Comparative Binding Energy Analysis of the Substrate Specificity of Haloalkane Dehalogenase from *Xanthobacter autotrophicus* GJ10[†]

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ABSTRACT: Comparative binding energy (COMBINE) analysis was conducted for 18 substrates of the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (DhlA): 1-chlorobutane, 1-chlorohexane, dichloromethane, 1,2-dichloroethane, 1,2-dichloropropane, 2-chloroethanol, epichlorohydrine, 2-chloroacetonitrile, 2-chloroacetamide, and their brominated analogues. The purpose of the COMBINE analysis was to identify the amino acid residues determining the substrate specificity of the haloalkane dehalogenase. This knowledge is essential for the tailoring of this enzyme for biotechnological applications. Complexes of the enzyme with these substrates were modeled and then refined by molecular mechanics energy minimization. The intermolecular enzyme−substrate energy was decomposed into residue-wise van der Waals and electrostatic contributions and complemented by surface area dependent and electrostatic desolvation terms. Partial least-squares projection to latent structures analysis was then used to establish relationships between the energy contributions and the experimental apparent dissociation constants. A model containing van der Waals and electrostatic intermolecular interaction energy contributions calculated using the AMBER force field explained 91% (73% cross-validated) of the quantitative variance in the apparent dissociation constants. A model based on van der Waals intermolecular contributions from AMBER and electrostatic interactions derived from the Poisson−Boltzmann equation explained 93% (74% cross-validated) of the quantitative variance. COMBINE models predicted correctly the change in apparent dissociation constants upon single-point mutation of DhlA for six enzyme−substrate complexes. The amino acid residues contributing most significantly to the substrate specificity of DhlA were identified; they include Asp124, Trp125, Phe164, Phe172, Trp175, Phe222, Pro223, and Leu263. These residues are suitable targets for modification by site-directed mutagenesis.

Haloalkane dehalogenases are microbial enzymes that catalyze dehalogenation reactions (*1*−*3*), which are important for the degradation of environmental pollutants (*4*−*6*). Halogenated aliphatic compounds are among the most frequently occurring pollutants. Large quantities of these compounds are widely used as pesticides, solvents, fire retardants, hydraulic and heat transfer fluids, and cleaning agents. They are environmentally dangerous and are hazardous to humans due their toxic, genotoxic, teratogenic, and irritating effects. Unfortunately, wild type enzymes often do not acquire sufficiently high activity or specificity for degradation of environmental pollutants. Protein design can be used to improve the catalytic properties of such enzymes. To tailor the enzyme for improved substrate specificity, the amino acid residues that participate in substrate binding must be identified so that they can be modified by site-directed mutagenesis. Comparative binding energy (COMBINE)[1] analysis has been shown to be a useful technique for deriving quantitative structure−activity relationships from a set of three-dimensional structures of enzyme−ligand complexes (*7*−*12*). Here, we use COMBINE analysis to derive a predictive model for substrate binding specificity in which

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[1] Abbreviations: BUW, block unscaled weights; COMBINE, comparative binding energy; DhlA, haloalkane dehalogenase from *X. autotrophicus* GJ10; E_{ele}^{ES}, enzyme−substrate electrostatic interaction energy in the presence of the surrounding solvent derived from the Poisson−Boltzmann equation; $E_{desolv-sur}$, surface area dependent term of the desolvation energy; E_{desolv}^{S}, desolvation energy of a substrate; E_{desolv}^{E}, desolvation energy of an enzyme; FFD, fractional factorial design; ΔG_{ele}, overall electrostatic free energy change upon binding; G_{ele}^{ES}, electrostatic energy for all atoms in the enzyme−substrate complex; G_{ele}^{S}, electrostatic energy for substrate atoms; G_{ele}^{E}, electrostatic energy for enzyme atoms; ΔG_{desolv}^{S}, change in desolvation energy of the substrate upon binding; ΔG_{desolv}^{E}, change in desolvation energy of the enzyme upon binding; ΔH_{vap}, enthalpy of vaporization; $INTC_{Q^2}$, intercept of the permutation plot for Q^2; K_m, enzyme−substrate dissociation constant; PLS, partial least-squares; Q^2, cross-validated correlation coefficient; R^2, correlation coefficient; SDEC, standard deviation of error of calculation for the working or training set; $SDEP_{int}$, standard deviation of error of (internal) predictions; $SDEP_{ext}$, standard deviation of error of (external) predictions; UV, scaling to unit variance; ΔU, total binding energy.

