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Switching from an Esterase to a Hydroxynitrile Lyase Mechanism Requires Only Two Amino Acid Substitutions

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SUMMARY

The α/β hydrolase superfamily contains mainly esterases, which catalyze hydrolysis, but also includes hydroxynitrile lyases, which catalyze addition of cyanide to aldehydes, a carbon-carbon bond formation. Here, we convert a plant esterase, SABP2, into a hydroxynitrile lyase using just two amino acid substitutions. Variant SABP2-G12T-M239K lost the ability to catalyze ester hydrolysis (<0.9 mU/mg) and gained the ability to catalyze the release of cyanide from mandelonitrile (20 mU/mg, k_{cat}/K_M = 70 $min^{-1}M^{-1}$). This variant also catalyzed the reverse reaction, formation of mandelonitrile with low enantioselectivity: 20% ee (*S*), *E* = 1.5. The specificity constant for the lysis of mandelontrile is 13,000-fold faster than the uncatalyzed reaction and only 1300-fold less efficient (k_{cat}/K_M) than hydroxynitrile lyase from rubber tree.

INTRODUCTION

Superfamilies are groups of enzymes created by divergent evolution and related by both similar protein folds and a common mechanistic strategy for lowering the free energies of the rate-limiting transition states in the reactions they catalyze (Babbitt and Gerlt, 1997; Gerlt and Babbitt, 2001; Glasner et al., 2006; Poelarends et al., 2008). The α/β-hydrolase superfamily contains enzymes with the α/β-hydrolase fold (Ollis et al., 1992; Holmquist, 2000) that catalyze nucleophilic additions to carbonyl compounds. Most α/β-hydrolases are indeed hydrolases (E.C. 3 enzyme classification) and use a nucleophile-histidine-aspartate catalytic triad together with an oxyanion hole for catalysis (Figure 1A). The nucleophile is serine for the esterases and lipase, aspartate for the epoxide hydrolase and dehalogenases, and cysteine for dienelactone hydrolase.

Many hydroxynitrile lyases (E.C. 4.1.2.11 and 4.1.2.37) also belong to the α/β-hydrolase superfamily, but their catalytic mechanism differs from that for esterase in three important ways (Figure 1B) (Gruber et al., 2004). First, the hydroxynitrile lyase reaction does not involve an acyl enzyme intermediate or another covalent complex with the enzyme. It has a single transition state, not the two for esterases (one for formation of the acyl enzyme, the other for release of acyl enzyme). Second, the role of serine differs. Instead of being a nucleophile, it is a hydrogen bond donor. The serine interacts not with the carbonyl carbon, but with the carbonyl oxygen because a threonine in hydroxynitrile lyase blocks the oxyanion hole forcing the aldehyde carbonyl to position differently in the active site. Third, hydroxynitrile lyases do not use hydrogen bonds from the oxyanion hole to activate the carbonyl group of the substrate.

In spite of these major mechanistic changes, X-ray crystal structures show that the active sites of esterases and hydroxynitrile lyases share the catalytic triad of serine, histidine, and aspartate and share an oxyanion hole. The difference is that hydroxynitrile lyases contain a threonine residue whose side chain partially blocks the oxyanion hole and a lysine residue whose side chain hydrogen bonds to cyanide. We hypothesized that one could convert an esterase into a hydroxynitrile lyase and make major mechanistic changes in catalysis with only two amino acid substitutions.

RESULTS

Structure and Sequence Alignment of Esterases with Hydroxynitrile Lyases

The three-dimensional structures of hydroxynitrile from *Hevea brasiliensis* (rubber tree; *Hb*HNL, pdb code 1YB6) (Wagner et al., 1996) and from *Manihot esculenta* (cassava; pdb code 1EB9) (Lauble et al., 2002) are highly similar to each other. The structures superimpose with a root-mean-square deviation between 256 equivalent α-carbon atoms of only 0.4 Å. The z-score of this structure alignment using DALI (Holm and Park, 2000) is also very high at 46.8. Values above 3 indicate some structural similarity.

The structures of several esterases are also similar to these hydroxynitrile lyases. Salicylic acid binding protein 2 from *Nicotiana tabacum* (tobacco; SABP2) is an esterase involved in plant defense signaling. Its structure (pdb code 1Y7I) (Forouhar et al., 2005) superimposed with the structure of *Hb*HNL aligned

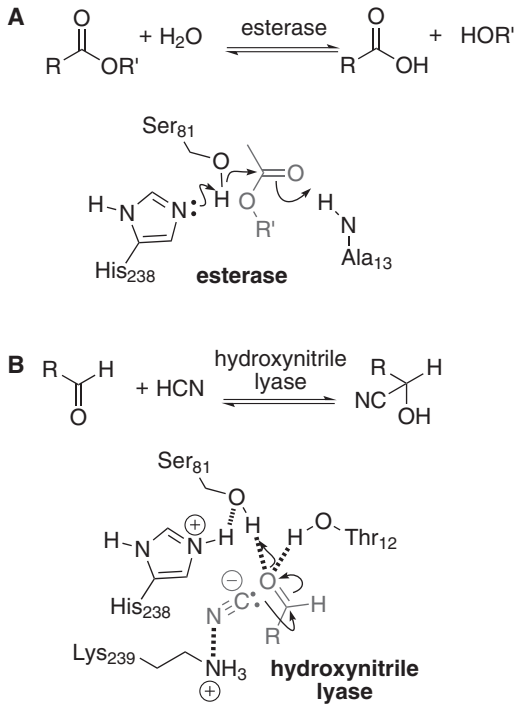

Figure 1. Hydroxynitrile Lyase Mechanism Differs Significantly from that for Ester Hydrolysis

(A) The first step of ester hydrolysis involves nucleophilic attack of the active site serine at the carbonyl carbon. Binding of the carbonyl oxygen in the oxyanion hole (two main chain N-H; only one is shown for clarity) positions the ester group.

