Modification of Activity and Specificity of Haloalkane Dehalogenase from *Sphingomonas paucimobilis* UT26 by Engineering of Its Entrance Tunnel*

Radka Chaloupková, Jana Sýkorová, Zbynek Prokop, Andrea Jesenská, Marta Monincová, Martina Pavlová, Masataka Tsuda, Yuji Nagata, and Jiří Damborský

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From the National Centre for Biomolecular Research, Masaryk University, Kotlarsko 2, 611 37 Brno, Czech Republic and the Graduate School of Life Sciences, Tohoku University, Katahira, Sendai 980-8577, Japan

Structural comparison of three different haloalkane dehalogenases suggested that substrate specificity of these bacterial enzymes could be significantly influenced by the size and shape of their entrance tunnels. The surface residue leucine 177 positioned at the tunnel opening of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26 was selected for modification based on structural and phylogenetic analysis; the residue partially blocks the entrance tunnel, and it is the most variable pocket residue in haloalkane dehalogenase-like proteins with nine substitutions in 14 proteins. Mutant genes coding for proteins carrying all possible substitutions in position 177 were constructed by site-directed mutagenesis and heterologously expressed in *Escherichia coli*. In total, 15 active protein variants were obtained, suggesting a relatively high tolerance of the site for the introduction of mutations. Purified protein variants were characterized by determination of specific activities with 12 halogenated substrates and steady-state kinetic parameters with two substrates. The effect of mutation on the enzyme activities varied dramatically with the structure of the substrates, suggesting that extrapolation of one substrate to another may be misleading and that a systematic characterization of the protein variants with a number of substrates is essential. Multivariate analysis of activity data revealed that catalytic activity of mutant enzymes generally increased with the introduction of small and nonpolar amino acid in position 177. This result is consistent with the phylogenetic analysis showing that glycine and alanine are the most commonly occurring amino acids in this position among haloalkane dehalogenases. The study demonstrates the advantages of using rational engineering to develop enzymes with modified catalytic properties and substrate specificities. The strategy of using site-directed mutagenesis to modify a specific entrance tunnel residue identified by structural and phylogenetic analyses, rather than combinatorial screening, generated a high percentage of viable mutants.

Haloalkane dehalogenases are microbial enzymes acting on halogenated compounds. The enzymes cleave the carbon-halogen bond and replace a halogen with a hydroxyl group from a water molecule (1). Activity and specificity of haloalkane dehalogenases is not optimal for industrial applications (2), and numerous studies have been conducted to improve their catalytic properties using *in vitro* techniques (3–13). Engineered enzymes can be used in biotechnology applications, such as detoxification of environmental pollutants and biogenic synthesis. Such technologies are already in use (14) or are under development (2, 15, 16). Furthermore, haloalkane dehalogenases have become an important model system for *in silico* study of molecular principles of enzymatic catalysis (12, 17–24).

Haloalkane dehalogenase LinB (25) is the enzyme isolated from a γ-hexachlorocyclohexane-degrading bacterium *Sphingomonas paucimobilis* UT26 (26). The LinB enzyme catalyzes conversion of 1,3,4,6-tetrachloro-1,4-cyclohexadiene to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol via 2,4,5-trichloro-2,5-cyclohexadien-1-ol. LinB has broad substrate specificity, and in addition to cyclic dienes, it also converts halogenated alkenes, cycloalkanes, alkenes, ethers, and alcohols (27).

The crystal structures of three different haloalkane dehalogenases, i.e. DhlA from Xanthobacter autotrophicus GJ10, DhaA from *Rhodococcus* sp., and LinB from *S. paucimobilis* UT26 have been determined (28–30). A comparison of these structures revealed that not only are the size, shape, and physicochemical properties of the active site important determinants of specificity but also the size and shape of the entrance tunnel (30). In this study we attempted to modify the specificity of haloalkane dehalogenase LinB by engineering of the tunnel connecting the protein surface with its active site cavity. Amino acid residue Leu177, which is positioned in the tunnel opening (see Fig. 1), was replaced, and the effect of the mutation on enzyme activity and specificity was studied.