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important interactions for binding are highlighted so that the model can be used to guide mutagenesis experiments to modify the enzyme's substrate specificity.

The haloalkane dehalogenase isolated from the soil bacterium *Xanthobacter autotrophicus* GJ10 (DhlA) is a soluble globular enzyme (*13*). DhlA is composed of 310 residues and has a molecular mass of ~36 kDa. It consists of two different domains: the α/β-fold domain (main domain) which is conserved for all α/β-hydrolases (*14*, *15*) and the so-called cap domain. The main domain is composed of eight β-sheets surrounded by six α-helices, whereas the cap domain is composed of five additional α-helices. The active site of the enzyme is located between these two domains in an internal, predominantly hydrophobic cavity that can be reached from the solvent through a tunnel. The catalytic residues form a catalytic triad (nucleophile, base, and acid) that is highly conserved among all of the α/β-hydrolases known to date. The mechanism of dehalogenation is hydrolytic and requires the substrate and a water molecule in the active site; no other cofactor is necessary. During the hydrolytic dehalogenation, a carbon−halogen bond in the substrate is cleaved and the corresponding alcohol is formed. Details of the reaction mechanism have been investigated by crystallography (*16*−*18*), kinetic measurements (*19*−*23*), site-directed mutagenesis (*24*−*31*), and molecular modeling (*32*−*38*).

Previous theoretical studies were focused on the reaction mechanism of haloalkane dehalogenases (quantum mechanical calculations) and their conformational behavior (molecular dynamic simulations). The study presented here, on the other hand, deals with the substrate specificity of DhlA, and its aim is to construct a predictive model for estimation of the binding affinities for mutant proteins. To this end, a COMBINE analysis was carried out to identify the protein residues responsible for the differences in binding affinities of 18 chlorinated and brominated aliphatic substrates of DhlA. The effects of different scaling and variable selection procedures on the quality of the models were studied. The best model explained 93% (74% cross-validated) of the quantitative variance in binding constants and enabled identification of the residues that contribute most to the binding specificity; these are candidates for site-directed mutagenesis aimed at altering the substrate specificity of DhlA.

METHODS

Experimental Data. Apparent dissociation constants (K_m) were used as the measure of binding affinities for a set of 18 substrates. The binding affinities of these compounds vary over 4 orders of magnitude. The K_m values determined by Schanstra et al. (*39*) were logarithmically transformed (Table 1). Experimental activities were measured using steady-state kinetic analysis with purified DhlA. The K_m values for dichloromethane, 2-chloroethanol, and 2-chloroacetamide were fixed at the highest measured concentrations since the exact dissociation constants were not reported (*39*).

Overview of COMBINE Analysis. Binding energies are calculated for the set of enzyme−substrate complexes using a molecular mechanics force field. The total binding energy, ΔU, may be assumed to be given by the sum of five terms: (i) the sum of intermolecular interaction energies (Δu_i) between the substrate and each enzyme residue, E_{inter}^{ES}, (ii) the change in the intramolecular energy of the substrate upon binding to the enzyme, ΔE^S, (iii) the change in the intramolecular energy of the enzyme upon substrate binding, ΔE^E, (iv) the desolvation energy of a substrate, E_{desolv}^S, and (v) the desolvation energy of the enzyme, E_{desolv}^E.

Table 1: Steady-State Dissociation Constants of Haloalkane Dehalogenase[a]

	compound	log K_m (mM)		compound	log K_m (mM)
1	1-chlorobutane	0.34	**10**	1,2-dibromopropane	0.11
2	1-chlorohexane	0.15	**11**	2-chloroethanol	2.60
3	1-bromobutane	−1.22	**12**	2-bromoethanol	1.04
4	1-bromohexane	−0.52	**13**	epichlorohydrine	1.68
5	dichloromethane	2.00	**14**	epibromohydrine	0.34
6	1,2-dichloroethane	−0.28	**15**	2-chloroacetonitrile	0.80
7	dibromomethane	0.38	**16**	2-bromoacetonitrile	−0.31
8	1,2-dibromoethane	−2.00	**17**	2-chloroacetamide	2.00
9	1,2-dichloropropane	1.11	**18**	2-bromoacetamide	1.30

[a] From ref *39*.

$$\Delta U = E_{inter}^{ES} + \Delta E^S + \Delta E^E + E_{desolv}^S + E_{desolv}^E \quad (1)$$

The second and third terms, describing changes in intramolecular energies upon binding, were neglected in the study presented here because the DhlA substrates are rather small molecules and there is no evidence for large differences in the structure of DhlA when different substrates are bound. Intermolecular energy contributions were decomposed into van der Waals and electrostatic interactions.

