Published in final edited form as:

Chembiochem. 2014 September 5; 15(13): 1931–1938. doi:10.1002/cbic.201402081.

Increasing the reaction rate of hydroxynitrile lyase from *Hevea brasiliensis* towards mandelonitrile by copying active site residues from an esterase that accepts aromatic esters

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Abstract

The natural substrate of hydroxynitrile lyase from rubber tree (HbHNL, *Hevea brasiliensis*) is acetone cyanohydrin, but synthetic applications usually involve aromatic cyanohydrins such as mandelonitrile. To increase the activity of HbHNL toward this unnatural substrate, we replaced active site residues in HbHNL with the corresponding ones from esterase SABP2 (salicylic acid binding protein 2). Although this enzyme catalyzes a different reaction (hydrolysis of esters), its natural substrate (methyl salicylate) contains an aromatic ring. Three of the eleven single-amino-acid-substitution variants of HbHNL reacted faster with mandelonitrile. The best was HbHNL-L121Y, with a 4.2-fold higher k_{cat} and high enantioselectivity. Site-saturation mutagenesis at position 121 identified three other improved variants. We hypothesize that the smaller active site orients the aromatic substrate more productively.

Keywords

hydroxynitrile lyase; esterase; protein engineering; promiscuous activity

Introduction

Protein engineering can improve enzyme properties, but predicting the needed changes is difficult.^[1, 2] For example, increasing reactivity toward a chosen substrate requires predicting the transition state orientation and charge distribution and which amino acid substitutions would favor it.^[3] There are too many possible conformations of substrate and enzyme to exhaustively search all of them.^[4] Researchers usually use molecular dynamics simulations to find likely conformations, but this approach misses conformations that

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require crossing energy barriers and thus may make incorrect predictions. For example, the predicted and observed active site side chain orientations differ in an engineered aldolase.^[5]

Even if one predicts the correct changes for the design goal, they must be compatible with existing enzyme properties. The changes should not affect protein stability, pH optimum, stereoselectivity and other enzyme properties. The incompatibility of the designed changes with stability or folding often causes insolubility or poor protein expression of designed protein variants.

A protein engineering approach that avoids some of these difficulties is to copy solutions from homologous enzymes, Figure 1. For example, to engineer more stable proteases, researchers compared the structures to that of more stable homologs and copied selected features into the enzyme to be stabilized.^[6, 7] Although choosing which features to copy still requires insight and modeling, the chances of a correctly folded enzyme was much higher because the features already work in a homolog.

One common protein engineering goal is to increase the activity of an enzyme toward unnatural substrates. Such improvements are often needed for the synthesis of pharmaceutical and agrochemical intermediates and fine chemicals. Since the substrate is unnatural, applying the feature-copying approach is difficult because there is no enzyme with the desired activity that could be used as a template. In this paper, we describe an approach to solve this problem. Homologous enzymes may act on substrates similar to the unnatural one, so their active sites could serve as templates, even when they catalyze different reactions.

Researchers previously used data from homologous enzymes to create smarter libraries.^[8, 9] Instead of random substitutions, researchers limited the choices to those found in corresponding positions in homologous proteins. The fraction of improved proteins was higher in these smart libraries than in random libraries. The target enzyme likely tolerates amino acid substitutions that occur in homologs over substitutions that occur rarely or never. In these engineering experiments, the homolog information created a menu of possible choices, but since the homologs did not have the desired properties, one did not know which of these choices would improve the enzyme, only that these substitutions would be tolerated. Our approach differs in that it attempts to copy specific features from a homolog to a target enzyme.

In this paper, we improve the substrate specificity of hydroxynitrile lyase from rubber tree (*Hevea brasiliensis*, *HbHNL*) toward mandelonitrile, a typical aromatic cyanohydrin. Plants use hydroxynitrile lyases to defend themselves against insects. The HNL-catalyzed cleavage of cyanohydrins, Scheme 1, releases the toxic hydrogen cyanide. The natural substrate for *HbHNL* is acetone cyanohydrin, but many synthetic applications such as the synthesis of various pyrethroids, e.g. deltamethrin, cypermethrin and tralomethrin, involve aromatic cyanohydrins, which are poorer substrates.^[10] The k_{cat} for acetone cyanohydrin is 2.7-fold higher than for mandelonitrile (65 vs. 25 s⁻¹) and acetone cyanohydrin binds to *HbHNL* 8 times more tightly ($K_M = 0.40$ vs. 3.3 mM), so the specificity constants, k_{cat}/K_M , differ by a factor of 22.

HbHNL belongs to the α/β -hydrolase superfamily.^[11] Another hydroxynitrile lyase in this superfamily is *MeHNL* (HNL from *Manihot esculenta*), which has 77% amino acid sequence identity with *HbHNL*) and whose natural substrate is also acetone cyanohydrin.^[12, 13] Interestingly, the natural substrate of hydroxynitrile lyase from almond is mandelonitrile, but almond HNL belongs to the glucose-methanol-choline oxidoreductase family, so its protein fold differs from that for *HbHNL*.^[14] One cannot expect that features would be compatible between different fold.