(B) Hydroxynitrile lyase catalysis involves a single step without formation of an acyl enzyme intermediate. The orientation of aldehyde carbonyl group differs because the threonine side chain blocks the oxyanion hole. The active site serine donates a hydrogen bond to the oxygen of the carbonyl. (Table S1 summarizes molecular modeling that predicts mutations needed to switch from an esterase to a hydroxynitrile mechanism.)

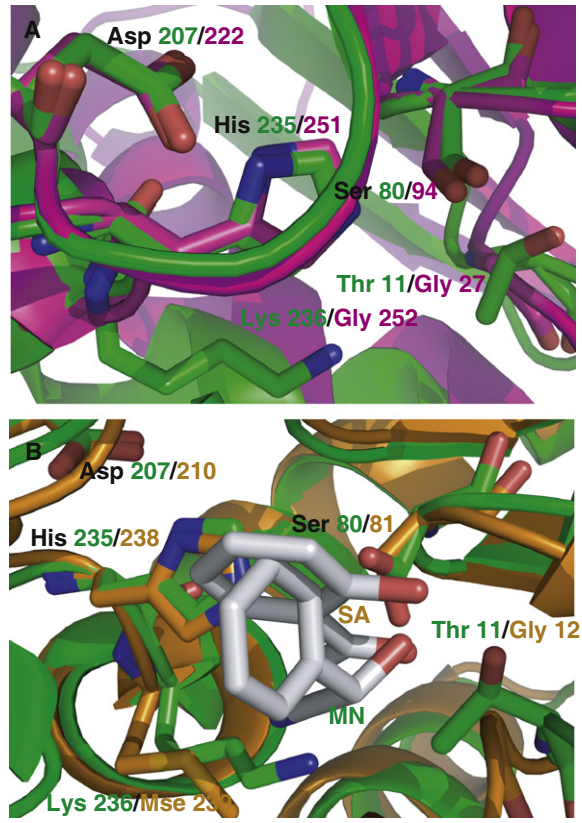

Figure 2. Superposition of the Active Sites of *Hb*HNL with Esterases PFE and SABP2

(A) The side chains of three active site residues (Ser, His, Asp) of HbHNL (green) and esterase PFE (magneta) superpose well, but the esterase lacks the catalytically essential Thr and Lys of the hydroxynitrile lyase. Adding these residues requires the following two substitutions: Gly27Thr and Gly252Lys.

(B) The side chains of three active site residues of HbHNL (green) and esterase SABP2 (gold) also superpose well, but the esterase requires the following two substitutions to include the catalytically essential residues of the hydroxynitrile lyase: Gly12Thr, Met239Lys. The substrates mandelonitrile (MN) and product salicylic acid (SA) in gray align approximately. The reacting carbonyl groups are both deep in the active site but have different orientations as expected from the reaction mechanism. The substrate phenyl groups lie toward the surface with different orientations. (Figure S1 shows additional structure comparisons.)

length of 256 and with a root-mean-square deviation between equivalent α-carbon atoms of 1.3 Å and gave a z-score of 41.7. The structure of aryl esterase from *Pseudomonas fluorescens* (PFE; pdb code 1VA4) (Cheeseman et al., 2004) is also similar to the structure of *Hb*HNL with aligned length of 220, but to a lesser degree: 2.9 Å rms deviation and a z-score of 21.6.

The amino acid sequences of these structurally related enzymes change more rapidly than structures during evolution so an amino acid sequence comparison reveals more differences. In spite of the highly similar structures, the two hydroxynitrile lyases share only 77% identical amino acid residues, while SABP2 shares 45% identical with *Hb*HNL and 40% identical with *Me*HNL. The microbial esterase PFE shares only 17% identical amino acid residues with *Hb*HNL and 16% identical with *Me*HNL. A structure-based alignment of the amino acid sequences using VAST (Madej et al., 1995; Gibrat et al., 1996) (see Figure S1 available online) reveals only 19 residues identical in all four sequences (red), including the catalytic triad of Ser, Asp, and His (cyan highlight). As expected, the two esterases lack the threonine and lysine residues that are catalytically important for hydroxynitrile lyases (green highlight). The corresponding residues in PFE are two glycines and in SABP2 they are glycine and methionine.

Superposition of the active site of *Hb*HNL with PFE (Figure 2A) and SABP2 (Figure 2B) show a close alignment of both the main chain and the side chains. The side chains of the catalytic triad (Asp, His, and Ser) superpose closely. The esterases lack the catalytic threonine and lysine of *Hb*HNL, but main chains of the corresponding residues align well. For selenomethionine 239 of SABP2, the side-chain orientation is similar to the side chain in Lys236 (Figure 2B). The remaining three corresponding residues, Gly27 and Gly252 of PFE and Gly12 of SABP2, have no side chain, so no conclusion can be made about side-chain orientation. The X-ray structures also show the substrates of *Hb*HNL and SABP2 bound in similar regions of the active site. The reacting groups (hydroxyl of mandelonitrile or acid carbonyl of salicylic acid) are both deep in the active site. The orientations differ as required by the mechanisms as discussed above. The phenyl

groups are in the region that corresponds to the acyl binding site of the esterase, but have different orientations. The structure of *Hb*HNL also shows a salt bridge between the catalytic Lys236 and Glu79 (not shown in Figure 2 for clarity). The residue corresponding to Glu79 in SABP2 is His80 and in PFE is Phe93.

Computer Modeling of Predicted Variants

To predict whether amino acid substitutions would enable hydroxynitrile lyase activity, we used computer modeling. The computer modeling predicted first whether the substrate hydroxynitrile would bind to the new active site, second, whether the catalytically essential hydrogen bonds would form, and third, whether the folded protein was stable. This modeling identified problems with the double and triple substitution mutants of PFE and with the loop mutant of SABP2 (see below) but suggests that the double and triple substitution mutants of SABP2 may show hydroxynitrile lyase activity.