In the first step of COMBINE analysis, a set of structures of enzyme−substrate complexes is prepared and the total binding energy is calculated for each of these complexes. The following step is the decomposition of the enzyme−substrate interaction energy on a per residue basis for each of the complexes. A matrix is then constructed in which the rows represent the different compounds studied and the columns contain the residue-based energy information, which is separated into two blocks (van der Waals and electrostatic), plus an additional column containing the experimental binding affinities. Further columns can contain additional energy terms such as the substrate desolvation energy terms. This matrix is then projected onto a small number of orthogonal "latent variables" using partial least-squares (PLS) analysis (*40*, *41*), and the original energy terms are given weights, w_i, according to their importance in the model, in the form of PLS pseudocoefficients. The higher these coefficients are, the more significant they are for explaining the variance in the experimental data. Thus, in the simplest form, the COMBINE model for binding affinity, ΔG, is of the following form (C is a constant term):

$$\Delta G = \sum w_i \Delta u_i + C \quad (2)$$

Parametrization of Halogenated Substrates. The all-atom AMBER molecular mechanics force field (*42*) was used throughout, and consistent parameters for the haloalkanes were derived to describe the bonded and nonbonded interactions. For each molecule, molecular electrostatic potentials (MEPs) were calculated from the corresponding ab initio wave functions (RHF MP2//6-31G*) using Gaussian94 (*43*)

following full energy minimization. Partial atomic charges were then derived by fitting each MEP to a monopole−monopole expression using the RESP methodology (*44*, *45*). One conformation of each molecule (trans) was employed in the fit, except for 1,2-dichoroethane for which both the gauche and trans conformations were considered. Atom types for carbon atoms in the haloalkanes (CT) were taken from the AMBER database. Equilibrium bond lengths and angles for chlorinated and brominated hydrocarbons were obtained by averaging equivalent terms from the ab initio 6-31G(d) energy-minimized structures (Table 1). Dihedral parameters involving halogens were adjusted so as to reproduce in the molecular mechanics force field the torsional barriers calculated ab initio. For this purpose, the SPASMS module in AMBER (*46*) was employed. Nonbonded parameters for halogen atoms were developed and tested following a previously reported procedure (*47*) with some modifications. In brief, periodic cubic boxes (27 Å × 27 Å × 27 Å) containing 149 solvent molecules of 1,2-dichloroethane, bromoethane, and acetonitrile were constructed to reproduce the density and enthalpy of vaporization (ΔH_{vap}) of these liquids at 300 K. The compressibility values (in 10^{-6} bar^{-1}) that were used were 84.6, 142.3, and 107.0, respectively (*48*). Molecular dynamics simulations were carried out at 300 K using the SANDER module in AMBER. Both the temperature and the pressure were coupled to thermal and pressure baths with relaxation times of 0.2 and 0.6 ps, respectively. In a 20 ps heating phase, the temperature was gradually increased under constant-volume conditions, and the velocities were reassigned at each new temperature according to a Maxwell−Boltzmann distribution. This was followed by an equilibration phase of 200 ps at 300 K, and by a 300 ps sampling period at constant pressure during which system coordinates were saved every 50 ps. All bonds involving hydrogens were constrained to their equilibrium values by means of the SHAKE algorithm (*49*), which allowed an integration time step of 2 fs to be used. A nonbonded cutoff of 10 Å was employed, and the lists of nonbonded pairs were updated every 25 steps. Density values were provided directly by the SANDER module. ΔH_{vap} values were calculated according to the equation

$$\Delta H_{vap} = RT - E_{inter} \quad (3)$$

where E_{inter} is the interaction energy of the system, which encompasses both the electrostatic and van der Waals components obtained directly from the SANDER output, divided by the number of molecules in each box.