The esterase SABP2 (salicylic acid binding protein 2 from to-bacco) is an α/β -hydrolase with 45% amino acid identity with *HbHNL*.^[13] The natural substrate for SABP2 is methyl salicylate, Scheme 1, which contains an aromatic ring. We hypothesized that the replacing of the substrate-binding residues in *HbHNL* with the corresponding residues in SABP2 will improve the fit of an aromatic substrate like mandelonitrile. The backbones of the two enzymes align closely (rmsd of 1.3 Å) and the aromatic ligands bind similarly, Figure 2. The overlay of the substrate mandelonitrile (from *HbHNL*, pdb: 1YB6) and product salicylic acid (from SABP2, pdb: 1Y7I), show that both bind in the same place, but the orientation of the aromatic rings differ slightly.^[13]

Although the active sites of *HbHNL* and SABP2 are similar, their catalytic mechanisms differ significantly, Scheme 1. Both enzymes contain a catalytic triad of serine, histidine and aspartate. Both enzymes bind a carbonyl compound and catalyze a nucleophilic attack on the carbonyl. However, the esterase mechanism involves the formation and cleavage of an acyl enzyme, while the hydroxynitrile lyase acts in a single step without an intermediate covalently-bound to the enzyme. In addition, esterases use an oxyanion hole to stabilize intermediates, while the oxyanion hole is blocked in *HbHNL* to change the binding orientation of the substrate. Despite these differences in mechanism, exchanging catalytic residues between *HbHNL* and SABP2 also exchanged the catalytic activities^[13] showing that catalytic features can be exchanged between these enzymes.

Results

Substrate-binding site differences in *HbHNL* and SABP2

Comparison of the active sites of *HbHNL*, *MeHNL* and SABP2 identified 18 amino acid residues within 4 Å of the substrates, Table 1. Four of these residues contribute to catalysis in either the esterase or hydroxynitrile lyases (11, 80, 235, 236; indicated by 'c', *HbHNL* numbering; yellow sticks in Figure 2) and three other residues are identical in the three enzymes (His14, Trp128, Leu157; blue sticks). The remaining 11 amino acid residues (bold in Table 1, grey in Figure 2: Ile12, Cys81, Val106, Leu121, Phe125, Tyr133, Leu146, Leu148, Leu152, Ile209 and Phe210) likely account for the preference of SABP2 for its aromatic substrate and of *HbHNL* and *MeHNL* for acetone cyanohydrin. These substrate-binding residues lie behind the substrate (as seen from Ser80 and Thr11) and away from the catalytic residues. Consistent with the both *HbHNL* and *MeHNL* having the same natural substrate, nine of these eleven residues are identical in these two enzymes and the two residues that differ are similar: Val or Leu at 106 and Leu or Met at 146.

We excluded two other hydroxynitrile lyases in the α/β -hydrolase superfamily from this comparison: *BmHNL* from *Baliospermum montanum* (a medicinal shrub from India)^[15] and *AtHNL* from *Arabidopsis thaliana*,^[16] (see supporting information). The amino acid sequences of these HNL are 57% and 47%, respectively, identical to *HbHNL*, but neither plant contains cyanogenic glycosides, so the enzymes' natural function is unknown. Both enzymes catalyze cleavage of mandelonitrile, but there is not enough substrate specificity data to know if they are optimized for this substrate. Finally, *AtHNL* favors the (*R*)-mandelonitrile in contrast to *HbHNL*'s preference for the (*S*)-enantiomer, so the substrate orientations likely differs significantly.^[17]

We hypothesized that substitution of the amino acid residues in *HbHNL* with the corresponding ones in SABP2 may improve the fit of aromatic substrates in *HbHNL*. Besides the eleven substitutions predicted above, we also tested a twelfth substitution at Ile209. SABP2 contains a glycine at this position, but a similar SABP2-esterase from *Nicotiana benthamiana* (95% sequence identity with SABP2 from *N. tabacum*) contains an alanine. We tested both glycine and alanine.

Wild type *HbHNL* catalyzed cleavage of mandelonitrile at a rate of 25 s^{-1} (k_{cat}) with a K_{M} -value of 3.3 mM, which corresponds to a specificity constant ($k_{\text{cat}}/K_{\text{M}}$) of $7.4 \text{ s}^{-1}\cdot\text{mM}^{-1}$. We compared variants both by comparing k_{cat} values and $k_{\text{cat}}/K_{\text{M}}$ values. Biocatalysis applications typically use substrate concentrations much higher than K_{M} , so k_{cat} is a better measure of performance. For faster purification we added a C-terminal His-tag to wild-type *HbHNL*. This addition did not alter the activity or enantioselectivity of wild type *HbHNL*.