**MATERIALS AND METHODS**

**Sequence Analysis—** Retrieval and multiple alignment of protein sequences of biochemically characterized and putative haloalkane dehalogenases has been published (31). In brief, the sequences of putative haloalkane dehalogenases were identified by iterative searches of non-redundant databases using PSI-BLAST algorithm (32) and BLOSUM62 substitution matrix. The protein sequences of known haloalkane dehalogenases served as the query sequences. Top scoring sequences were downloaded from the SWISS-PROT database and aligned using CLUSTALX v1.8 (33) with manual refinement. Amino acid residues located in the active site and entrance tunnel of LinB protein were identified from its crystal structure (protein Data Bank identification code 1CV2)(30), and the number of substitutions/position were counted in all sequences of the alignment.

**Structure Analysis—** The crystal structure of LinB protein (30) was downloaded from the Protein Data Bank (identification code 1CV2) and...
visualized by the INSIGHT II v95 modeling package (Biovysio/MSI, San Diego, CA). The molecular surface of the active site pocket was calculated in the program Python Molecule Viewer 1.0 (Scripps Research Institute) using the probe radius 1.4 Å.

**Site-directed Mutagenesis**—Mutagenesis of LinB was performed using three different methods. The plasmid pULBH6 (34) was used as a template. All resulting LinB mutants had 6× histidyl tail at the C terminus. The first method used the principle of long and accurate PCR in vitro mutagenesis kit (TaKaRa Shuzo Co., Kyoto, Japan) according to the manufacturer’s protocol provided, except for using Pyrobest DNA polymerase (TaKaRa Shuzo Co.) or expand high fidelity PCR system (Roche Applied Science). Seven LinB mutants (L177A, L177C, L177F, L177G, L177K, L177T, and L177V) were constructed using this technique. In the second strategy, two parts of the gene were amplified independently by PCR. One fragment was amplified with the M13 primer 5′-CAG GAA ACA GCT GTG-3′ and the oligonucleotide carrying a mutation in the position 177 (see below), whereas the second fragment was amplified using the M13 primer 5′-GTT TTC CCA GTC GAC AC-3′ and the oligonucleotide 5′-GCC TTA AGC GAC GGG GAG-3′. Both fragments were purified by the gel extraction kit (Qiagen), treated with T4-polynucleotide kinase (TaKaRa Shuzo Co.) and ligated. Full-length genes were amplified with M13 primers RV and RV2, and the mutants were confirmed by the dideoxy-chain terminated method (35). The oligonucleotides that were used to introduce mutation in the LinB gene were amplified with an automated DNA sequencer ABI PRISM™ 310 (Applied Biosystems). The plasmid pULBH6 (34) was used as a template. The oligonucleotides that were used to introduce mutation in position 177 are as follows: L177A (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177C (5′-GCC CAG GAT ACA TCC GGG GAG-3′), L177D (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177F (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177G (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177H (5′-GCC CAG GAT GGT TCC GGG GAG-3′), L177I (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177K (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177L (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177M (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177N (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177Q (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177R (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177S (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177T (5′-GCC CAG GAT GCC TGC GGG GAG-3′), L177V (5′-GCC CAG GAT GCC TGC GGG GAG-3′), and L177Y (5′-GCC CAG GAT GCC TGC GGG GAG-3′).

**Circular Dichroism Spectra**—Circular dichroism spectra were recorded on a Jasco J-810 spectrometer (Jasco). The reaction was performed at 37 °C (Eq. 2).

**Growth and Dehalogenation kinetic assay**—Transformants were grown under the conditions of induction of the lacI gene. The enzymatic reaction was initiated by adding 200 μl of enzyme solution (0.004 mg/ml) into 10 ml of substrate solution (6.4 μl of halogenated compound dissolved in a glycerine buffer, pH 8.6). The mixture was incubated at 37 °C and analyzed. The progress of the reaction was monitored by withdrawing 0.5-ml samples at 0, 10, 20, 30, and 60 min using a syringe needle to reduce evaporation of the substrate from the reaction mixture. The reaction mixture samples were mixed with 0.5 ml of methanol to terminate the reaction. The reaction mixture without enzyme served as an abiotic control.

