# A Molecular Modeling Study of the Catalytic Mechanism of Haloalkane Dehalogenase. 2. Quantum Chemical Study of Complete Reaction Mechanism

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Haloalkane dehalogenase is a bacterial enzyme, which catalyzes hydrolytic cleavage of the carbon−halogen bond of haloalkanes. Quantum mechanical calculations at the semiempirical level using the MOPAC/DRIVER methodology were applied to study the enzymatic hydrolysis of 1,2-dichloroethane to 2-chloroethanol. In our previous study, the first $S_N2$ step of dehalogenation reaction was investigated (Damborský, J.; Kutý, M.; Němec, M.; Koča, J. A Molecular Modeling Study of the Catalytic Mechanism of Haloalkane Dehalogenase: 1. Quantum Chemical Study of the First Reaction Step. *J. Chem. Inf. Comput. Sci*. **1997**, *37*, 562−568). The present contribution explores the complete three-step reaction to determine the rate-limiting reaction step and to investigate the importance of active site residues for the kinetics and thermodynamics of the hydrolysis. The nucleophilic addition ($Ad_N$) step has the highest energy barrier, which is in qualitative agreement with experimental rates, assigning the second hydrolytic step as the rate-limiting one. In order to establish the catalytically important active-site residues, Mulliken charges of selected active-site atoms were monitored along the reaction pathway. A significant change in charges on the hydrogen atoms of $Trp^{125}$, $Trp^{175}$, and $Phe^{172}$ active-site residues was observed. These residues interact with the halide ion released during the $S_N2$ step. Changes in charges on the hydrogen atoms of $Trp^{125}$ and $Glu^{56}$ prove the significance of those residues in the stabilization of the partial charge developed on the oxygen atom of the nucleophilic aspartate ($Asp^{124}$). The same methodology confirmed the importance of the *charge relay system* ($Asp^{124}$, $His^{289}$, and $Asp^{260}$ residues) in the base-hydrolysis reaction ($Ad_N$ step).

## INTRODUCTION

The theoretical study of biomolecules is becoming an important research area.[1] Due to the fact that effective molecular modeling algorithms and computer technology have been developed, the number of computational studies performed on the most important biomolecules—nucleic acids and proteins—have increased during last few years. The most common application of such biomolecular modeling is in medicinal chemistry.[2] In our study, we focused on molecular modeling of environmentally interesting degradation process where the haloalkane dehalogenase enzyme is involved.

The haloalkane dehalogenase from soil bacteria *Xanthobacter autotrophicus* GJ10[3] was intensively investigated from the aspects of structure,[4,5] genetics,[6,7] catalytic mechanism,[8−12] and substrate specificity.[13] The enzyme is a globular protein with molecular weight of 36 kD and comprises 310 amino acid residues. The protein consists of two domains: a main-domain composed of eight $\beta$-sheets surrounded by six $\alpha$-helices and a cap-domain composed of an additional five $\alpha$-helices.[3] The active site is positioned between these two domains (see Figure 1), it is predominantly hydrophobic and can be reached from the solvent through a tunnel. The enzyme hydrolyses terminally chlorinated alkanes with a chain length up to four carbon atoms and brominated alkanes with a chain length up to ten carbon atoms to primary alcohols.[6] The optimal activity for the catalysis occurs at a pH of 8.2 and a temperature of 37 °C. The catalytic constant $k_{cat}$ for the natural dehalogenase substrate 1,2-dichloroethane has a value of 6 $s^{-1}$.[13]