Site-directed mutagenesis to make *HbHNL*'s substrate-binding site more SABP2-like

Site-directed mutagenesis yielded all twelve single substitution mutants. All of the twelve single substitutions *HbHNL* mutants yielded soluble proteins, Table 2, but three mutants (I12A, I209G and F210I) yielded mostly insoluble protein, suggesting that the substitutions hindered protein folding. Three variants showed higher values of k_{cat} : C81I, 33 s^{-1} (1.3-fold increase), F125T, 66 s^{-1} (2.6-fold increase) and L121Y, 106 s^{-1} (4.2 fold increase). Four variants showed higher specificity constants ($k_{\text{cat}}/K_{\text{M}}$): L152F ($11 \text{ s}^{-1}\cdot\text{mM}^{-1}$, 1.5-fold increase), I209A ($29 \text{ s}^{-1}\cdot\text{mM}^{-1}$, 3.9-fold increase), F125T ($33 \text{ s}^{-1}\cdot\text{mM}^{-1}$, 4.5-fold increase) and L121Y ($39 \text{ s}^{-1}\cdot\text{mM}^{-1}$, 5.3-fold increase). Two of these variants – F125T and L121Y – improved according to either measure. The changes in specific activity, Table 2, were similar to the changes in the k_{cat} , because the substrate concentration in the assay (5 mM) was above the K_{M} value in most cases.

Combining the best single substitutions did not yield additive improvements, but variants that were worse than either single variant. These results indicate negative cooperativity, that is, the substitutions interfered with each other. The k_{cat} value for L121Y-F125T-*HbHNL* was 43 s^{-1} (1.7 fold increase over wild type) and the specificity constant was $14 \text{ s}^{-1}\cdot\text{mM}^{-1}$ (1.9-fold increase over wild type). These substitutions lie close to each other on the same helix approximately one turn apart, so they may interfere with each other. Other double substitutions also yielded improvements over the wild type, but none were better than L121Y-*HbHNL*, Table 2.

Similarly, both triple substitution variants tested were better than wild-type *HbHNL*, but not better than L121Y-*HbHNL*. Adding the L146M substitution to the double substitution variant changed catalytic behavior only slightly. Similarly, adding the L146M substitution to wild type *HbHNL* had only slight effects. In contrast, adding the V106F substitution in wt *HbHNL* decreased catalytic performance, but adding it to L121Y-F125T-variant increased performance. The k_{cat} value for V106F-L121Y-F125T-*HbHNL* was 82 s^{-1} (3.3 fold increase over wild type; 0.77 of the value for L121Y-*HbHNL*) and the specificity constant was $41 \text{ s}^{-1} \cdot \text{mM}^{-1}$ (5.5-fold increase over wild type; 1.04-fold increase over L121Y-*HbHNL*). Although the specificity constant was marginally better than the one for L121Y-*HbHNL*, the lower k_{cat} value makes this triple substitution variant inferior to L121Y-*HbHNL*. The behavior of the V106F substitution again shows the non-additive effect of these substitutions, likely because all substitutions lie close within the active site. Both triple substitution variants were significantly less stable than L121Y-*HbHNL*, so we focused further efforts on this variant.

Consistent with the notion that these substitutions improve the reactivity of aromatic substrates, but not aliphatic substrates, the specific activity of L121Y-*HbHNL* toward acetone cyanohydrin was slightly lower than wild-type *HbHNL*: $2.6 \text{ U} \cdot \text{mg}^{-1}$ versus $3.8 \text{ U} \cdot \text{mg}^{-1}$.

A smaller binding site increases activity

To identify why the tyrosine substitution increased catalytic activity, we created all possible amino acid substitutions at Leu121 using site saturation mutagenesis^[18] and measured their effect, Table 3. Amino acids with charged and polar side chains yielded either no soluble protein (Asp, Lys, Arg) or variants with low specific activity (Glu, His, Ser, Thr, Asn, Gln): 0.2 to $6 \text{ U} \cdot \text{mg}^{-1}$ as compared to $33 \text{ U} \cdot \text{mg}^{-1}$ for wild type. Glycine and proline at position 121 also yielded variants with low activity: 1.4 and $0.1 \text{ U} \cdot \text{mg}^{-1}$, respectively.

The effect of amino acids with hydrophobic side chains varied with the size of side chain, Figure 3, suggesting an optimum size. For alanine, cysteine and valine, the k_{cat} was similar to wild type (leucine), but for larger amino acids (Ile, Met, Phe, Tyr) the k_{cat} increased roughly in order of their size. Tyr was the best. Activity decreased to about 60% of the wild type value for the largest amino acid, Trp, suggesting that it was too big. When comparing $k_{\text{cat}}/K_{\text{M}}$ values, Phe was the best amino acid residue. The K_{M} for variant L121F-*HbHNL* was low: 1.5 mM as compared to 2.7 mM for L121Y-*HbHNL* and 3.3 mM for wild type. The specificity constant ($k_{\text{cat}}/K_{\text{M}}$) for L121F-*HbHNL* was $51 \text{ s}^{-1} \cdot \text{mM}^{-1}$ which is higher than for L121Y-*HbHNL* ($39 \text{ s}^{-1} \cdot \text{mM}^{-1}$) and is a 6.9-fold increase over the wild type.