**Steady-state Kinetics Measurements**—Steady-state kinetic constants of LinB mutants with two substrates, 1-chlorobutane and 1,2-dibromoethane, were measured by determination of the substrate and product concentrations using a gas chromatograph Triospac GC 2000 (Finnigan, San Jose, CA) equipped with a flame ionization detector and a capillary column DBFFAP 30 m × 0.25 mm × 0.25 μm (J & W Scientific). The reaction was conducted in 25-ml Reacti-Flasks closed by Mininert Valves. The enzymatic reaction was initiated by adding 200 μl of enzyme solution. The reaction mixture samples were measured in triplicate. The steady-state kinetic constants (kcat, Km, and Kd) were calculated using the computer program Eiz-Fit version 3.3 (46). The Michaelis-Menten equation was used to calculate the kinetic parameters (Eq. 1).

**Results**—Design and Construction of L177 Variants—The amino acid in position 177 was identified as a probable determinant of the substrate specificity of haloalkane dehalogenase LinB by structural analysis and comparison of the primary sequence of LinB with protein sequences of other family members. Leu177 is positioned at the mouth of the largest entrance tunnel leading
to the enzyme active site and points directly into the tunnel (Fig. 1). At the same time it is the most variable pocket residue of the haloalkane dehalogenase-like proteins, showing nine different substitutions in 14 proteins (Fig. 2). Seven different amino acid residues Ala, Cys, Phe, Gly, Lys, Thr, and Trp were introduced in the position 177 of LinB by site-directed mutagenesis (hereafter to be referred to as the first set of mutants) to investigate the role of Leu177 on catalytic efficiency and substrate specificity. The protein variants were overexpressed in E. coli, purified to homogeneity, and kinetically characterized. Kinetic data were analyzed using the PCA to establish relationships between the physicochemical properties of the introduced amino acids and catalytic properties of individual protein variants. The low statistical significance of the developed model caused by insufficient variability in the data set led us to construct and characterize the protein variants carrying the rest of the possible substitutions in 14 proteins (Fig. 2). Significant differences in circular dichroism spectra of the entrance tunnel leading to the enzyme active site.

Substrate Specificity of Leu177 Variants—Substrate specificity of constructed mutants was assayed by determination of specific activities with 12 halogenated substrates representing different chemical groups: mono-, di-, and tri-halogenated; chlorinated, brominated, and iodinated; aliphatic and cyclic; and saturated and unsaturated compounds. Three of these substrates, 1,2-dichloroethane, 1,2-dichloropropane, and 1,2,3-trichloropropane, are industrially interesting chemicals toward which the wild type enzyme does not exhibit detectable activity. The specific activities were measured with purified proteins in an excess of substrate (Table I). Simple visual inspection of the data reveals that: (i) without exception, all of the mutants exhibited modified activities compared with the wild type enzyme, (ii) the impact of the mutations on activity toward different substrates was different, and (iii) none of the mutants exhibited activity toward substrates that were not attacked by the wild type enzyme. Systematic exploration of the data was done by the PCA, which was applied to the data matrix of 17 proteins and specific activities measured with eight substrates (for description of data matrix see “Materials and Methods”). The analysis resulted in two biologically interpretable principal components, which explained 63% of the data variance. The first statistically significant component explained 44% of the data variability and sorted the proteins mostly according to the size of the amino acid residue introduced in position 177. The second principal component explained 19% of data variability and sorted the proteins according to the polarity of amino acid residue introduced in position 177 (Fig. 4A). The proteins are ordered according to their overall activity toward selected substrates along the diagonal. The most active proteins (L177A, L177F, and L177M) are positioned in the top right corner, and the least active mutants (L177R, L177D, and L177H) are positioned in the bottom left corner. The overall activity is not a linear function of the size and polarity of the mutated residue. For example, L177F has large and nonpolar residue in the position 177 but is still highly active with most of the substrates. “Outlying” activities of some of the mutants toward certain substrates is obvious from a comparison of the score plot (Fig. 4A) with the loading plot (Fig. 4B). The score and loading plots should be viewed in parallel, when the positions of mutants and their favorite substrates correspond to each other and vice versa. For instance, high activity of L177W (positioned in the top left corner of the score plot) toward 1-chlorobutane (positioned in the top left corner of the loading plot) but lowered activity with almost all other substrates, or exceptionally high activity of L177T, L177S, and L177Q (positioned in the bottom right corner of the score plot) with 1-iodobutane and 1-bromobutane (positioned in the bottom right corner of the loading plot). As expected, the wild type enzymes from the first (wt1) and the second (wt2) set of mutants are positioned close to each other, confirming good homogeneity of both sets of experiments. The substrates are clustered according to their ability to undergo the dehalogenation reaction (Fig. 4B) with at least three obvious clusters: (i) 1-chlorobutane separated clearly from the rest of the substrates, (ii) 1,3-diiodopropane and 3-chloro-2-methylpropene, and (iii) 1-bromobutane and 1-iodobutane. 1-Chlorobutane exhibits a very different dehalogenation pattern compared with other substrates, especially because of its high conversion rates by L177C and resistance to dehalogenation by L177T (in two independent experiments; detection limit 0.0005 μmol·s⁻¹·mg⁻¹ of enzyme). On the other hand similar dehalogenation patterns were observed for the pairs, 1-bromobutane and 1-iodobutane ($R^2 = 0.97$, $n = 17$) and 1,3-diiodopropane and 3-chloro-2-methylpropene ($R^2 = 0.72$, $n = 17$). Omitting individual groups of substrates from the analysis in a stepwise manner did not alter the model, thereby largely confirming its robustness.