Using high resolution X-ray crystallography at varying pH and temperatures the three-step reaction mechanism of hydrolysis of 1,2-dichloroethane was observed[8] (see Figure 2). Noncovalently bound substrate in the enzyme crystal has been obtained in soaking experiments at pH 5 and 4 °C. In the Michaelis complex, one of the chlorine atoms of the substrate binds to the nitrogen-bound hydrogen atoms of the tryptophan residues $Trp^{125}$ and $Trp^{175}$, the second chlorine atom weakly interacts with $Phe^{128}$, $Phe^{168}$, and $Phe^{174}$ residues.[8] At room temperature the nucleophilic oxygen atom of the side chain of aspartate residue $Asp^{124}$ attacks the carbon atom of the substrate (Figure 2, $S_N2$ step). The observable products of the first reaction step are the 2-chloroethyl-enzyme ester intermediate and the released chlorine anion. Based on X-ray crystallography and fluorescence quenching experiments,[9] site-directed mutagenesis experiments,[14] and our previous theoretical study,[15] two tryptophanes, $Trp^{125}$ and $Trp^{175}$, have been assigned to play an important role in stabilization of the released halide ion. The two tryptophane aromatic rings are positively charged and thus may interact with small anions, whereas the putative character of $\pi$-electron clouds of aromatics causes the negatively charged atoms or anions to be located in the aromatic plane. When the pH is reduced to a value of pH 6.2 at the room temperature the second reaction step (Figure 2, $Ad_N$ step) proceeds. In this step, the substrate−enzyme

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**Figure 1.** Stereoplot of the α-carbon backbone of dehalogenase. The active site is positioned between two domains and can be reached from the solvent through a tunnel. The substrate molecule of 1,2-dichloroethane bound in the active site is also shown.

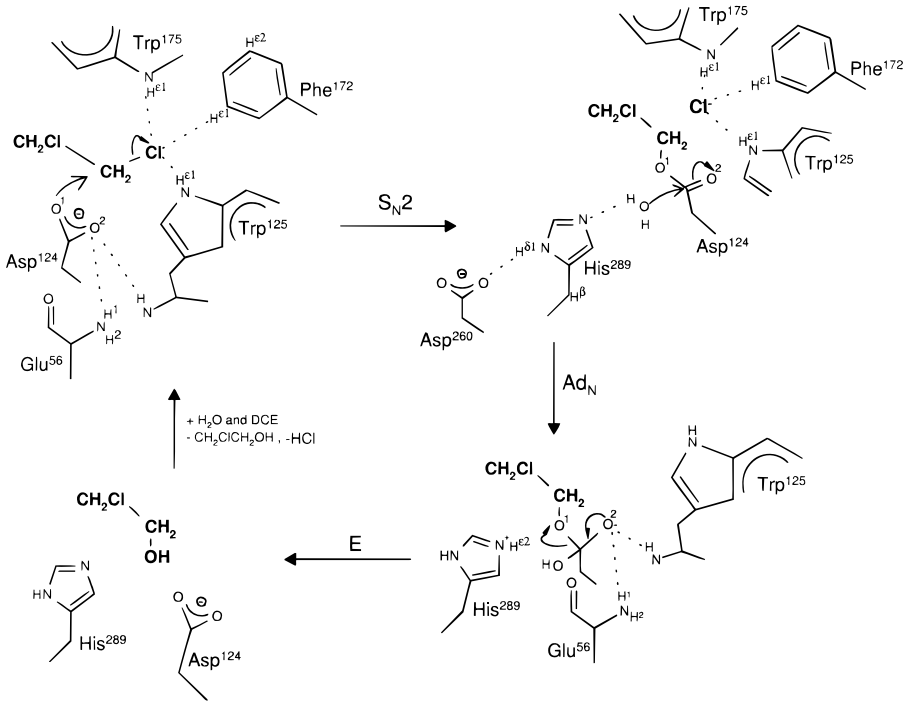


**Figure 2.** Reaction mechanism of the hydrolytic process on the enzyme active site deduced from the crystal structures.[8] In the three-step catalysis ($S_N2$, nucleophilic substitution; $Ad_N$, nucleophilic addition; and E, elimination step), the substrate molecule (bolded) of 1,2-dichloroethane is hydrolyzed into 2-chloroethanol. Nonbonding substrate−enzyme electrostatic interactions (denoted by the dotted lines) stabilize the transition states and intermediate structures, having an impact on kinetics and thermodynamics of the hydrolysis.

