478), 96-97.
- 437 (17) Chen, V. B.; Arendall, W. B.; Headd, J. J.; Keedy, D. A.; Immormino, R. M.;
438 Kapral, G. J.; Murray, L. W.; Richardson, J. S.; Richardson, D. C. *Acta Crystallogr.*
439 *Sect. D: Biol. Crystallogr.* **2009**, *66* (1), 12-21.
- 440 (18) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. *J.*
441 *Chem. Phys.* **1983**, *79* (2), 926-935.
- 442 (19) Brooks, C. L.; Karplus, M. *J. Chem. Phys.* **1983**, *79* (12), 6312-6325.
- 443 (20) Ahlrichs, R.; Bär, M.; Häser, M.; Horn, H.; Kölmel, C. *Chem. Phys. Lett.* **1989**,
444 *162* (3), 165-169.
- 445 (21) Burke, K.; Werschnik, J.; Gross, E. K. U. *J. Chem. Phys.* **2005**, *123* (062206),
446 1-9.
- 447 (22) Smith, W.; Forester, T.R. *J. Mol. Graphics.* **1996**, *14* (3), 136-141.
- 448 (23) MacKerell, A. D.; Bashford, D.; Bellott, M.; Dunbrack, R. L.; Evanseck, J. D.;
449 Field, M. J.; Gao, J.; Guo, H.; Ha, S.; Joseph-McCarthy, D.; Kuchnir, L.; Kuczera, K.;
450 Lau, F. T. K.; Mattos, C.; Michnick, S.; Ngo, T.; Nguyen, D. T.; Prodhom, B.; Reiher,

- 451 W. E.; Roux, III, B.; Schlenkrich, M.; Smith, J. C.; Stote, R.; Straub, J.; Watanabe, M.;
452 Wiórkiewicz-Kuczera, J.; Yin, D.; Karplus, M. *J. Phys. Chem. B* **1998**, *102* (18),
453 3586-3616.
- 454 (24) Sherwooda, P.; Vriesa, A. H. D.; Guesta, M. F.; Schreckenbacha, G.; Catlowb, C.
455 R. A.; Frenchb, S. A.; Sokolb, A. A.; Bromleyb, S. T.; Thielc, W.; Turnerc, A. J.;
456 Billeterc, S.; Terstegenc, F.; Thielc, S.; Kendrickd, J.; Rogersd, S. C.; Cascie, J.;
457 Watstone, M.; Kinge, F.; Karlsef, E.; Sjovollf, M.; Fahmif, A.; Schaferg, A.;
458 Lennartzg, C. *J. Mol. Struc-Theochem.* **2003**, *632* (1-3), 1-28.
- 459 (25) Bakowies, D.; Thiel, W. *J. Phys. Chem.*, **1996**, *100* (25), 10580-10594.
- 460 (26) de Vries, A. H.; Sherwood, P.; Collins, S. J.; Rigby, A. M.; Rigutto, M.; Kramer,
461 G. J. *J. Phys. Chem. B* **1999**, *103* (29), 6133-6141.
- 462 (27) Billeter, S. R.; Turner, A. J.; Thiel, W. *Phys. Chem. Chem. Phys.* **2000**, *2*,
463 2177-2186.
- 464 (28) Baker, J. *J. Comput. Chem.* **1986**, *7* (4), 385-395.
- 465 (29) Liu, D. C.; Nocedal, J. *Math. Program.* **1989**, *45* (3), 503-528.
- 466 (30) Zhang, D.; Zhang, R. *J. Am. Chem. Soc.* **2002**, *124* (11), 2692-2703.
- 467 (31) Lei, W.; Derecskei-Kovacs, A.; Zhang, R. *J. Chem. Phys.* **2000**, *113* (13),
468 5354-5360.
- 469 (32) Zhao, J.; Khalizov, A.; Zhang, R.; McGraw, R. J. *Phys. Chem. A* **2009**, *113* (4),
470 680-689.
- 471 (33) Lu, H. P.; Xun, L. Y.; Xie, X. S. *Science* **1998**, *282* (5395), 1877-1882.
- 472 (34) Min, W.; English, B. P.; Luo, G.; Cherayil, B. J.; Kou, S. C.; Xie, X. S. *Acc.*

- 473 *Chem. Res.* **2005**, 38 (12), 923-931.
- 474 (35) Tang, X. W.; Zhang, R. M.; Zhang, Q. Z.; Wang, W. X. *RSC Adv.* **2016**, 6 (5),
475 4183-4192.
- 476 (36) Lonsdale, R. Harvey, J. N.; Mulholland, A. J. *J. Phys. Chem. B* **2010**, 114 (2),
477 1156-1162.
- 478 (37) Li, Y. W.; Shi, X. L.; Zhang, Q. Z.; Hu, J. T.; Chen, J. M.; Wang, W. X. *Environ.*
479 *Sci. Technol.* **2014**, 48 (9), 5008-5016.
- 480 (38) Li, Y. W.; Zhang, R. M.; Du, L. K.; Zhang, Q. Z.; Wang, W. X. *Catal. Sci.*
481 *Technol.* **2016**, 6 (1), 73-80.
- 482 (39) Otyepka, M.; Banáš, P.; Magistrato, A.; Carloni, P.; Damborský, J. *Proteins:*
483 *Struct., Funct., Bioinf.* **2008**, 70 (3), 707-717.
- 484 (40) Prokop, Z.; Monincova, M.; Chaloupkova, R.; Klvana, M.; Nagata, Y.; Janssen,
485 D. B.; Damborsky, J. *J. Biol. Chem.* **2003**, 278 (46), 45094-45100.
- 486 (41) Tang, X. W.; Zhang, R. M.; Zhang, Q. Z.; Wang, W. X. *Chem. Phys. Lett.*, **2015**,
487 637, 97-102.