The binding energies of hydroxynitrile to the enzyme suggest that the PFE variants will not bind the substrate lactonitrile, but the SABP2 variants would bind mandelonitrile (acetate esters and methyl salicylate are the preferred substrates for PFE and SABP2, respectively; hence the closest cyanohydrin analogs were modeled). Interaction energies were calculated using combined quantum mechanics/molecular mechanics (QM/MM). The interaction energy is given by $E_{interaction} = E_{complex} - E_{ligand} - E_{protein}$. The energy of the complex was modeled using molecular mechanics for the protein and quantum mechanics for the ligand. This approach yielded an interaction energy of −124 kcal/mol for the *Hb*HNL-(*S*)-mandelonitrile complex (Table S1). The PFE variant-lactonitrile complexes had interaction energies of only −55 to −67 kcal/mol. This less negative value indicates much weaker, if any, binding. In contrast the SABP2 variant-mandelonitrile complexes showed large interaction energies (−184 to −193 kcal/mol), suggesting that the new substrates will bind well. The SABP2 loop mutant showed much lower interaction energies (−82 kcal/mol for [*S*], −116 for [*R*]), so this variant may not bind substrate. This loop mutant contains five additional substitutions besides the threonine and lysine required for catalysis (SABP2 G12T-A13I-H80E-L82C-M239K-A240L-M241Q). These substitutions lie in the loops that hold the catalytic residues may orient them more precisely.

The hydroxynitrile-enzyme complexes formed the catalytically essential hydrogen bonds in most cases, suggesting possible catalytic activity (Supplemental Experimental Procedures and Table S1). The hydrogen bonds were measured after geometry optimization of the complexes using molecular mechanics. The PFE G27T-G252K variant complexed with (*S*)- and (*R*)-lactonitrile lacked two hydrogen bonds, the Thr-OH and Ser-His hydrogen bonds. The PFE G27T-F93E-G252K variant complexed with (*R*)-lactonitrile also lacked two hydrogen bonds, but the complex with (*S*)-lactonitrile contained all key hydrogen bonds, suggesting possible catalytic activity. According to the experimental design, we modeled the complexes with (*R*)- and with (*S*)-mandelonitrile (Supplemental Experimental Procedures and Figure S1) which contained all key hydrogen bonds. The SABP2 G12T-M239K variant complexed with (*S*)-mandelonitrile contained all catalytically essential hydrogen bonds (Table S1 and Figure S1), while the complex with (*R*)-mandelonitrile lacked one hydrogen bond.

Since the PFE G27T-G252K mutant both showed a poor interaction energy and lacked two catalytically essential hydrogen bonds, it is unlikely to show hydroxynitrile lyase activity. The SABP2 G12T-M239K and G12T-H80E-M239K mutants showed more negative interaction energies that suggest better binding of mandelonitrile than even wild-type HNL. The SABP2 G12T-M239K variant formed all catalytically essential bonds (Table S1 and Figure S1); thus, the calculations predict catalytic activity. Hydrogen bond calculations were not completed for the triple mutant or for the loop mutant since we met with protein expression problems, see below.

To check if the variant proteins would fold properly, we calculated the free energy of unfolding for the different variants using the FoldX force field. (Figure S2) (Guerois et al., 2002). This calculation predicted that all the variant proteins were less likely to fold than wild-type. In general, the variants with larger numbers of amino acid substitutions were most destabilized, while those with fewer substitutions were less destabilized. These predictions correlated approximately with the amount of soluble protein isolated for the variants (Supplemental Experimental Procedures). FoldX predicted that the triple substitution mutants of PFE and SABP2 as well as the loop mutant of SABP2 were the least stable. In agreement with this prediction, our experiments below did not yield enough soluble protein to measure activity.

Preparation of Variants

Site-directed mutagenesis on the PFE or SABP2 gene yielded the double and triple mutants, while gene synthesis yielded the SABP2 loop mutant (Supplemental Experimental Procedures). The PFE and SABP2 variants were overexpressed in *Escherichia coli*. The wild-type proteins and SABP2-G12T gave good expression of soluble protein (≥10 mg protein/l), but the other variants expressed mainly as insoluble inclusion bodies (Figure S2). Although these inclusion bodies could be refolded to yield soluble proteins, none of these refolded proteins showed catalytic activity. Refolded wild-type PFE or SABP2 showed <5% of their original esterase activity indicating that the refolding procedure, while yielding soluble protein, yielded a catalytically inactive conformation. Hence refolded protein (except the loop mutant) was not used rather only soluble catalytically active protein was used to measure both esterase and HNL activity. We optimized the protein expression conditions to maximize the amount of protein expressed in soluble form. Typical expression conditions were 17°C for 24 hr using 0.5% (w/v) rhamnose (PFE variants) or 1 mM isopropyl β-D-1-thiogalactopyranoside (SABP2 variants). The proteins were purified using Ni-agarose affinity chromatography, which yielded pure protein for the PFE variants, but the SABP2 variants required an additional gel-filtration chromatography to remove higher molecular weight contaminants (Figure S2). All single and double amino acid substitution variants could be isolated in soluble form, albeit in very low yield for the double ones (~1 mg/l broth). The triple amino acid substitution variant PFE-G27T-F93E-G252K (0.1 mg/l broth) and SABP2 G12T-H80E-M239K (0.17 mg/l broth) formed in even lower yields. The SABP2 loop mutant formed only inclusion bodies. Hence the HNL activity of these three proteins was not measured. Protein refolding was not successful in the production of catalytically active soluble protein, which is likely due to incorrectly folded protein.