ester is attacked by nucleophilic water molecule as a cosubstrate to form a tetrahedral intermediate on the $C^{\delta}$ atom of $Asp^{124}$ residue. The mechanism of nucleophilic addition arises due the base character of $His^{289}$ residue. In particular, $His^{289}$ deprotonates the hydrolytic water nucleophile, while the $Asp^{260}$ residue simultaneously interacts with the nitrogen atom of the $His^{289}$ residue through a *syn* type hydrogen bond. A small pocket near the amide of the residue following the nucleophile ($Trp^{125}$) called the "oxyanion hole" [16] is proposed to stabilize the partial charge developed on the oxygen atom of the nucleophilic aspartate ($Asp^{124}$-$O^2$) (see Figure 2, stabilization by NH of $Trp^{125}$ and $NH^1$ of $Glu^{56}$ is denoted by the dotted line). In the last step of hydrolysis (Figure 2, E step), the tetrahedral intermediate decomposes, and the haloalcohol (2-chloroethanol) together with a chlorine anion are released. A new water molecule enters the cavity as a new cosubstrate in the next catalytic cycle.

Experimental methods are usually not capable of determining in detail the entire energy profile for the reaction under study because, for example, the lifetime of the transition state is too short. If the structure of the enzyme is known, it is possible to perform a molecular modeling study of the reaction pathway to calculate the geometry of the transition states from which the enthalpy or free-energy of the system can be estimated. A knowledge of the energies obtained for ground state and transition states structures can be used for prediction of the rate-limiting step of the reaction under study and for the identification of interactions important for stabilization of the transition state. In our previous paper,[15] the semiempirical quantum-chemical calculations

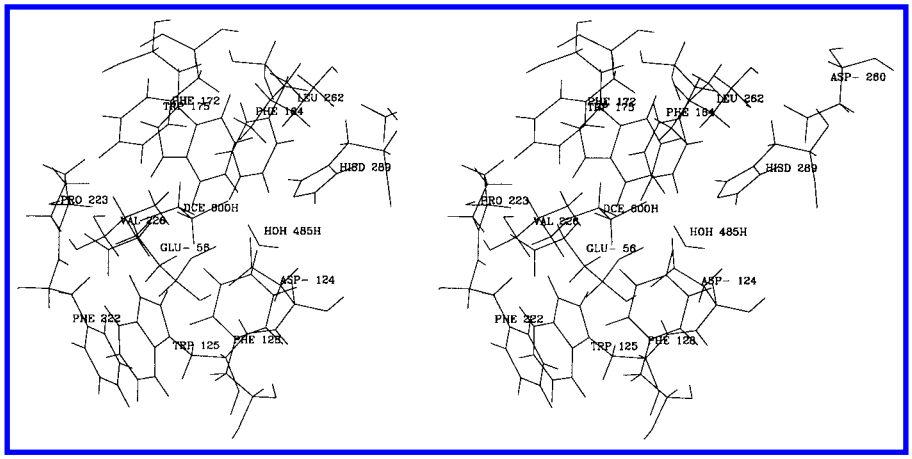


**Figure 3.** Stereomicroscopic model of the haloalkane dehalogenase active site: Glu[56], Asp[124], Trp[125], Phe[128], Phe[164], Phe[172], Trp[175], Phe[222], Pro[223], Val[226], Asp[260], Leu[262], His[289] and substrate 1,2-dichloroethane (DCE). All residues suggested to be important[4] for the mechanism of the dehalogenation reaction were included in the semiempirical MOPAC/DRIVER calculations.

have been used to study the kinetics and thermodynamics of the $S_N2$ reaction step. A detailed study of the entire reaction mechanism is described in this contribution. A similar study has been performed also by another group.[17]

## METHODS

**Software and Hardware.** For displaying the molecules and preparation of input data for calculations a comprehensive graphic molecular modeling program InsightII[18] has been used. A new graphically oriented software Triton[19] was used to prepare Z-matrices of MOPAC[20] input files and for the fixation of the peptide backbone. The semiempirical quantum chemical package MOPAC was applied for mapping the reaction pathway and for Mulliken charges calculations.

The DRIVER method[21] (implemented as a part of MOPAC) was used to calculate reaction pathways and to estimate transition states as the highest energy points along the path between reactant and product. In this method all the internal coordinates, which are expected to be the reaction coordinates—distances, angles, and dihedral angles—may be driven, decreased, or increased monotonically, while the energy of the system with respect to all other coordinates is simultaneously minimized.