Catalytic Properties of Leu177 Variants—Catalytic efficiency of mutant enzymes were assessed by determination of the
steady-state kinetic constants for 1-chlorobutane and 1,2-dibromoethane conversion. 1-Chlorobutane is often used as a reference compound for comparison of different haloalkane dehalogenases, and 1,2-dibromoethane is one of the best substrates for this family of proteins. A typical increase in the velocity of LinB reaction was observed when 1-chlorobutane concentration increased, whereas a deviation from the relationship of velocity on substrate concentration was observed for 1,2-dibromoethane. The kinetics of 1,2-dibromoethane conversion shows a decrease after the maximum velocity is reached, indicating substrate inhibition at high substrate concentration. The steady-state kinetic parameters of the wild type and mutant haloalkane dehalogenases are presented in Table II. Except for L177V (0.06 mM), all of the mutants showed a higher $K_m$ compared with the wild type enzyme (0.23 mM). L177D exhibited the highest $K_m$ (21.9 mM) and L177F the highest $k_{cat}$ (3.23 s$^{-1}$) for 1-chlorobutane conversion. Compared with wild type enzyme (1.11 s$^{-1}$), the $k_{cat}$ for 1-chlorobutane conversion remained exchanged in L177G, L177K, L177D, and L177H; decreased in L177C, L177V, and L177Y; and increased for L177A, L177F, L177W, L177M, L177Q, and L177S; but generally did not change greatly. Only one mutant, L177V, was catalytically more efficient with 1-chlorobutane (8.87 mM$^{-1}$s$^{-1}$) compared with the wild type enzyme (4.83 mM$^{-1}$s$^{-1}$), mostly because of the significant improvement of $K_m$. L177T was inactive with 1-chlorobutane, whereas its $K_m$ for 1,2-dibromoethane (18.3 mM) was the highest among all of the mutants tested. This indicates that the inability of L177T to exhibit activity toward 1-chlorobutane may be related to poor binding of substrate to the active site. Like the wild type enzyme, all of the mutants without exception exhibited substrate inhibition with 1,2-dibromoethane. The substrate inhibition constant ($K_{si}$) changed in the case of only two mutants; an 8-fold increase of $K_{si}$ was determined for L177G, and a 6-fold decrease was determined for L177Y. The extent of this change in $K_{si}$ for L177G is however uncertain because of a poor fit of the substrate inhibition model to the experimental data. The lowest $K_m$ was observed for conversion of 1,2-dibromoethane by L177W (0.12 mM), but at the same time a sizable decrease in the $k_{cat}$ was also observed (0.61 s$^{-1}$). Compared with the wild type enzyme ($K_m = 5.54$ mM, $k_{cat} = 29.33$ s$^{-1}$), a higher $k_{cat}$ for 1,2-dibromoethane conversion was observed for L177A, L177F, L177T, L177M, L177Q, L177R, and L177S.