Table 1. Esterase and HNL Activity of PFE, SABP2, and Selected Variants

Enzymes	Esterase Activity[a] (mU/mg)	Fold Decreased Esterase Activity	HNL Activity[b] (mU/mg)	HNL/Esterase Activity
*Me*HNL[c]	0.31 ± 0.07	na	2310 ± 50	7500
PFE	15,800 ± 980	1 (PFE)	<0.7[d]	<0.00004
PFE- S94A	0.9	17,500 (PFE)	nd	na
PFE-G27T	75	200 (PFE)	<0.7[d]	<0.009
PFE- F93E	580	27 (PFE)	<0.7[d]	<0.001
PFE- G252K	1940	8 (PFE)	<0.7[d]	<0.0004
PFE-G27T-G252K	67.4 ± 1.6	235 (PFE)	<0.7[d]	<0.010
PFE-G27T-F93E-G252K	2510	6 (PFE)	np	na
SABP2	460 ± 30	1 (SABP2)	<0.7[d]	<0.0015
SABP2-G12T	0.24	2000 (SABP2)	<0.7[d]	<0.3
SABP2- M239K	8.9	53 (SABP2)	<0.7[d]	<0.08
SABP2- G12T-M239K	0.9 ± 0.1	550 (SABP2)	20[e]	22
SABP2- G12T-H80E-M239K	3.4	140 (SABP2)	np	na
Loop mutant[f]	0.4	1200 (SABP2)	np	na

See also Figure S2. One unit of enzyme activity corresponds to formation on one μmol of product per minute. The values in this table are in mU and thus correspond to formation of one nmol of product per minute. na = not applicable, nd = not determined, np = not enough protein available for testing.
[a] *p*-Nitrophenyl acetate hydrolysis (pNPAc).
[b] Release of benzaldehyde from racemic mandelonitrile.
[c] (*S*)-selective hydroxynitrile lyase from *Manihot esculenta* (71811) from Fluka.
[d] Below detection limit of 0.7 mU/mg.
[e] Depending on purity three other different batches gave 7, 12 and 64 mU/mg, but the 20 mU/mg protein was used for kinetics experiments. The Vmax value for this sample was higher 31 U/mg because this assay condition used 15 mM mandelontrile, which does not saturate this enzyme (K_M = 13 mM).
[f] Refolded protein that is likely in a non native conformation, all other proteins in the table are soluble proteins in native conformation.

Esterase Activity

Esterase activity was measured using *p*-nitrophenyl acetate (pNPAc). The natural substrate of PFE is unknown, but aryl acetates like pNPAc are among the best substrates (Liu et al., 2001). The natural substrate of SABP2 is methyl salicylate, but strong product inhibition by salicylic acid (K_d of 90 nM) (Forouhar et al., 2005; Du and Klessig, 1997) complicates measurements with this substrate. The activity of SABP2 is also 2.7-fold higher with pNPAc as the substrate (480 mU/mg) than with methyl salicylate (180 mU/mg).

As expected, all the variants of PFE and SABP2 engineered to increase hydroxynitrile lyase activity showed decreased esterase activity (Table 1). The true hydroxynitrile lyase, *Me*HNL, showed only 0.3 mU/mg esterase activity. In comparison, the PFE S94A variant where the active site serine is replaced by alanine, showed a specific activity of 0.9 mU/mg. The single amino acid substitution that caused the largest decrease in esterase activity is the glycine to threonine replacement on the oxyanion loop. The PFE-G27T variant showed 200-fold less esterase activity than the wild-type PFE and the SABP2-G12T variant showed 2000-fold less. The most likely explanation is that the side chain of the threonine blocks access to the oxyanion hole (Figure S3). This blocking is essential to the catalytic mechanism of the hydroxynitrile lyase since the aldehyde carbonyl oxygen should not orient in the oxyanion hole.

The other single amino acid substitutions also decreased esterase activity, but to a smaller extent. PFE-F93E and PFE-G252K showed ~27 and ~8-fold decreases in esterase activity, respectively, as compared with wild-type PFE. SABP2-M239K showed ~53-fold decrease in esterase activity as compared with wild-type SABP2. The decreased activity might be due to the introduction of a charged group in the hydrophobic active site.

Combinations of the substitutions did not behave in an additive fashion on esterase activity. Additivity requires that each substitution act independently, but these substitutions are near each other with the side chains pointing toward each other, so interaction between them are expected. The PFE-G27T-G252K showed ~235-fold lower esterase activity, while an additive effect would predict 200 × 8 or a 1600-fold lower activity. The lack of additivity was more dramatic for the triple mutant PFE-G27T-F93E-G252K, which was only 6-fold less active than the wild-type. Additivity of the single substitutions would predict this variant to be 200 × 27 × 8 or 43,000-fold less active. The cancellation of the charges by placing a glutamate and a lysine residue near one another may account for some of the nonadditive behavior. SABP2-G12T-M239K showed ~550-fold lower esterase activity while additivity predicts 106,000-fold and SABP2-G12T-H80E-M239K showed 140-fold less. The single substitution variant SABP2-H80E was not available for testing, so an additivity prediction is not possible for this variant.

Steady-state kinetic measurements for hydrolysis of pNPAc show a 4000-fold drop in catalytic efficiency (k_{cat}/K_M) for SABP2-G12T as compared with wild-type (Table 2; Figure S3). This drop is consistent with the notion that the threonine side chain blocks the oxyanion hole. In 7% acetonitrile, the limited solubility of pNPAc prevented measurement of k_{cat} and K_M separately. In 25% acetonitrile, the drop in catalytic efficiency of SABP2-G12T was only 6.5-fold as compared with wild-type.