Construction of Enzyme−Substrate Complexes and Energy Analysis. The complexes were modeled with AMBER 5.0 (*50*) using the structure (*16*) of DhlA complexed with the substrate 1,2-dichloroethane (DCE) (PDB entry 2DHC) as a template. The WHATIF 5.0 program (*51*) was used for adding the polar hydrogen atoms. His289 was singly protonated in the δ-position in accordance with its catalytic function. Nonpolar hydrogen atoms were added using the AMBER 5.0 graphic interface xLEaP. The substrates were manually docked in the enzyme active site and aligned so that the $X-C_1-C_2$ angle (where X is a halogen atom) of each substrate could be superimposed on that of the DCE molecule. These initial structures of the complexes were refined using the molecular mechanics force field of Cornell et al. (1994) implemented in AMBER 5.0. One hundred steps of steepest descent were followed by conjugate gradient energy minimization until the root-mean-square value of the potential energy gradient was less than 0.1 kcal mol^{-1} Å^{-1}. A nonbonded cutoff of 10.0 Å and a distance-dependent dielectric constant ($\epsilon = 4r_{ij}$) were used. The ANAL module of AMBER 5.0 was used for energy decomposition of the refined complexes.

Estimation of Surface Desolvation Energy. The surface desolvation energy ($E_{desolv-sur}$) of a substrate was calculated as a sum of atomic surface accessibilities multiplied by hydrophobicity coefficients for specific atom types. Atomic surface accessibility was calculated using the NACCESS 2.1.1 program (*52*). This program is an implementation of the method of Lee and Richards. Appropriate hydrophobicity coefficients were taken from the literature (*53*, *54*) as follows: carbon-containing group, 18; neutral oxygen or nitrogen, −9; sulfur, −5; charged nitrogen, −38; and charged oxygen, −37. In this context, we assigned a value of 1 to the hydrophobicity coefficient of halogen atoms.

Estimation of the Electrostatic Contributions to the Free Energies of Binding. Continuum Electrostatics Calculations. The overall electrostatic free energy change upon binding (ΔG_{ele}) can be calculated from the total electrostatic energy of the system by running three consecutive calculations on the same grid (*55*, *56*): one for all the atoms in the complex (G_{ele}^{ES}), one for the substrate atoms alone (G_{ele}^{S}), and a third one for the enzyme atoms alone (G_{ele}^{E}). Since the grid definition is the same in the three calculations, the grid energy artifact cancels out when the electrostatic contribution to the binding free energy is expressed as the difference in energy between the bound and the unbound molecule:

$$\Delta G_{ele} = G_{ele}^{ES} - (G_{ele}^{S} + G_{ele}^{E}) \quad (4)$$

An alternative method, which allows partitioning at the residue level, considers a different description of the binding process. This consists of first desolvating the apposing surfaces of both the substrate and enzyme and then letting the charges of the two molecules interact. It is then possible to separate the change in electrostatic free energy on molecular association (ΔG_{ele}) into three components (*55*−*57*): (i) the enzyme−substrate interaction energy in the presence of the surrounding solvent (E_{ele}^{ES}), (ii) the change in desolvation energy of the substrate upon binding (ΔG_{desolv}^{S}), and (iii) the change in desolvation energy of the enzyme upon binding (ΔG_{desolv}^{E}):

$$\Delta G_{ele} = E_{ele}^{ES} + (\Delta G_{desolv}^{S} + \Delta G_{desolv}^{E}) \quad (5)$$

This decomposition is exact, contains all cross terms, and can be profitably used in COMBINE analysis. The first term in eq 5, that is, the electrostatic energy of interaction between the group of *E* atoms in the enzyme and the group of *S* atoms in the substrate, can be described (in kilocalories per mole) by

$$E_{ele}^{ES} = \sum_{i=1}^{E} q_i \phi_i \quad (6)$$

where *q* represents the atomic point charges of the *i*th atom of the enzyme and ϕ_i is the electrostatic potential at the *i*th