Molecular modeling suggests that the larger hydrophobic side chains prevent catalytically non-productive orientations, Figure 4. Wild-type *HbHNL* has a large space for the substrate, which permits catalytically non-productive orientations that may break key hydrogen bonds such as between the nitrile and Lys236.^[20] Nonproductive binding of the substrate reduces the k_{cat} value. In contrast, the substrate-binding site of L121Y-*HbHNL* eliminates the empty regions where a nonproductively oriented mandelonitrile would bind. Although the modeled position of the aromatic ring is almost identical in both active sites, mandelonitrile cannot move into a suboptimal position in the L121Y variant. An improved binding of

mandelonitrile is also consistent with the slightly lower K_M values with the L121F and L121Y variants.

Lower stability, but the same enantioselectivity, of L121Y-*HbHNL*

Protein unfolding measurements in urea indicate that the L121Y-*HbHNL* variant is ca. 1.5 kcal·mol⁻¹ less stable than wild type, Figure S3. The tryptophan fluorescence of wild-type *HbHNL* increased in increasing concentrations of urea consistent with protein unfolding to expose tryptophan side chains to solvent. The halfway point, which corresponds to equal amounts of folded and unfolded protein, was 2.3 M urea. Extrapolation of this unfolding data to 0 M urea indicated that the folded form of *HbHNL* is favored by 4.2 kcal·mol⁻¹ (ΔG_{H_2O}) in water. Similar measurement with the L121Y-*HbHNL* variant showed a halfway point at 1.5 M urea. This unfolding at lower concentrations of urea indicates a less stable folded form than the wild-type. Extrapolation of the unfolding data yielded a ΔG_{H_2O} of 2.7 kcal·mol⁻¹, which corresponds to a loss of ~1.5 kcal·mol⁻¹ relative to wild type. Although the L121Y-*HbHNL* variant is less stable, we encountered no experimental difficulties due to this lower stability.

Variant L121Y-*HbHNL* showed the same high (*S*)-enantioselectivity as wild-type *HbHNL*. The L121Y-*HbHNL*-catalyzed addition of hydrogen cyanide to benzaldehyde in a two phase mixture of *tert*-butyl methyl ether and citrate buffer (pH 4.5) yielded (*S*)-mandelonitrile with >98% e.e.. Wild type *HbHNL* yielded identical enantiomeric excess.

Increased promiscuous nitroaldolase activity

Wild type *HbHNL* also catalyzes the cleavage 1-phenyl-2-nitroethanol to nitromethane and benzaldehyde.^[21] In this promiscuous reaction, the larger and less acidic nitromethane (pK_a = 10) replaces hydrogen cyanide (pK_a = 9.2). This promiscuous reaction is slow (0.13 U·mg⁻¹); only 0.3% of the specific activity for cleavage of mandelonitrile (33.4 U·mg⁻¹). Variant L121Y-*HbHNL* catalyzed the cleavage of 1-phenyl-2-nitroethanol 4.8-fold faster (specific activity 0.62 U·mg⁻¹) than wild type, Table 4, which is similar to the 4.5-fold increase in mandelonitrile cleavage. The similar increase for both reactions supports the suggestion above that the increase is due to improved orientation of the benzaldehyde portion, which is common to both substrates. Various double and triple substitutions yielded only slight improvements. The best was a triple substitution L121Y-F125T-L146M, which showed a specific activity of 0.71 U·mg⁻¹ (5.5-fold increase over wild type). None of the variants showed esterase activity (<0.5 mU·mg⁻¹ using 4-nitrophenyl acetate). Although we copied substitutions from an esterase, none of the copied residues were catalytic residues so we did not expect to increase esterase activity.

Discussion

A comparison of the active sites of HNL from *Hevea brasiliensis* and SABP2-esterase from *Nicotiana tabacum* suggested twelve amino acid substitutions that might improve the activity of *HbHNL* toward the aromatic substrate mandelonitrile. Three to four were improved variants and the best substitution was L121Y, which improved k_{cat} 4.2-fold (106 vs. 25 s⁻¹) and the specificity constant (k_{cat}/K_M) 5.3-fold (39 vs. 7.4 s⁻¹·mM⁻¹). The effects

of combined substitutions were less than the sum of the individual substitutions indicating negative cooperativity. Site saturation mutagenesis found another substitution L121F which improved k_{cat} 3.1-fold (77 vs. 25 s⁻¹) and the specificity constant (k_{cat}/K_M) 6.9-fold (51 vs. 7.4 s⁻¹·mM⁻¹). Modeling indicated that these substitutions reduce the size of the substrate-binding site and favor a productive orientation of the substrate. Similar effects were found for the promiscuous nitroaldolase activity of *HbHNL*. The specific activity improved up to 5.5-fold.

This transfer of substrate specificity succeeded despite the different catalytic activities of the two enzymes. The advantages of this active site redesign approach over random mutagenesis or rational design are the simplicity of choosing these substitutions and the high success rate ($\sim 4/12 = 25\%$). It is surprising that improving the fit to a large substrate involved making the active site smaller. Acetone cyanohydrin is smaller than mandelonitrile, so a naive approach would have been to make the active site larger. The use of SAPB2 as a template revealed that the opposite approach could succeed.