Table 2. Steady-State Kinetic Constants for Esterase Activity of Wild-Type SABP2 and SABP2-G12T

Enzyme (Reaction Conditions)	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
SABP2 (7% MeCN)	15	0.32	45
SABP2-G12T (7% MeCN)	Nd[a]	>2[b]	0.011[c] (4000-fold decrease)
SABP2 (25% MeCN)	5.3	4.0	1.3
SABP2-G12T (25% MeCN)	1.7	8.7	0.20 (6.5-fold decrease)

See also Figure S3. Acetonitrile was added to increase the solubility of the substrate *p*-nitrophenyl acetate (pNPAc) whose hydrolysis was detected spectrophotometrically at 404 nm. For comparison the kinetic parameters for wild type SABP2 with methyl salicylate in 10 vol% ethanol: k_{cat}: 0.45 s^{-1}, K_M: 8.6 μM, k_{cat}/K_M: 3140 min^{-1} mM^{-1}.[8]

[a] nd = not determined.

[b] The solubility limit for pNPAc of ~1.8 mM prevented reaching concentrations high enough to saturate the enzyme.

[c] k_{cat}/K_M was measured from the slope of the reaction rate with increasing substrate concentration at low substrate concentration.

Table 3. Competitive Inhibition Constants of Benzaldehyde and Salicylic Acid toward SABP2 and Two Variants

	K_i (Benzaldehyde), μM	K_i (Salicylic Acid), μM
SABP2	25,000	0.090[a]
SABP2-G12T	78	160
SABP2-G12T-M239K	160	13,000

See also Figure S4. The reaction was the SABP2-catalyzed hydrolysis of *p*-nitrophenyl acetate.

[a] From Du and Klessig (1997).

Both a decrease in k_{cat} and an increase in K_M for SABP2-G12T contributed to the lower catalytic efficiency. The change from 7% to 25% acetonitrile also perturbed the enzyme since the wild-type catalytic efficiency decreased 35-fold from 45 to 1.3 min^{-1} mM^{-1}.

Substrate Binding

The amino acid substitutions also changed substrate binding as expected for new function (Table 3). We measured the competitive inhibition of *p*-nitrophenyl acetate hydrolysis by benzaldehyde and by salicylic acid. Benzaldehyde was a poor inhibitor of the wild-type SABP2, K_i = 25,000 μM, but was a 160- to 320-fold better inhibitor of both SABP2-G12T (78 μM, Figure S4) and SABP2-G12T-M239K (160 μM). The competitive behavior of this inhibition indicates that benzaldehyde binds at the same site as *p*-nitrophenyl acetate and the low value for the inhibition constant indicates strong binding. As the binding of benzaldehyde became tighter, the binding of salicylic acid became weaker. The apparent dissociation constant of salicylic acid from wild-type SABP2 is 0.090 μM (Du and Klessig, 1997). In SABP2-G12T, the competitive inhibition constant for salicylic acid becomes 1800-fold weaker (K_i = 160 μM) and in SABP2-G12T-M239K, the competitive inhibition constant for salicylic acid becomes 140,000-fold weaker, 13,000 μM (Figure S4). This better binding of benzaldehyde and poorer binding of salicylic acid is consistent with the notion that the G12T substitution disrupts the oxyanion hole as it does in the hydroxynitrile lyase. The poorer binding of the salicylic acid to SABP2-G12T-M239K as compared with SABP2-G12T is likely due to the smaller substrate-binding site. The side chain of methionine contains four nonhydrogen atoms, while the side chain of lysine contains five nonhydrogen atoms.

Hydroxynitrile lyase Activity

The HNL activity assay detected benzaldehyde release from racemic mandelonitrile by the increase in absorbance at 280 nm. Spontaneous lysis of mandelontrile set the detection limit of the assay at 0.7 mU/mg under our experimental conditions. Commercial (*S*)-selective *Me*HNL showed ~2300 mU/mg of HNL activity (Table 1). Neither wild-type PFE nor SABP2 showed detectable HNL activity (<0.7 mU/mg), nor did the PFE variants G27T, G252K and G27T-G252K, nor SABP2 variants G12T and M239K and the loop mutant. SABP2-G12T-M239K is the only variant that showed HNL activity, ~20 mU/mg. Poor expression of soluble protein for the triple mutants PFE-G27T-F93E-G252K and SABP2-G12T-H80E-M239K did not yield enough protein for the HNL assay.

The ratio of hydroxynitrile lyase to esterase activity identifies an enzyme as an HNL or an esterase (Table 1). For *Me*HNL, this ratio is 7460, which is greater than one indicating it is a hydroxynitrile lyase. For PFE and SABP2, the ratio is 0.000044 and 0.0015, respectively, which is less than one indicating that they are esterases. For SABP2-G12T-M239K this ratio is 22 indicating that it is a hydroxynitrile lyase. The esterase activity of this variant decreased 550-fold as compared with wild-type, while the hydroxynitrile lyase activity increased >29-fold compared with wild-type. The combination of these two changes is a >16,000-fold shift in the catalytic activity caused by the two substitutions.

The sample of SABP2-G12T-M239K for measuring the steady-state kinetic parameters had a specific activity of 20 mU/mg. Other samples of this variant showed 7, 12, and 64 mU/mg of HNL activity because the protein purity differed (Figure S5) and because the variant could lose activity due to inactivation. The HNL activity in SABP2-G12T-M239K cannot come from an HNL contaminant for three reasons. First, SABP2-G12T-M239K and two HNLs show different K_Ms for mandelonitrile. Second, other SABP2 variants did not show HNL activity. A contaminant would be expected in several samples. Finally, the enantioselectivity of SABP2-G12T-M239K was low (E = 1.5) in contrast to the high enantioselectivity of the HNLs.