The results from DRIVER process may be examined as a step-by-step animation of the whole reaction mechanism using Xmol[22] and Triton. Turbomole 95.0/3.0.0[23] was applied for single point *ab initio* energy calculations for several points along reaction pathway. All semiempirical and *ab initio* calculations were carried out on SGI PowerChallenge XL multiprocessor supercomputer running under the IRIX Release 6.2 operating system; the structural data were prepared/manipulated using an SGI *Indigo*[2] workstation under IRIX Release 5.3 platform.

**Calculation Level.** Many molecular modeling studies on macromolecules use time efficient and for many purposes sufficiently accurate empirical molecular mechanic force fields (molecular mechanics). However, the study of chemical reactions molecular mechanics is usually not applicable because it is mainly parameterized for the structures in the ground state and not for transition states which typically have unusual bond lengths and angles. Quantum mechanical methods, on the other hand, allow for the description of such bond-breaking/making processes.[24] The accuracy of results, computer time, and hardware requirements depend on the level of theory used. Semiempirical procedures such as AM1 (which was used in this study) or PM3 yield results which often appear to be of an accuracy equivalent to results from *ab initio* calculations with small split-valence basis sets while at the same time requiring less computational effort. The semiempirical approach was carried out using Linear Combination of Atomic Orbitals Molecular Orbital Method (MO LCAO), restricted Hartree−Fock (RHF), Austin Model 1[25] (AM1) Hamiltonian, and Broyden-Fletcher-Goldfarb-Shanno[26−29] (BFGS) quasi-Newton geometry optimization algorithm.

**Active Site Model.** The X-ray structure of the enzyme was used as the basis for an active site model. Cartesian coordinates of the enzyme−substrate complex (1,2-dichloroethane as a substrate) at various pH and temperature conditions were obtained from the Brookhaven Protein Databank (PDB with accession codes lede, 2dhc, and 2dhe representing the substrate free enzyme, enzyme−substrate complex, and enzyme−product complex, respectively). All the active site residues in direct contact with 1,2-dichloroethane substrate and some others implicated to be important for the mechanism of dehalogenation reaction have been included in the calculations. Thirteen catalytic residues Asp[124], Asp[260], Glu[56], His[289], Leu[262], Phe[128], Phe[164] Phe[172], Phe[222], Pro[223], Trp[125], Trp[175], Val[226], and a single water molecule were included in the modeling study (Figure 3). The overall charge of the system was set to −2 due to negatively charged aspartic acids which are deprotonated at the beginning of the catalysis.[8] The carbon and nitrogen terminals of the residues have been assigned hydrogen atoms to saturate their valencies. The α carbons of the amino acid residues were fixed in the same relative orientations during the driving along reaction pathway. Molecular dynamics trajectory of the solvated haloalkane dehalogenase (GROMOS forcefield; 500 ps) has shown that α carbon atoms of the residues included in the QM calculations did not deviate significantly from their positions determined in the crystal structure (Linssen, T.; Damborský, J., Berendsen, H. J. C. unpublished results).

**Reaction Pathway Modeling.** The reaction pathway of the three reaction steps was calculated using MOPAC/DRIVER[21] methodology.