atom of the enzyme created by the

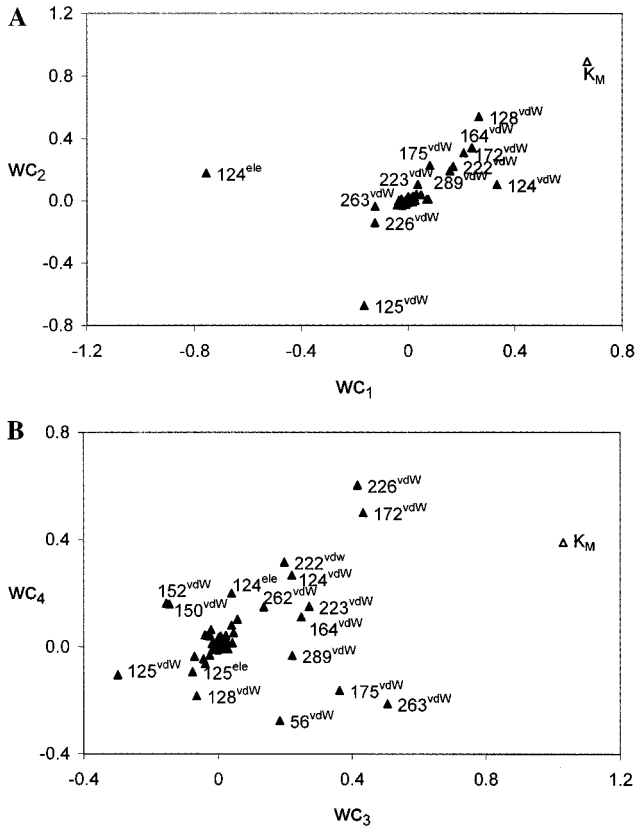

FIGURE 4: Loading plots of wc_1 vs wc_2 (A) and wc_3 vs wc_4 (B) for model 4. Selected variables (energy contributions) are numbered according to the DhlA sequence.

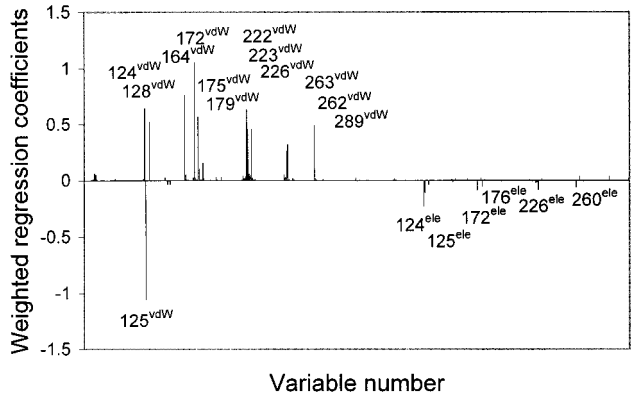

FIGURE 5: Plot of weighted regression coefficients for model 4. Selected variables (energy contributions) are numbered according to the DhlA sequence.

The van der Waals interactions with positive coefficients can also be explained on a physical basis. Better van der Waals interactions result in better binding affinity. Most of the residues with positive van der Waals contributions line the active site cavity (Table 4 and Figure 7). Those further away may be explained by general improvement in packing. These residues explain most of the third and fourth principal components but also contribute to the first and second components.

A favorable van der Waals interaction of Asp124 is observed for the substrates 1-chlorohexane, 1-bromohexane, 1,2-dibromoethane, and 1,2-dibromopropane, while unfavorable interactions are observed with 2-chloroethanol, 2-bromoethanol, and 2-bromoacetamide in the first component. Asp124 is a nucleophile that initiates the dehalogenation

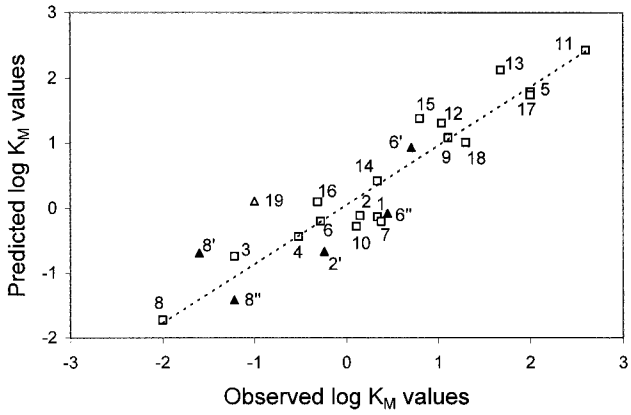

FIGURE 6: Plot of observed vs predicted K_m values for model 4. The objects (compounds) are numbered according to Tables 1 and 5. Compounds from the working (training) set are represented as white squares, and compounds from the prediction set are represented as triangles (the white triangle represents a prediction made for a complex of a new substrate *and* a new mutant enzyme). A single prime corresponds to the Phe172Trp mutant, and a double prime corresponds to the Trp175Tyr mutant.

reaction by nucleophilic attack on the carbon atom bonded to halogen in a substrate molecule (*16*, *25*). This attack leads to formation of a covalent alkyl−enzyme ester and a halide ion. Asp124 is positioned on a nucleophile elbow (*14*) and points toward the active site cavity.