A disadvantage was the lower stability of the variants as compared to wild type. Guo *et al.* estimated that the average decrease in stability by a random substitution in a protein is 1.1 kcal·mol⁻¹,^[22] so the 1.5 kcal·mol⁻¹ decrease for the L121Y substitution is not unusual. Further engineering should stabilize the protein if needed.^[23]

Experimental Section

General

Water was purified by a Milli-Q-water-system (Millipore, Billerica, MA (USA), 18 MΩ purity). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Hampton, NH, USA) or VWR (Radnor, PA, USA) in highest available purity and used as received unless otherwise noted. Benzaldehyde was distilled and stored at -18 °C before use. Mandelonitrile was aliquoted into portions and stored at -18 °C to minimize decomposition to benzaldehyde and hydrogen cyanide. The DNA polymerase *Pfu* and endonuclease *DpnI* were obtained from Promega (Madison, WI, USA). Protein concentrations were measured with the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) at 595 nm using bovine serum albumin to make the calibration curve. LB-media (MP Biomedicals, Santa Anna, CA, USA) was used as suggested. Solutions of ampicillin and isopropyl β-D-1-thiogalactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO, USA) were prepared in water, filter-sterilized, and stored at -18 °C. Protein electrophoresis gels were run with NuPage gradient 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and included a BenchMark protein ladder (Life Technologies). *HbHNL* appeared at 29–30 kDa consistent with its mass of 29.2 kDa.^[24] Plasmids were purified from *E. coli* cultures with the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and sequenced by ACGT, Inc. (Wheeling, IL, USA). Protein solutions were concentrated with centrifuge concentrator tubes Vivaspın 15R and VS15RH02 from Sartorius (Göttingen, Germany).

His-tagged *HbHNL*

The gene for *HbHNL* was initially in the pSE420 vector (Invitrogen, amp⁺ selectable marker, trc promoter, lac operator, *HbHNL* gene, 774 bp, inserted at *HcoI-HindIII* restriction sites from Prof. Helmut Schwab, TU Graz, Austria). This gene was transferred to the pET21a+ vector (Novagen, amp⁺ selectable marker, a C-terminal 6× His-tag, and T7 promoter, lac operator) at restriction sites *BamHI-XhoI* by copying the gene using PCR with the following primers: forward: 5'-CGA TTA AAT AAG GAG GAA GGA TCC ATG GCA TTC GCT CAT TTT GTT-3'; reverse: 5'-AAC AGC CAA GCT TCA AAG CTC GAG ATT ATA GGT ATC AGC CAC C-3'. These primers introduced the *BamHI-XhoI* restriction sites and added two amino acids, lysine and glutamate, before the His tag. PCR was run with pSE420 *HbHNL* WT template (1 µL, 70–150 ng/µL), AccuPrime *Pfx* DNA polymerase (1 µL, Invitrogen), and 50 nmol of forward and reverse primers. The PCR product (20 µL) and pET21a+ were digested with *BamHI* and *XhoI* in *BamHI* buffer, purified by gel electrophoresis, isolated from the gel using QIAquick spin columns and ligated (16 °C, 4 h) using equimolar *HbHNL* gene to pET21a+ with 2 µL ligase buffer and 1 µL T4 ligase. An aliquot (5 µL) was transformed to *E. coli* DH5α chemically competent cells and plated on LB-amp agar plates. Several colonies were picked, cultured in LB-amp media, plasmids were isolated and sequenced with sequencing primer (5'-CGA CTC ACT ATA GGG GAA TTG TGA GC-3'). Later experiments used the His-tagged proteins, while earlier ones used non-tagged proteins as described below.

Site directed mutagenesis

Site-directed mutagenesis was done using whole plasmid mutagenesis similar to the approach in QuikChange. A high fidelity DNA polymerase (*Pfu*) extended a pair of overlapping mutagenic primers (Supplementary Table S1) to yield linear strands with complementary overlapping ends that can cyclize to yield a nicked circular DNA. The template was pET21a(+) containing the wild-type *HbHNL* gene described above. The thermocycling reaction mixture contained ~100 ng DNA template, forward and reverse primer (1 µL each of 10 µM), 10× PCR buffer (5 µL), dNTPs (0.9 µL of 10 mM), *Pfu* (1 µL) and autoclaved water to a final volume of 50 µL. Since two primers overlap, the thermocycling reaction linearly amplifies the plasmid; it is not a chain reaction and should not be called a PCR. The thermocycling program for plasmid amplification was 95 °C for 1.5 min, 25–35 cycles of 95 °C for 45 s, × °C for 30 s and 72 °C for 8 min, with a final extension at 72 °C for 5 min and storage at 10 °C for a few hours if needed. The annealing temperature (× °C) was 5–7 °C below the melting point of the primers. After confirming with gel electrophoresis that the amplification succeeded, the methylated parental DNA strands were fragmented with a restriction enzyme specific for methylated DNA (*DpnI*) by adding 5 units of *DpnI* and incubating at 37 °C for 2.5 h. An aliquot (5 µL) was transformed into *E. coli* DH5α competent cells (50 µL) by heat shock. The mixture was transferred to LB-media (1 mL, without ampicillin), incubated at 37 °C for 1 h, centrifuged, 850 µL of the supernatant discarded and the remaining 150 µL spread on an LB-media-plate containing 100 µg/mL ampicillin and incubated at 37 °C overnight. Colonies that appeared were picked and grown in LB-ampicillin media (3 mL) overnight. Plasmid DNA was isolated from this

culture using QiaPrep MiniPrep kit and sequenced to confirm that the DNA contained the desired mutation.