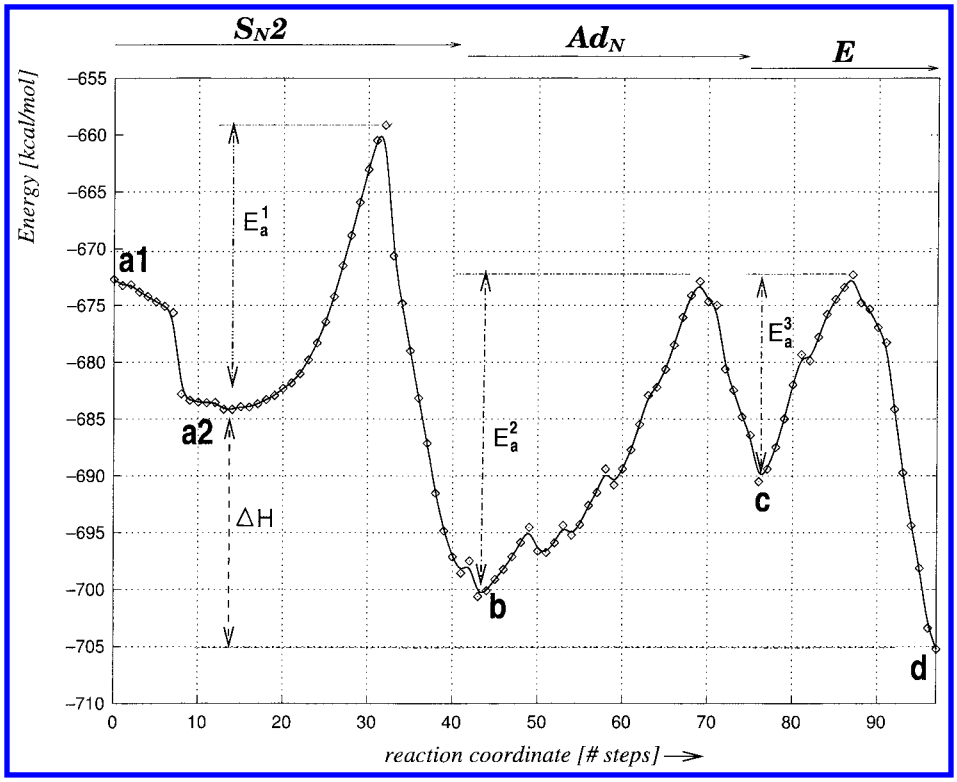


**Figure 4.** Three-step MOPAC/DRIVER energy profile for the catalytic hydrolysis of 1,2-dichloroethane. The energy was calculated by the AM1 semiempirical method, step numbers refer to the steps in the MOPAC/DRIVER calculation. The important points along the reaction pathway are as follows: free enzyme (X-ray structure) (a1) and its optimized form of noncovalently bound enzyme−substrate (a2), the enzyme−substrate ester structure (b), tetrahedral intermediate (c), products of the hydrolysis (d), and transition states referring to the 32nd, 69th, and 88th step. The values of the activation energies ($E_a^1$ = 24.9, $E_a^2$ = 27.7, and $E_a^3$ = 18.2 kcal·mol$^{-1}$) are in a good qualitative agreement with experimentally determined rates,[14] assigning the $Ad_N$ reaction as the rate-limiting reaction step. The energy of the alkyl−enzyme intermediate (b) was considerably lower to that calculated for the tetrahedral intermediate (c). The heat of reaction ($\Delta E$ = −21.1 kcal·mol$^{-1}$) corresponds to the exothermic nature of the hydrolysis.

**First Reaction Step.** In the first $S_N2$ step, the substantial internal reaction coordinate of this process—the distance between oxygen $O^1$ of the aspartic acid (Figure 2) and the carbon of the substrate—was driven (decreased) by a defined increment of 0.05 Å while allowing the remainder of the system to be minimized (except for constrained α carbons).

**Second Reaction Step.** The final structure obtained from the calculation of the first reaction step was used as an input structure for modeling of the second step. A new reaction coordinate for this step was chosen. The distance between the oxygen atom of water and the aspartic acid carbon atom was driven (decreased by 0.050 Å), and simultaneously, the distance between the hydrogen atom of the water molecule and the $His^{289}$ nitrogen was also reduced (by increments of 0.035 Å in order to fit exactly the ground state interatomic distances in the products of this step).

**Third Reaction Step.** The resulting negatively charged tetrahedral intermediate from the second reaction step was used as a starting structure in the modeling of the last catalytic step. A new reaction coordinate was chosen in which the distance between the aspartic acid carbon atom and $O^1$ was driven (increased by 0.05 Å) together with the distance between hydrogen $H^{\epsilon 2}$ of $His^{289}$ and oxygen atom $O^1$ (by increment 0.05 Å).