DISCUSSION

The haloalkane dehalogenases are enzymes with broad substrate specificity. At least three different specificity classes can be distinguished in this protein family (25, 36). A comparison of representative structures of enzymes belonging to different specificity classes suggested an important role for the entrance tunnel in determining substrate specificity (30, 37). This study aims to validate the above proposal by engineering the entrance tunnel of haloalkane dehalogenase LinB. The residue Leu$^{177}$ located in the tunnel opening was selected for mutagenesis based on structural (Fig. 1) and phylogenetic (Fig. 2) analysis and was shown to significantly influence the substrate specificity of LinB enzyme. Two independent studies conducted in parallel to our project attempted to engineer the related haloalkane dehalogenase, DhaA, by the directed evolution approach and established the importance of the equivalent resi-
Position 177 of LinB was found to be highly tolerant to introduction of different amino acid residues because 15 active protein variants could be obtained by site-directed mutagenesis with 1-chlorobutane that is 2 orders of magnitude higher than the Km for enzyme activity. Gray et al. (9) constructed mutant proteins by standard error prone PCR, whereas Bosma et al. (11) used a combination of DNA shuffling and error-prone PCR. Reconstruction of mycobacterial dehalogenase Rv2579 from LinB by cumulative mutagenesis underlined the prominent role of Leu177 for catalytic activity (13). That the importance of an equivalent residue was established by four independent studies conducted on two different enzymes emphasizes its unique functional role. An important message of the current study is that the role of this residue could be deduced by a combination of structural and phylogenetic analysis, which clearly indicated that the residue equivalent to Leu177 is the most variable among the proteins of haloalkane dehalogenase family (Fig. 2). The fact that both rational and directed evolutionary studies came to the same conclusion proves that knowledge-driven designs are competitive to the combinatorial screening and remains the essential toolbox for protein engineering projects (for discussion see Refs. 38 and 39). The advantage of introducing substitutions into the protein structure in a rational and systematic manner is the possibility of deriving new knowledge about the structure-function relationships, here the relationships between the size/polarity of introduced residues and activity of mutant proteins.

Fifteen active proteins were kinetically characterized by determination of their specific activities with 12 different substrates (Table I) and steady-state kinetic parameters with two substrates: 1-chlorobutane and 1,2-dibromoethane (Table II). An important discovery was that the effect of mutation on enzyme activities varied with the structure of the substrates. This observation questions the usual practice of characterizing broad specificity enzymes using only a few substrates. For example, substitution of Leu177 by Thr completely inactivated the enzyme toward the substrate 1-chlorobutane, whereas activities with all other substrates were either the same (1,2-dibromoethane, 1,3-diiodopropane, and 3-chloro-2-methylpropene) or even higher (1-chlorohexane, 1-bromobutane, and bromocyclohexane) than the wild type enzyme. This is probably due to the fact that 1-chlorobutane does not bind efficiently to the active site of L177T at the concentration used in the assay. We note that the wt enzyme has a $K_m$ with 1-chlorobutane that is 2 orders of magnitude higher than the $K_m$ with 1-chlorohexane, yet these two compounds differ “only” by two carbon atoms in length. The active site of LinB is apparently too large for 1-chlorobutane, and a further increase of its size by L177T mutation coupled with increased polarity of the active site tunnel prevents the efficient binding of 1-chlo-

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**Table I**

| Protein | Position 177 | Activity (relative to wild type) |
|---------|--------------|----------------------------------|
| 1-Chlorobutane | Leu | 100 |
| 1-Chlorohexane | Ile | 100 |
| 1-Bromobutane | Leu | 100 |
| 1,2-Dibromoethane | Leu | 100 |
| 1,3-Diiodopropane | Leu | 100 |
| 3-Chloro-2-methylpropene | Leu | 100 |

* Specific activities (in units) for the wild type enzyme in the set of mutants are $0.0287$ (1,2-dibromoethane), $0.0351$ (1-chlorobutane), and $0.1312$ (1-chlorohexane).

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**Table II**

| Protein | Position 177 | Activity (relative to wild type) |
|---------|--------------|----------------------------------|
| 1-Chlorobutane | Leu | 100 |
| 1-Chlorohexane | Ile | 100 |
| 1-Bromobutane | Leu | 100 |
| 1,2-Dibromoethane | Leu | 100 |
| 1,3-Diiodopropane | Leu | 100 |
| 3-Chloro-2-methylpropene | Leu | 100 |

* Specific activities (in units) for the wild type enzyme in the set of mutants are $0.0287$ (1,2-dibromoethane), $0.0351$ (1-chlorobutane), and $0.1312$ (1-chlorohexane).