Protein expression and purification

For protein expression, the desired plasmid was transferred to *E. coli* BL21. One colony of *E. coli* BL21 containing the desired plasmid was used to inoculate LB-Amp-medium (5 mL) and grown overnight (15 h) at 37 °C. An aliquot (2.5 mL) of this culture was used to inoculate 250 mL of LB-Amp-medium and grown 37 °C to a OD₆₀₀ of 0.7–0.9. The culture was cooled to room temperature, protein expression was induced by addition of IPTG (1 mM) and the culture was incubated for 22 h at 17 °C. The culture was centrifuged, the supernatant discarded and the cells resuspended in sodium phosphate buffer (100 mL, 50 mM pH 7.0). The suspension was centrifuged, the supernatant discarded and resuspended in lysis buffer (10 mL, for non-His-tagged proteins: 50 mM Tris HCl-buffer pH 7.5 and for His-tagged-proteins: 50 mM sodium phosphate buffer pH 7.5 with 10 mM imidazole). The cells were lysed by sonication, centrifuged to remove cell debris and the soluble protein purified from the supernatant. *For non-His tagged protein (C81L, V106F, L121Y, F125T, Y133F, L146M, V106F-L125T, V106F-L146M)*: Enzyme solution (5 mL) was added to anion exchange resin (4 mL, Macro-Prep High Q anion exchange from BioRad, Hercules, Ca (USA)) and eluted with 6 column volumes of TrisHCl-buffer (50 mM, pH 7.5) followed by 6 column volumes each of 90 mM, 110 mM and 130 mM NaCl in the same buffer. The target protein usually eluted within the 110 mM NaCl-fraction. The protein was further purified by gel filtration through two XK–16 GE columns in series (length: 300 mm each, 150 mL total volume, resin: Sephadex G-75), eluted with 50 mM TrisHCl-buffer, pH 7.5, 300 mM NaCl, and concentrated with a centrifuge concentrator tube, and washed twice with Tris HCl-buffer (30 mL, 50 mM, pH 7.5) to remove the sodium chloride. *For His-tagged protein (all others)*: Enzyme solution (5 mL) was added to an Ni-affinity-column (Ni-NTA Agarose from Qiagen, Hilden, Germany, 2.5 mL resin volume) and eluted with sodium phosphate buffer (10 mL, 50 mM, pH 7.5) containing increasing concentrations of imidazole (20 mM, 30 mM, 50 mM, 70 mM, 125 mM and 160 mM). The target protein eluted at 125 mM imidazole, and was concentrated with a centrifuge concentrator tube, and washed twice with Tris HCl-buffer (30 mL, 50 mM, pH 7.5) to remove the imidazole. In a separate experiment, imidazole (100 mM, pH 5.0) did not catalyze cleavage of mandelonitrile. The enzyme activity of His-tagged and non-His-tagged variants of HbHNL showed no significant differences.

Enzyme assays

Hydroxynitrile lyase and nitroaldolase activity were measured spectrophotometrically at 280 nm by monitoring the release of benzaldehyde ($\epsilon_{280\text{ nm}} = 1352\text{ L}/(\text{mol}\cdot\text{cm})$) from *rac*-mandelonitrile or *rac*-2-nitro-1-phenylethanol, respectively. *rac*-2-Nitro-1-phenylethanol was synthesized according to a literature method.^[25] Assay conditions: (190-x) μL 50 mM citrate buffer pH 5.0, x μL 100 mM stock solution of *rac*-mandelonitrile (HNL) or *rac*-2-nitro-1-phenylethanol (nitroaldolase) in 10 mM citric acid to suppress spontaneous cleavage and 10 μL enzyme sample (200 μL total volume within a microtiter plate; path length of 0.6 cm). If required, up to 50% acetonitrile can be added to the substrate stock solution to dissolve the substrate. All rates were measured in triplicate and the spontaneous reaction

(also in triplicate) was subtracted. One unit of enzyme activity is defined as the formation of 1 μmol benzaldehyde per minute. Steady state kinetic parameters were obtained by fitting the rates of reaction at different substrate concentrations to the Michaelis-Menten equation using Origin 6.0 (OriginLab, Northhampton, MA, USA).