## RESULTS AND DISCUSSION

**Three-Step MOPAC/DRIVER Energy Profile of the Catalytic Mechanism of 1,2-Dichloroethane Hydrolysis.** The results of MOPAC/DRIVER calculations are represented by the reaction pathway (Figure 4) as a function of the enthalpy (heat of formation) on the reaction coordinate. The starting X-ray structure (a1) was optimized to the noncovalently bound enzyme−substrate (a2). The product of the $S_N2$ first reaction step is the enzyme−substrate ester structure (b). The other local energy minima of the pathway represent the tetrahedral intermediate (c) and the final products of hydrolysis (d). The values of the energy barriers (approximation of activation energies) $E_a^1$ =24.9, $E_a^2$ = 27.7, and $E_a^3$ = 18.2 kcal·mol$^{-1}$ suggest that $Ad_N$ is the rate-limiting step. This result is in a good qualitative agreement with the experimentally determined rates,[14] 50 s$^{-1}$ for the $S_N2$ step and 14 s$^{-1}$ for the second ($Ad_N$) step. The third reaction step was not observed in kinetic measurements, most probably due to its very high reaction rate compared to the time scale of the experiment. The energy of the alkyl−enzyme intermediate (b) was considerably lower to that calculated for the tetrahedral intermediate (c). The reaction enthalpy ($\Delta H$ = −21.1 kcal·mol$^{-1}$) is consistent with the exothermic nature of hydrolysis.

**Monitoring of Charges on the Active-Site Atoms during the Catalytic Process.** Charge monitoring method has successfully been applied in our previous study[15] dealing with the first reaction step. Additionally, reliability of AM1/Mulliken charges has been tested against DFT[(VWN)/6-31G**]/Mulliken single point calculations. We did the comparison for two points on the reaction pathway (point **b**