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J. Damborský, K. Hynkova, and Y. Nagata, unpublished data.
robustane. It is of note that 1-iodobutane is a much better substrate than 1-chlorobutane because iodine possesses a wider van der Waals radius, resulting in a substrate molecule with a larger volume and thus better complementarity with the enlarged active site. The mutant L177T also showed significantly lowered affinity for 1,2-dibromoethane, the $K_m$ of 18.3 mM being the highest from all tested mutants. These results demonstrate that extrapolation from one substrate, even for seemingly related substrates like chloro-, bromo-, and iodobutane, may be misleading.

Leu$^{177}$ is positioned at the tunnel opening, but it makes van der Waals contacts with some of the substrates bound to the Michaelis complex. This could be one of the reasons for different effects of mutations on activities measured with different substrates. For example, the lower $K_m$ observed for conversion of 1,2-dibromoethane by L177W (0.12 mM), which correlated with a decrease in the $k_{cat}$ (0.61 s$^{-1}$), suggests stabilization of the Michaelis complex, but not the transition state, for this substrate. The correlated changes in $K_m$ and $k_{cat}$ values, resulting in equally catalytically efficient enzymes, have been previously observed for five mutants of LinB (13). PCA of the specific activities measured for eight halogenated substrates. The arrows in the score plot indicate that the protein variants are ordered approximately by the size of amino acid introduced to the position 177 along the first principal component, by the polarity of amino acid introduced to the position 177 along the second principal component, and by the overall activity along the diagonal.

Table II

| Steady-state kinetic parameters of purified wild type and mutant haloalkane dehalogenases |
|-----------------------------------------------|
| | First set | Second set |
| | wt1<sup>a</sup> | L177A | L177C | L177G | L177F | L177K | L177T | L177W |
| 1-Chlorobutane $K_m$ (mM) | 0.23 ± 0.04 | 3.34 ± 2.14 | 0.60 ± 0.18 | 0.81 ± 0.93 | 0.78 ± 0.11 | 0.99 ± 0.20 | 2.22 ± 0.27 |
| $k_{cat}$ (s<sup>-1</sup>) | 1.11 ± 0.05 | 2.67 ± 0.75 | 0.49 ± 0.06 | 1.28 ± 0.84 | 3.23 ± 0.19 | 0.98 ± 0.09 | 2.52 ± 0.17 |
| $k_{cat}/K_m$ (mM$^{-1}$s<sup>-1</sup>) | 4.83 | 0.80 | 0.82 | 1.58 | 4.14 | 0.99 | 1.14 |
| 1,2-Dibromoethane $K_m$ (mM) | 5.54 ± 0.49 | 87.5 ± 0.70 | 3.82 ± 0.77 | 4.03 ± 0.84 | 5.03 ± 0.43 | 6.23 ± 1.31 | 18.3 ± 1.62 |
| $k_{cat}$ (s<sup>-1</sup>) | 29.33 ± 1.19 | 47.20 ± 1.59 | 14.00 ± 1.15 | 12.35 ± 1.34 | 39.60 ± 1.65 | 16.67 ± 2.95 | 38.67 ± 1.81 |
| $k_{cat}/K_m$ (mM$^{-1}$s<sup>-1</sup>) | 5.29 | 5.39 | 3.66 | 3.06 | 7.87 | 7.68 | 2.11 |
| | wt2<sup>b</sup> | L177D | L177H | L177I | L177M | L177P | L177Q | L177R |
| 1-Chlorobutane $K_m$ (mM) | 0.24 ± 0.02 | 2.90 ± 0.51 | 1.70 ± 0.06 | 0.94 ± 0.09 | 1.15 ± 0.02 | 1.49 ± 0.04 | 2.05 ± 0.11 |
| $k_{cat}$ (s<sup>-1</sup>) | 1.00 ± 0.03 | 0.84 ± 0.02 | 0.96 ± 0.02 | 0.96 ± 0.02 | 0.96 ± 0.02 | 0.96 ± 0.02 | 0.96 ± 0.02 |
| $k_{cat}/K_m$ (mM$^{-1}$s<sup>-1</sup>) | 4.32 | 0.04 | 0.62 | 0.96 | 3.43 | 0.04 | 0.96 |
| 1,2-Dibromoethane $K_m$ (mM) | 5.76 ± 0.60 | 14.00 ± 0.91 | 5.49 ± 0.89 | 8.36 ± 0.72 | 10.37 ± 1.05 | 10.36 ± 1.04 | 13.20 ± 1.69 |
| $K_m$ (mM) | 2.17 ± 0.22 | 5.32 ± 0.39 | 0.98 ± 0.15 | 1.68 ± 0.14 | 1.12 ± 0.10 | 0.82 ± 0.08 | 1.04 ± 0.13 |
| $k_{cat}$ (s<sup>-1</sup>) | 23.42 ± 1.17 | 18.67 ± 0.59 | 26.33 ± 2.07 | 39.33 ± 1.69 | 48.80 ± 2.26 | 41.73 ± 2.15 | 45.33 ± 3.07 |
| $k_{cat}/K_m$ (mM$^{-1}$s<sup>-1</sup>) | 4.07 | 1.33 | 4.47 | 4.70 | 4.71 | 4.93 | 3.03 |