Cyanohydrin synthesis assay

A two-phase 1:1 mixture (total volume 2.5 mL) of *tert*-butyl-methyl ether and citrate buffer (50 mM, pH 4.5) contained 25 mM benzaldehyde in the organic phase, and 250 mM hydrogen cyanide and 30 U/mL (37.5 U) enzyme in the aqueous phase. The reaction progress and enantiomeric excess of the product cyanohydrin was measured by HPLC equipped with a Chiralcel OD-H column and a diode array detector (210 nm) and eluted with 90% *n*-hexane / 10% isopropanol.^[13]

Protein unfolding in urea

All experiments followed procedures suggested by Pace *et al.*^[26] Within a black microtiter plate, protein solutions (200 μL , 0.2 mg·mL⁻¹ with 0–5 M urea in 5 mM BES buffer pH 7.5) were incubated for 16 h at 4 °C. Fluorescence was measured for triplicate samples by excitation at 278 nm and measurement of emission at 329 nm (further details are given in the supporting information).

Shape of substrate-binding site

Starting from the x-ray crystal structure of wt-*HbHNL* containing mandelonitrile (PDB-ID: 1YB6^[27]), the structure was prepared for modeling by the Protein Wizard function in Maestro (Schrodinger). This function added hydrogen atoms to the x-ray data, adjusted the protonation state to pH 7.0, optimized the geometry of first the heavy atoms, then the entire structure to a convergence threshold of 1.0 kcal/mol/Å with a maximum of 2000 iterations. The OPLS (Optimized Potential for Liquid Simulations) 2005 force field^[28] was used for geometry optimization. The binding site was defined using the software SiteMap v 2.9 (Schrodinger),^[29] which mapped the space accessible to a sphere with a 1.6 Å radius (size of a water molecule). A model of the L121Y-*HbHNL* variant was created by replacement of Leu121 with Tyr and geometry optimization as above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the US National Science Foundation (CBET-0932762, CHE-1152804), US National Institutes of Health (1R01GM102205-01), the 'Postdoc-Program of the German Academic Exchange Service' (DAAD – Deutscher Akademischer Austauschdienst) (fellowship for JvL) for financial support, the Minnesota Supercomputing Institute for use of computers and software, and Prof. Dr. Helmut Schwab (Technische Universität Graz, Austria) for the plasmid for expressing HbHNL, Prof. Antony Dean (U. of Minnesota) for useful discussions, Bryan J. Jones (U. of Minnesota) for the help with protein unfolding experiments in urea and Titu Devamani (U. of Minnesota) for the determination of esterase activity.

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copy features from

Figure 1.

One protein engineering strategy is to copy features from a homolog. For example, copying features (circle) from a thermostable enzyme (pentagon) into an unstable enzyme (square) may stabilize it. The advantage is that features from a homolog are less likely than random changes to disrupt protein folding and other properties in the target enzyme. The difficulty of this approach is identifying which amino acids should be copied to transfer properties.

Figure 2.

Superposition of the x-ray structures of *HbHNL* (red) and *SABP2* (green) show significant similarities between both proteins. The main chains, shown as ribbons, overlay within a rmsd of 1.3 Å, top structure. The enlargement below shows the side chains of residues within 4 Å of the bound mandelonitrile (red sticks) or salicylic acid (green sticks). Yellow sticks indicate the catalytic residues, blue indicates residues identical in both proteins, and orange or gray indicate the residues that differ in *HbHNL* and *SABP2* and may contribute to substrate binding. The orange sticks mark the best two substitutions that increased the

reaction rate of *HbHNL* toward mandelonitrile. For clarity, Leu157 (identical in both enzymes) is not shown.

Figure 3.

HbHNL variants with larger hydrophobic amino acids (Phe, Tyr) at position 121 catalyzed the cleavage of mandelonitrile faster than wild-type, which has leucine at this position. The line, added only a guide to the eye, suggests that there is an optimum size for this residue. The valine variant was only 50% pure, so the true k_{cat} value lies higher as suggested by the arrow.

Figure 4.

Wild-type *HbHNL* A) has a larger substrate binding site (mesh) that allows nonproductive orientations of the substrate (mandelonitrile in green sticks), but a model of L121Y-*HbHNL* B) has a smaller binding site that favors a productive orientation. The catalytic residues (Thr11, Ser80 and Lys236) are shown as yellow sticks and Leu121/Tyr121 is shown as red sticks. The mandelonitrile and side chain orientations were optimized by using the OPLS force field.

Scheme 1.

Both SAPB2 esterase (top) and hydroxynitrile lyase (bottom) contain a catalytic triad of Ser, His and Asp (Asp not shown for clarity), but the reactions and key steps of the catalytic mechanisms differ significantly. In the esterase, the substrate carbonyl binds in an oxyanion hole (shown as semicircle for clarity) and the catalytic serine acts as a nucleophile. The reaction involves an acyl enzyme intermediate. For the hydroxynitrile lyase, the oxyanion hole is blocked so the carbonyl orients differently. The serine donates a hydrogen bond and the reaction occurs in one step without intermediates.