The catalytic efficiency for hydroxynitrile lyase activity of SABP2-G12T-M239K is modest compared with the natural enzymes (Table 4). The turnover number, k_{cat}, was 0.9 min^{-1}, which is ~120- and ~1470-fold lower than for *Hb*HNL (Bauer et al., 1999) and *Me*HNL (Yan et al., 2003), respectively. The spontaneous cleavage of mandelonitrile occurs with a first order rate constant of 7.0×10^{-5} min^{-1} under our experimental conditions, which is similar to a previously reported value (Willeman et al., 2000). The rate acceleration of the SABP2-G12T-M239K catalyzed reaction, k_{cat}/k_{uncat} is 13,000, which corresponds to 5.7 kcal/mol of stabilization of the transition state. The K_M value

Table 4. Steady-State Kinetic Constants for Hydroxynitrile Lyase Activity

Enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
*Hb*HNL[a]	110	1.2	93
*Me*HNL[a]	1300	1.4	950
SABP2-G12T-M239K	0.90	13	0.072

See also Figure S5. Release of benzaldehyde from racemic mandelonitrile at pH 5.5 detected spectrophotometically at 280 nm.
[a] Literature values Bauer et al. (1999) and Yan et al. (2003).

of 13 mM for mandelonitrile with SABP2-G12T-M239K is 170-fold higher than the 78 μM inhibition constant for benzaldehyde measured for SABP2-G12T. The double mutant is ~1300-fold less catalytically efficient (k_{cat}/K_M) than *Hb*HNL for HNL catalysis and ~13,000-fold less than *Me*HNL.

A comparison of SABP2-G12T-M239K as an engineered enzyme for carbon-carbon bond cleavage to a designed aldolase (Jiang et al., 2008) shows the SABP2-G12T-M239K has a 100-fold higher k_{cat} (0.9 min^{-1} versus 0.009 min^{-1} for the aldolase). For biocatalysis, k_{cat} is the most important kinetic constant because reactions typically take place at high substrate concentrations where k_{cat} determines the overall rate of reaction. Despite the low value of k_{cat} for the aldolase, the value of k_{cat}/k_{uncat} was similar (23,000) because the uncatalyzed rate of the aldol cleavage is 100-fold lower that the uncatalyzed rate of mandelonitrile cleavage. The k_{cat}/K_M for the designed aldolase was lower by only a factor of two (0.043 mM^{-1} min^{-1}) because it bound its substrate more tightly.

Enantioselectivity

SABP2-G12T-M239K also catalyzed the reverse reaction, synthesis of mandelonitrile from benzaldehyde and hydrogen cyanide, on an HPLC scale. Combining the enzyme (791 μg, 10 mU) in citrate buffer with benzaldehyde and HCN in di-isopropyl ether yielded ~1 μmol of mandelonitrile (~10% conversion) after 3 hr according to peak areas in the HPLC. The product showed only 20% ee (*S*), which corresponds to a poor enantioselectivity of E = 1.5. The HPLC column contained a chiral stationary phase that separated the enantiomers of mandelonitrile (Figure S5). Both peaks had nearly equal area indicating poor enantioselectivity. We confirmed the low enantioselectivity in the cleavage of mandelonitrile using (*R*)- and (*S*)-mandelonitrile separately. The specific activity for cleavage of (*R*)-, (*S*)-, and racemic mandelonitrile was 12.6, 15.6, and 20 mU/mg, respectively. The computer modeling above made contradictory predictions for the enantioselectivity of SABP2-G12T-M239K. The hydrogen bonds prediction suggested that it would favor (*S*)-mandelonitrile, but the similar interaction energies of both enantiomers suggests no enantioselectivity.

DISCUSSION

Only two amino acid substitutions can dramatically change the reaction type. Previous reports of altered catalytic mechanisms involved reactions with common mechanistic steps, but even these more modest changes often required extensive changes in the protein. For example, changing from hydrolysis of a thioester to hydrolysis of a β-lactam required insertion, deletion, and substitution of loops as well as amino acid substitutions (Park et al., 2006), changing from hydrolysis of an ester to an epoxide required several substitutions and a loop exchange (Jochens et al., 2009), and changing from a dehalogenase to a crotonase required eight amino acid substitutions (Xiang et al., 1999). A few examples also show a single amino acid substitution converted a racemase to an aldolase (Seebeck and Hilvert, 2003), a decarboxylase to a racemase (Terao et al., 2006), and restored the glutathione transferase activity in the yeast prior protein Ure2 (Zhang et al., 2008) and two site-directed mutations converted a sugar dehydratase to an aminotransferase (Cook et al., 2009). In the current work, there are no common mechanistic steps in the two reactions and even a different substrate orientation, but the change required only two amino acid substitutions.

Of the 18 examples of new catalytic activity in an enzyme (summary in Table S2), this k_{cat} value is in the middle of the group. The highest value was 50,000 min^{-1} for an oxidation of a sulfide to sulfoxide by a tungstate-substituted thermolysin. The value reported here is more than 5000-fold higher than the slowest reported successful switches of catalytic activity.The turnover number for HNL catalytic activity of SABP2-G12T-M239K is 0.9 min^{-1}, which corresponds to a rate acceleration of 13,000 over the uncatalyzed spontaneous reaction. This catalytic activity is less than that for the natural hydroxynitrile lyases but is 100-fold higher than a designed aldolase, which also cleaves a carbon-carbon bond. The HNL from *Manihot esculenta* does not have a lysine residue in the active site, but the SABP2-G12T variant, which also lacks the lysine, did not show any hydroxynitrile lyase activity (30-fold or more lower than SABP2-G12T-M239K). An improper fit of benzaldehyde may be the reason for the lack of catalytic in the PFE variants. The ester substrate of SABP2 is methyl salicylate where the aryl group is in the acyl portion of the ester. In contrast, the best substrate for PFE is phenyl acetate where the aryl group is in the alcohol group of the ester. The superposition of X-ray structures of *Hb*HNL and SABP2 show the aryl group in similar locations, although the aryl ring orientation differs. The aryl ring of phenyl acetate must orient in a different region in PFE suggesting that benzaldehyde binds to the PFE variant in non-SABP2-like orientation. This different orientation may be catalytically nonproductive. This reasoning suggests that the PFE double mutant might be a catalyst for addition of cyanide to acetaldehyde since acetaldehyde may mimic the binding of the acetyl part of phenyl acetate. Initial experiments to test this hypothesis were inconclusive.