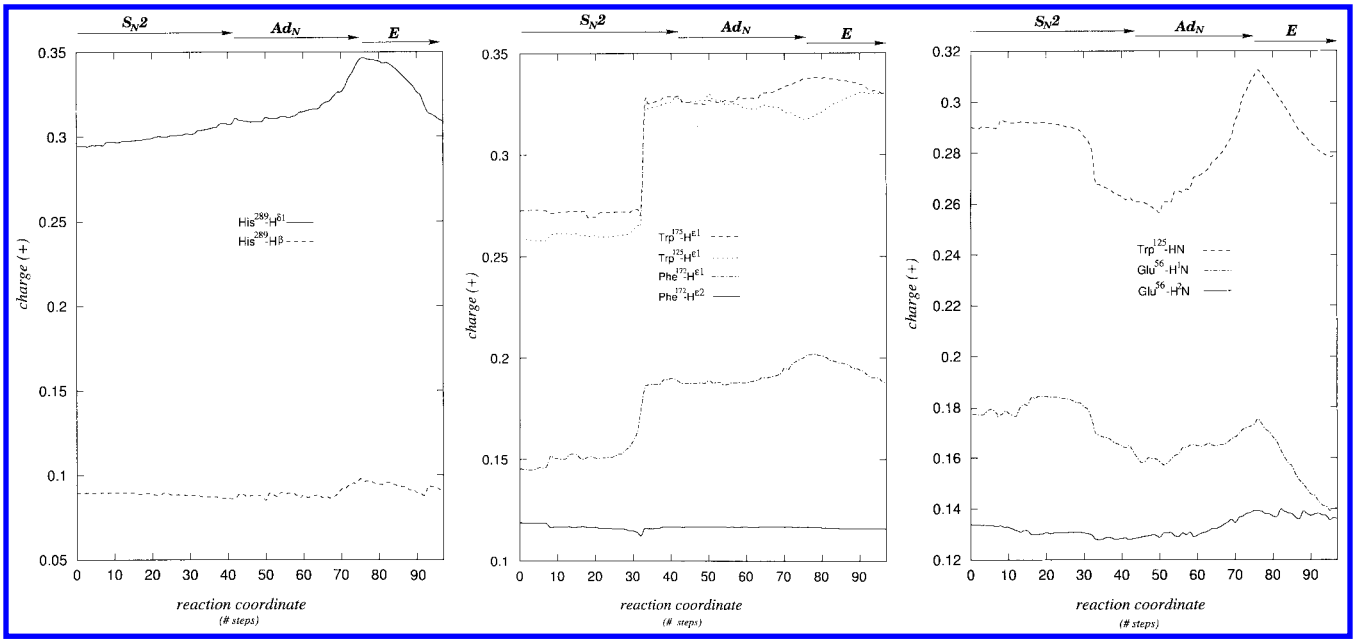


**Figure 5.** Charge development on selected atoms along the reaction pathway. For numbering of reaction coordinate steps, see Figure 4. (a) Two tryptophanes, $Trp^{125}$ and $Trp^{175}$, have been assigned to play an important role in stabilization of the released halide ion, based on X-ray crystallography,[9] fluorescence quenching experiments,[9] and site-directed mutagenesis experiments.[14] A significant change in charges on the hydrogen atoms interacting with halide ion released during the $S_N2$ reaction was observed, *i.e.*, the polarized hydrogen atoms became more positive. The additional hydrogen atom $H^{\epsilon 1}$ of $Phe^{172}$ residue involved in halide ion stabilization has been identified.[15] Note the constant charge on the hydrogen atom $H^{\epsilon 2}$ of $Phe^{172}$ where no interaction was expected. (b) Haloalkane dehalogenase has a small pocket near the amide of the residue following the nucleophile ($Trp^{125}$) called the oxyanion hole.[16] The proposed function of this hole is to stabilize the partial charge developed on the oxygen atom of the nucleophilic aspartate ($Asp^{124}$-$O^2$) during the dehalogenation reaction (see Figure 2, stabilization by NH of $Trp^{125}$ and $NH^1$ of $Glu^{56}$ is denoted by the dotted line). Changes in the atomic charges of the hydrogen atoms interacting with $Asp^{124}$-$O^2$ observed for all three reaction steps clearly support the proposed function (note the similar development of charges on HN of $Trp^{125}$ and H1N of $Glu^{56}$ during three reaction steps, but constant charge on the hydrogen atom H2N of $Glu^{56}$ where no interaction was expected). (c) Charge monitoring on the $H^{\delta 1}$ and $H^{\beta}$ atoms of $His^{289}$ residue confirmed the importance of $His^{289}$ and $Asp^{260}$ for the $Ad_N$ reaction step. Note the charge development on the $H^{\delta 1}$ due the *syn* type $H^{\delta 1}$-$O^{Asp260}$ hydrogen bond interaction is opposite to the constant course for $H^{\beta}$ where no interaction was expected.

and the transition state between **b** and **c**, *cf.* Figure 4). The charges obtained from these two methods were very similar (the largest difference was about $10^{-2}$). Charges on the active-site atoms were monitored to investigate the importance of residues in the stabilization of the transition state. A significant change in charges on the $Trp^{175}$-$H^{\epsilon 1}$ and $Trp^{125}$-$H^{\epsilon 1}$ hydrogen atoms interacting with halide ion released during the $S_N2$ reaction was observed (Figure 5a), *i.e.*, the polarized hydrogen atoms became more positive. Additionally a hydrogen atom ($H^{\epsilon 1}$ of $Phe^{172}$) involved in halide ion stabilization has been identified.[15] Changes in the atomic charges of the $Trp^{125}$-HN and $Glu^{56}$-$H^1N$ hydrogen atoms interacting with $Asp^{124}$-$O^2$ were observed for all three reaction steps (Figure 5b) and support the idea that they are involved in the stabilization of the tetrahedral intermediate. The importance of the histidine-$Asp^{260}$ *syn* type hydrogen bond interaction in the $Ad_N$ reaction step calculations was confirmed by the changes in the charges on the $H^{\delta 1}$ atom of $His^{289}$ residue (Figure 5c). Animation of the entire catalytic process provides qualitative information regarding the flexibility of both the ligand and the side chains of the protein active site.

## CONCLUSIONS

Quantum mechanical calculations at the semiempirical level using the MOPAC/DRIVER methodology were applied to study the enzymatic hydrolysis of 1,2-dichloroethane to 2-chloroethanol. The applicability of the MOPAC/DRIVER technology for multidimensional driving along the potential energy hypersurface was verified by this study. The activation barriers along the reaction pathway were calculated and compared to determine the rate-limiting step of the three-step reaction. Nucleophilic addition $Ad_N$ has been identified as the rate-limiting step, which is in qualitative agreement with experimental observations. The changes in partial charges on the atoms of the active site residues were monitored to identify those amino acid residues which are directly involved in the reaction mechanism. The charge monitoring appears to be a promising tool to discover parts of reaction system that participate on the reaction. This is important, for example, for prediction of mutants with improved activity and/or selectivity.

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