Table 1

Natural substrates and amino acid residues in the active sites of SABP2, *HbHNL* and *MeHNL*.^a

Protein	natural substrate	11 c	12	14	80 c	81	106	121	125	128	133	146	148	152	157	209	210	235 c	236 c
SABP2	methyl salicylate	G	A	H	S	L	F	Y	T	W	F	M	F	F	L	G (A ^b)	I	H	M
<i>HbHNL</i>	acetone cyanohydrin	T	I	H	S	C	V	L	F	W	Y	L	L	L	L	I	F	H	K
<i>MeHNL</i>	acetone cyanohydrin	T	I	H	S	C	L	L	F	W	Y	M	L	L	L	I	F	H	K

^a Amino acids numbering according to *HbHNL*. A 'c' indicates catalytic residues in either the esterase or the hydroxynitrile lyases. The residues in bold are hypothesized to create the substrate binding site.

^b A similar SABP2-esterase from *Nicotiana benthamiana* (95% sequence identity with SABP2 from *N. tabacum*) has an alanine instead of glycine at this position. All other active site residues were identical.

Table 2HNL-activity of selected *HbHNL*-variants^[a]

mutation	k_{cat} [s ⁻¹]	K_{M} [mM]	$k_{\text{cat}}/K_{\text{M}}$ [s ⁻¹ ·mM ⁻¹]	spec. activity [U·mg ⁻¹]
wild type	25	3.3	7.4	33
I12A ^[b]	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	0.9
C81L	33	9.4	3.5	22
V106F	7	5.3	1.4	6.7
L121Y	106	2.7	39	149
F125T	66	2.0	33	92
Y133F	22	3.1	7.1	28
L146M	20	3.7	5.4	24
L148F	17	8.6	1.0	7.0
L152F	7	0.6	11	12
I209G ^[b]	3	0.7	4.0	5.1
I209A	23	0.8	29	42
F210I ^[b]	21	8.6	2.5	14
V106F-L121Y	55	1.4	39	88
L121Y-F125T	43	3.1	14	55
L121Y-L146M ^[c]	<i>n.d.</i>	6.7	<i>n.d.</i>	115
F125T-Y133F	32	2.1	15	52
F125T-L146M	32	1.5	21	53
V106F-L121Y-F125T	82	2.0	41	140
L121Y-F125T-L146M	45	3.6	12	64

^[a] All values are based on the cleavage of racemic mandelonitrile;^[b] significant lower protein yield in comparison to other variants (large amounts of protein were found within the insoluble fraction);^[c] moderate substrate inhibition;

n.d. – not determined; improvements are highlighted in bold

Table 3The effect of substitutions at position 121 within *HbHNL* on HNL-activity^[a]

Mutation	k_{cat} [s ⁻¹]	K_{M} [mM]	$k_{\text{cat}}/K_{\text{M}}$ [s ⁻¹ ·mM ⁻¹]	spec. activity [U·mg ⁻¹]
Amino acids with electrically charged side chains				
L121D	no soluble protein			
L121E ^[b]	n.d.	n.d.	n.d.	0.2
L121H	5	8.0	0.6	4.0
L121K	no soluble protein			
L121R	no soluble protein			
Amino acids with polar uncharged side chains				
L121N ^[b]	n.d.	n.d.	n.d.	0.7
L121Q	6	5.7	1.1	5.9
L121S	9	6.6	1.4	3.8
L121T	3	2.7	1.4	2.0
Special cases				
L121C	20	8.1	2.5	16
L121G	> 2.5	> 15	< 0.5	1.4
L121P ^[b]	n.d.	n.d.	n.d.	0.1
Amino acids with hydrophobic side chains (ordered by size) ^[19]				
L121A	23	2.8	8.3	30
L121V ^[c]	15	3.7	4.0	16
L121 (wt)	25	3.3	7.4	33
L121I	33	4.9	6.6	35
L121M	43	4.0	11	52
L121F	77	1.5	51	119
L121Y	106	2.7	39	149
L121W	15	5.6	2.6	14

^[a] Values are based on the cleavage of racemic mandelonitrile;^[b] significant lower protein yield in comparison to other variants;^[c] ca. 50% protein impurities (based on SDS-PAGE) – k_{cat} , $k_{\text{cat}}/K_{\text{M}}$ and spec. activity are approximately 100% higher;

n.d. – not determined; bold highlights the improvements; wt – wild type.

Table 4Nitroaldolase-activity of selected *HbHNL*-variants^[a]

mutation	k_{cat} [s ⁻¹]	K_{M} [mM]	spec. activity [U·mg ⁻¹]
wild type	0.15	6.1	0.13
C81L	> 0.2	> 5	0.15
V106F	0.1	1.4	0.17
L121Y	> 0.5	> 5	0.62
F125T	0.6	> 5	0.27
Y133F	0.3	7.1	0.29
L146M	> 0.5	> 5	0.32
V106F-L121Y	< 0.1	1.6	< 0.1
V106F-F125T	0.2	1.8	0.39
V106F-L146M	> 0.2	> 5	0.21
L121Y-L146M	> 0.1	> 5	0.13
F125T-Y133F	0.12	0.7	0.21
F125T-L146M	0.1	0.4	0.20
L121Y-F125T-Y133F	> 0.5	> 5	0.56
L121Y-F125T-L146M	> 0.5	> 5	0.71
V106F-L121Y-F125T	> 0.5	> 5	0.29

^[a] All values are based on the cleavage of racemic 2-nitro-1-phenylethanol;

n.d. – not determined