There are several possibilities for the lower HNL activity of SABP2-G12T-M239K as compared with wild-type HNLs. First, the substrates benzaldehyde and cyanide may not fit properly as mentioned above. The active site of SABP2 has evolved for ester hydrolysis not for addition of cyanide to benzaldehyde. The active sites of both the enzymes differ more than just the five catalytic amino acids. The two mutations introduced HNL activity into SABP2 protein but reduced the protein stability as evidenced by protein expression. The change in carbonyl activation from hydrogen bonds from the oxyanion hole to hydrogen bonds from the serine and threonine side chains requires a slightly different orientation. A second possible reason is that

additional, as yet unidentified residues, contribute to catalysis in HNLs and the SABP2 variants lacked these residues. These contributions may be protein motions, long-range electrostatics, or other effects.

Addition to a ketone or aldehyde carbonyl to make an sp^3-hybridized center and hydroxyl group requires adding a proton to the oxygen. The change from a reaction mechanism using hydrogen bonds from the oxyanion hole in hydrolases to a reaction mechanism using hydrogen bonds from the serine and threonine side chains allows adding a proton to the carbonyl oxygen. The oxyanion hole, while it can stabilize an oxyanion intermediate, does not include acids that can protonate it to make a hydroxyl. In contrast, the side chain of the serine is hydrogen bonded to the protonated histidine and thus can transfer a proton to the oxyanion to make a hydroxyl group. Thus, an oxyanion hole-based mechanism for addition of cyanide to a carbonyl compounds would form the less stable hydroxynitrile anion, not the hydroxynitrile. The oxyanion hole-based mechanism is perfect for hydrolases since hydrolysis reforms that carbonyl double bond, so there is no need to transfer a proton to the oxygen.

Support for this key difference in the two mechanisms comes from two promiscuous reactions catalyzed by hydrolases. *Candida antarctica* lipase B and several other hydrolases catalyze the aldol (Branneby et al., 2003; Branneby et al., 2004; Li et al., 2008) and Michael (Carlqvist et al., 2005; Torre et al., 2004; Kitazume et al., 1986) additions and epoxidation of α,β-unsaturated aldehydes by hydrogen peroxide (Svedendahl et al., 2008) using the oxyanion hole for catalysis. However, these reactions do not involve addition to carbonyl to make an sp^3-hybridized center and thus do not require protonation of the carbonyl oxygen (Figure 3). Thus, we hypothesize that promiscuous reactions that add to a ketone or aldehyde to make an sp^3-hybridized center and hydroxyl group should follow a hydroxynitrile lyase mechanism. Hydroxynitrile lyases are the most likely best candidates for such reactions. In accord with this prediction, researchers have reported the HNL-catalyzed addition of nitromethane to aldehydes to form hydroxy compounds (Purkarthofer et al., 2006).

One mechanism for evolution of new catalytic activities is neutral drift where random, selectively neutral mutations accumulate. These mutations do not significantly affect the function of the protein (e.g., hydrolysis of one ester) but may enable new activities (e.g., hydrolysis of another ester). Several groups showed that neutral drift can broaden the substrate range on enzymes (Bloom et al., 2007; Bershtein et al., 2008; Gupta and Tawfik, 2008), but these new activities likely follow mechanisms similar to the original. Neutral drift is not a viable mechanism for evolution of hydroxynitrile lyases from esterases or vice versa because the mechanisms differ. Enabling one mechanism disrupts the other mechanism. Another mechanism for evolution is gene duplication followed by point mutation in the copy. This mechanism is consistent with our results. A few point mutations can enable a dramatically different mechanism.

An evolutionary tree of the 30 closest structural relatives of *Hb*HNL shows that SABP2 is more closely related to the three hydroxynitrile lyases than to other hydrolases (Figure S1). The three hydroxynitrile lyases and SABP2 share a common ancestor. One reason for this similarity may be that these four

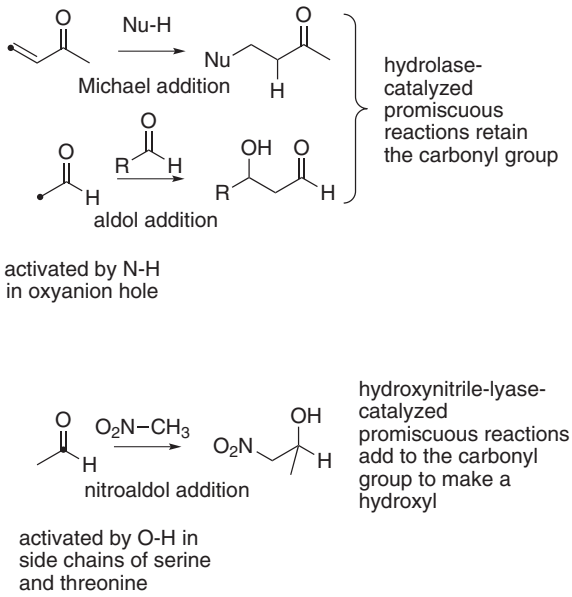

Figure 3. Promiscuous Reactions of Hydrolases Retain the Carbonyl Group, while Promiscuous Reactions of Hydroxynitrile Lyase Involve Addition to Carbonyl Group to Form an Alcohol

Lipase B from *Candida antarctica* catalyzes a promiscuous Michael and aldol additions, while hydroxynitrile lyase from *Hevea brasiliensis* catalyzes a promiscuous nitroaldol addition. In the aldol addition, the hydrolase oxyanion hole stabilized the enol, while in the nitroaldol addition the serine and threonine side chains stabilized the aldehyde component. The different mechanism allows hydroxynitrile lyases to protonate the substrate to form a hydroxyl group. The dot in the substrate marks the reaction site. (Table S2 compares the turnover numbers for engineered new catalytic activity to the turnover numbers in this work.)

enzymes are from plants, while the others are from microorganisms or animals. Another reason may be that SABP2 may be a transitional enzyme, a recently evolved esterase. SABP2 also lies between the (*R*)- (Andexer et al., 2007) and (*S*)-selective HNLs, which may account for its poor of enantioselectivity.