The aromatic ring of Phe128 displays steric hindrance with epichlorohydrine, epibromohydrine, 1,2-dichloropropane, 2-chloroacetamide, and 2-bromoacetamide in the second component. We noted that the much smaller Ala is present in the equivalent position of dehalogenases LinB and DhaA (*37*). Both enzymes exhibit better activity with β-substituted haloalkanes than DhlA. Substitution of Phe128 with a smaller amino acid may result in enzymes with improved affinity for β-substituted substrates.

van der Waals energies of Leu263, Phe172, Val226, Trp175, Trp125, Cys150, and Met152 are among the most significant interactions in the third component. Leu263 makes unfavorable contacts with the two largest substrates in the data set, namely, 1-bromohexane and 1-chlorohexane. A smaller amino acid in position 263 may improve the affinity for long-chain substrates. Both Cys150 and Met152 have the opposite effect on binding long-chain substrates. The van der Waals interaction energy of Cys150 with substrates **2** and **4** is 1 order of magnitude lower than with other substrates. Trp175 makes direct van der Waals contact with the halogen substituent of all substrates and provides stabilization in a manner similar to that of Trp125 (*28*). Most of the substrates interact with Trp175 favorably, but 1-bromohexane, 1-chlorohexane, 1-bromobutane, and 1-chlorobutane make unfavorable van der Waals contacts with Trp175 $HN_{\epsilon 1}$. Mutagenesis of Trp175 results in proteins with low activity (*19*, *28*); therefore, improved binding for these substrates can only be achieved by mutations in neighboring residues (helix 5). Nine of 12 in vivo mutants of DhlA with improved activity toward 1-chlorohexane (*30*) carried modifications in helix 5 or its close surroundings. Priest and coworkers suggested that this region is critical for the specificity of DhlA. This observation is in line with the COMBINE model which localizes seven highly significant interactions in helix 5 (Table 4 and Figure 7). The importance of Val226 could not be directly attributed to the binding of specific

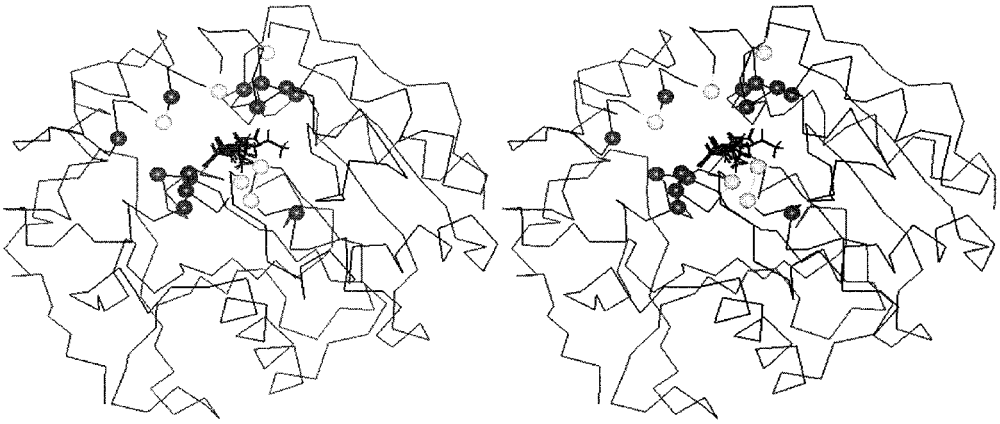

FIGURE 7: Stereoview of DhlA with assigned energy contributions. The protein backbone is represented by the Cα trace. The Cα atoms of the residues showing the most important energy contributions in model 4 are shown as balls (see Table 4). Dark-colored residues have only van der Waals energy contributions, while light-colored residues have both van der Waals and electrostatic energy contributions. The substrate molecules are in the positions obtained from energy minimization.