<sup>a</sup>The wild type enzyme in the first set of mutants.<br><sup>b</sup>The wild type enzyme in the second set of mutants.<br><sup>c</sup>Activity not detectable.
context of Leu\textsuperscript{177}. This residue partially blocks the entrance tunnel (Fig. 1), and it is expected that its size and polarity will influence binding of the substrate molecules to the active site. Especially poor binding was observed when a negatively charged residue was introduced in position 177 (K\textsubscript{as} for L177D is 21.9 mM with 1-chlorobutane and 14 mM with 1,2-dibromoethane). Positively charged (L177R) and polar residues (L177S and L177T) also disfavor binding. This may be due to electrostatic interactions between polar residues positioned in the mouth of the tunnel and the dipole moment of the halogenated substrates binding to the enzyme active site.

Requirements for small and nonpolar amino acid in the position equivalent to Leu\textsuperscript{177} in family members is obvious from the phylogenetic analysis showing that Gly and Ala are the most commonly occurring amino acids in this position (Fig. 2).

In the broader context of structure-function relationships, our studies demonstrate that activity and substrate specificity of enzymes with buried active sites can be modulated by the residues positioned far from the active site (distance of Leu\textsuperscript{177}–C\textsubscript{a} from the nucleophile Asp\textsuperscript{108}–C\textsubscript{a} is 12.5 Å) if they are a part of the entrance tunnel. Modification of the catalytic properties of enzymes using site-directed mutagenesis by specifically targeting such distant residues is attractive because the possibility of generating functional enzyme is much higher compared with mutating the active site residues. Studies have demonstrated that engineering of entrance tunnels is an appropriate approach for modification of substrate specificity. Huang and Rausch (40) engineered a blockage within the intermolecular tunnel of carbamoyl phosphate synthetase from E. coli which prevented the use of glutamine as a substrate. Capila \textit{et al.} (41) introduced substitution in the active site tunnel of chondroitin AC lyase from \textit{Flavobacterium heparinum} and obtained a mutant with stepwise endolytic and exolytic cleavage of the substrate oligosaccharide. Schmitt \textit{et al.} (42) mutated amino acids at different locations inside the tunnel of \textit{Candida rugosa} lipase and obtained mutants with different chain length specificity. Increasing the bulkiness of the amino acids inside the tunnel led to mutants with a strong discrimination toward chain lengths longer than C-14, whereas a mutation at the entrance of the tunnel had a strong impact on C-4 and C-6 substrates. The effects of mutations of substrate specificity could be explained by a simple mechanical model; the activity for a fatty acid sharply decreased as it became long enough to reach the mutated site.

Specific activities and steady-state kinetic constants obtained by systematic characterization of an exhaustive set of mutants in the position 177 of LinB represent a homogenous data set for future theoretical studies attempting to predict the effect of mutation on enzyme activity and substrate specificity. A quantitative structure-function relationship analysis (43) of this data set is currently being done in our laboratory. This analysis should not only relationships between the mutant structure but also substrate structure and enzymatic activity.

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