488 **Table 1** Calculated potential barrier for formation (ΔE_f) and decomposition (ΔE_d)
489 steps at the B3LYP/6-311++G(d,p)//CHARMM36 level and the disproportionate
490 effect in the formation step by considering all the ten snapshots (E_{de1}) and neglecting
491 the snapshot of 12.0 ns (E_{de2}).

snapshot	ΔE_f (kcal/mol)	E_{de1} E_{de2}		ΔE_d (kcal/mol)
10.5 ns	20.9	-0.4%	-0.3%	6.6
11.0 ns	22.5	-0.4%	-0.4%	2.9
11.5 ns	18.8	-0.4%	0.0%	4.8
12.0 ns	13.6	24.8%	*	1.9
12.5 ns	20.7	-0.4%	-0.3%	3.0
13.0 ns	29.9	-0.4%	-0.4%	2.1
13.5 ns	17.6	-0.4%	3.3%	2.1
14.0 ns	20.7	-0.4%	-0.3%	5.4
14.5 ns	22.0	-0.4%	-0.4%	4.5
15.0 ns	18.3	-0.4%	0.4%	6.6

Table 2 Selected internuclear distances (in Å) at the B3LYP/6-31G(d,p)//CHARMM36 level during the hydrolysis of β -HCH-LinB binary complex in ten snapshots.

snapshot	N ^e -H ¹					O ^o -H ¹					O ^o -C ^{α}					O ^l -C ^{α}					O ^l -H ¹				
	R	TS1	IM	TS2	P	R	TS1	IM	TS2	P	R	TS1	IM	TS2	P	R	TS1	IM	TS2	P	R	TS1	IM	TS2	P
10.5 ns	1.93	1.14	1.04	1.03	1.77	0.99	1.46	1.84	2.42	2.93	3.08	1.90	1.49	1.38	1.33	1.35	1.43	1.55	1.98	2.57	3.01	2.62	2.53	1.90	1.00
11.0 ns	1.91	1.12	1.04	1.05	1.75	0.99	1.54	1.94	2.76	2.84	3.18	1.92	1.54	1.39	1.35	1.35	1.43	1.56	2.07	2.61	3.14	2.71	2.55	1.70	1.00
11.5 ns	1.92	1.12	1.04	1.04	1.78	0.99	1.51	1.80	2.53	2.89	3.12	1.93	1.54	1.40	1.34	1.35	1.41	1.51	1.96	2.67	2.98	2.86	2.68	1.86	1.00
12.0 ns	1.87	1.12	1.03	1.03	1.81	1.00	1.49	1.93	2.63	2.87	3.05	2.02	1.53	1.39	1.33	1.35	1.39	1.50	1.94	2.75	3.01	2.88	2.76	1.87	1.01
12.5 ns	1.90	1.15	1.04	1.03	1.80	0.99	1.44	1.86	2.51	2.92	3.40	1.93	1.50	1.38	1.33	1.35	1.42	1.54	1.98	2.60	3.17	2.73	2.67	1.90	1.00
13.0 ns	1.84	1.10	1.04	1.05	1.77	1.00	1.57	1.89	2.65	3.00	3.08	1.97	1.55	1.40	1.34	1.35	1.42	1.52	1.95	2.62	3.07	2.77	2.56	1.73	1.01
13.5 ns	1.90	1.10	1.04	1.05	1.74	0.99	1.53	1.85	3.06	2.88	3.22	1.97	1.55	1.37	1.33	1.35	1.40	1.50	2.16	2.79	3.17	2.98	2.85	1.79	1.01
14.0 ns	1.78	1.08	1.04	1.06	1.72	1.00	1.61	1.84	2.67	2.99	3.32	1.92	1.56	1.39	1.35	1.35	1.43	1.51	2.05	2.61	3.34	2.81	2.63	1.65	1.01
14.5 ns	1.86	1.11	1.04	1.04	1.73	1.00	1.55	1.80	2.49	2.92	3.24	1.95	1.55	1.37	1.33	1.35	1.41	1.50	2.14	2.73	3.00	2.91	2.74	1.73	1.01
15.0 ns	1.92	1.11	1.04	1.06	1.73	0.99	1.51	1.90	2.73	3.00	3.16	1.93	1.51	1.37	1.34	1.35	1.40	1.53	2.13	2.61	3.00	2.86	2.56	1.67	1.01

Figure Captions

Scheme 1. Reaction pathway for the hydrolysis of β -HCH-LinB binary complex.

Several key atoms are numbered and the boundary between the QM and MM regions is indicated by wavy lines.

Figure 1. The three dimensional structures of R, TS1, IM, TS2 and P involved in the hydrolysis of β -HCH-LinB binary complex. The QM atoms including link hydrogen atoms are shown in ball and stick representation. The unit of bond distances is in Å.

Figure 2. Variations of five crucial internuclear distances (A) and atomic charges of five key atoms (B) along the hydrolysis of β -HCH-LinB binary complex.

Figure 3. ΔE^{i-0} values of thirteen individual residues toward the two elementary steps involved in the hydrolysis of β -HCH-LinB binary complex.

Scheme 1

Figure 1

A

B

Figure 2

Figure 3