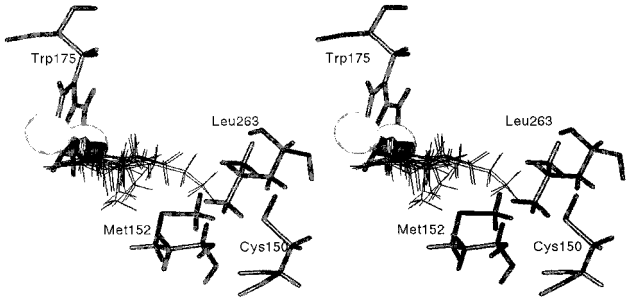

FIGURE 8: Stereoview of the substrate molecules docked in the enzyme active site. Unfavorable van der Waals interactions between long-chain substrates, i.e., butanes and hexanes, and Trp175 are represented by van der Waals surfaces of interacting atoms. Favorable interactions of the same long-chain molecules with Leu263, Met152, and Cys150 positioned on the opposite side of the active site can be seen.

substrates. This residue is not in direct contact with the substrate molecules, but makes important interactions with Trp125 and Phe172. The importance of these interactions has been experimentally demonstrated by Schanstra and co-workers (*29*). Many interactions significant for the third component also participate in the fourth component, for example, Val226, Phe172, Phe222, Glu56, Leu263, or Trp175. Phe172 contributes to stabilization of the transition state and the product (*32*, *62*). Schanstra and co-workers (*20*) performed mutational analysis at position 172 and constructed 16 different point mutants, some of which had modified activity and substrate range. Quantitative structure−function relationship (QSFR) analysis conducted with the same set of point mutants identified physicochemical properties critical for position 172: aromaticity, main-chain flexibility, refractivity, and bulkiness (*63*). Like Trp175 and Leu263, Phe172 also makes unfavorable van der Waals interactions with the long-chain substrates 1-bromohexane and 1-chlorohexane. Phe172 is among the most important residues of DhlA as it displayed both significant van der Waals and electrostatic interactions.

Two of the interactions with negative coefficients, $Asp124^{ele}$ and $Trp125^{vdw}$, are very important for the first and second component, respectively. $Asp124^{ele}$ together with the substrate electrostatic desolvation term dominates the first component of the model employing both AMBER-calculated electrostatic interaction energies and the change in the electrostatic desolvation energy of desolvation of the substrate and the enzyme upon complex formation (e.g., model 22). The variables 124^{ele} and $\Delta G_{desolv}{}^{S}$ are negatively correlated in this model and explain mainly the variability in the dissociation constants for the substrates 2-bromoacetamide, 2-chloroethanol, and 2-bromoethanol. Desolvation of these polar molecules is energetically demanding, resulting in poor binding affinity. The negative coefficient of Trp125 can be attributed to a different behavior of the energy changes associated with this residue in comparison with the rest of the variables. This can be observed in the partial weights and loading plots rather than in a different slope in the correlation with the external vector. The difference may have a structural origin, since Trp125 is located in a loop buried in the protein core, while most of the rest of the important interactions are associated with α-helices. Trp125 appears to be important for explaining differences between chlorinated and brominated derivatives. The essential role of Trp125 for binding of the halogen substituent, stabilization of the transition state, and halide ion release upon reaction has been postulated from crystallographic and fluorescence quenching studies (*64*), from site-directed mutagenesis experiments (*28*), and from molecular modeling (*32*, *36*). The electronegative aromatic indole nitrogens of the tryptophans provide polarization of the N−H bond, resulting in a slightly positive hydrogen that can interact with the halogen.

In summary, the most influential active site residues can be divided into two classes, with respect to their interaction with the substrates. The first class is formed by residues separating chlorinated derivates from brominated derivates. These residues include Trp125, Trp175, and Pro223, and form the halogen binding site in the protein, which is more selective for brominated derivatives. Mutations affecting these residues should be used to modulate the halogen specificity of the enzyme. The second set of residues discriminates substrates by their interactions with the substrate alkyl side chain. It includes Phe164, Phe172, Phe222, and Leu263, and there is a contribution from Asp124 as well. Mutations affecting these residues can be used to tune the activity of the enzyme for different side-chain specificities.

All amino acid residues discussed so far belong to the so-called first shell of residues, i.e., residues lining the active

site of DhlA. Their significance is not unexpected since these residues make direct contacts with the substrate molecules and their possible role could be inferred from the X-ray structure. The identification of second-shell residues might be more useful for protein design purposes. In DhlA, such residues were also identified with the COMBINE models and include Phe222, Leu179, Lys176, Lys224, Val165, and Pro57 (listed in order of their significance; see Table 4). These residues represent suitable targets for future site-directed mutagenesis experiments.

SUPPORTING INFORMATION AVAILABLE

Parameters derived for halogenated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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