SIGNIFICANCE

This work demonstrates that small changes in protein structure, only two amino acid substitutions, can dramatically change an enzymatic reaction mechanism. It identifies the key elements needed to catalyze an important reaction for synthesis, additions to an aldehyde. The engineered enzyme catalyzes a carbon-carbon bond formation at a rate (k_{cat}) only 120-fold slower than the natural enzyme from rubber tree. Further protein engineering may expand the reaction range of these enzymes to include a variety of additions to aldehydes and ketones.

EXPERIMENTAL PROCEDURES

Esterase Activity and Steady-State Kinetics

Esterase activity was measured by hydrolysis of *p*-nitrophenyl acetate (pNPAc) (Bernhardt et al., 2005) and was corrected for spontaneous hydrolysis. The assay performed in a 96-well microtiter plate with reaction volume of 100 μl (90 μl of assay solution [0.3 mM of pNPAc, 6.7% acetonitrile,

4.2 mM BES as final concentrations] and 10 μl of protein solution) monitored spectrophotometrically at 404 nm using $\varepsilon = 16.5 \times 10^3\ M^{-1}cm^{-1}$ and path length of light 0.29 cm. The steady-state kinetics for SABP2 G12T were measured in the same manner, but using 25 vol% acetonitrile to dissolve higher concentrations of pNPAc. The best fit of the data to the Michaelis-Menten equation was found using the Solver function of Microsoft Excel gave K_M = 8.7 mM and V_{max} = 0.058 mU/mg (Figure S5A). The inhibition of SABP2 G12T by benzaldehyde was measured by steady-state kinetics for hydrolysis of *p*-nitrophenol acetate in solutions of 1–100 μM benzaldehyde. The values of apparent K_M and V_{max} were determined at each benzaldehyde concentration. A plot of K_{Mapp}/V_{max} versus benzaldehyde concentration was a straight line and yielded an inhibition constant of 78 μM as the intercept with the x axis (Figure S5B). An Eadie-Hofstee plot showed that the inhibition was competitive because the V_{max} remained constant at different benzaldehyde concentrations (best fit lines intersect on the y axis, Figure S5C).

Hydroxynitrile Lyase Activity and Steady-State Kinetics

Hydroxynitrile lyase activity was measured at pH 5.0 by monitoring the release of benzaldehyde (ε_{280} = 13,800 M^{-1} cm^{-1}) from mandelonitrile spectrophotometrically at 280 nm. Each assay well in the microtiter plate contained mandelonitrile (15 mM, 10 μl of 300 mM mandelonitrile in citric acid buffer, 100 mM [pH 2.1]), (190-x) μl of assay buffer (50 mM citrate buffer [pH 5.0]), and x μl of enzyme in BES buffer (5 mM [pH 7.2]). The volume of enzyme solution added, x, varied depending on the protein concentration of the stock solution. The total assay volume was 200 μl (path length 0.60 cm) and the absorbance was monitored for 20 min. The blank was an identical solution, but the enzyme aliquot was replaced by an equal amount of BES buffer (5 mM [pH 7.2]) or an equal protein amount of wild-type SABP2. The spontaneous decomposition of mandelonitrile values were subtracted from measurements and set the detection limit of this assay at 0.7 mU/mg protein. The specific activity for (*R*)- and (*S*)-mandelonitrile (Supplemental Experimental Procedures) was 12.6 and 15.6 mU/mg, respectively, suggesting little if any enantioselectivity. Steady-state kinetics for hydroxynitrile lyase activity of SABP2-G12T-M239K were measured with 2, 4, 6, 8, 12, 15, 18, 20, 22, 24, 25, 26, 30, and 35 mM of racemic mandelonitrile in 50 mM citric acid pH 2.3 using the conditions above. Reactions were initiated by adding 5.05 μg of protein in a final volume of 200 μl. The rate of spontaneous decomposition of mandelonitrile was measured separately and subtracted from the rate to obtain the true enzymatic rate. One unit of HNL activity is defined as the formation of 1 μmol of benzaldehyde per minute. The best fit of the data to the Michaelis-Menten equation found using the Solver function of Microsoft Excel gave K_M = 12.5 mM and V_{max} = 30.6 mU/mg (Figure S10).

HPLC Scale Hydroxynitrile Synthesis and Enantioselectivity Determination

A mixture of SABP2-G12T-M239K protein (791 μg in 1.41 ml of 5 mM BES buffer [pH 7.2]), citrate buffer (590 μl, 100 mM [pH 4.75]), benzaldehyde (10 μmol, 50 μl of 200 mM in di-isopropyl ether), and hydrogen cyanide (~3 mmol, 1.5 ml of ~2M in di-isopropyl ether) was stirred in an ice bath for 3 hr. Control experiments contained everything except the enzyme (5 mM BES [pH 7.2] used instead) or an equal amount of wild-type SABP2 (rest of volume 5 mM BES [pH 7.2]). After the reaction, the organic layer was separated and aqueous layer was extracted with di-isopropyl ether (3 × 2 ml). The combined organics were dried over anhydrous sodium sulfate and the solvent was removed by a stream of air. The residue was dissolved in mixture of isopropanol (50 μl, HPLC grade) and hexane (960 μl, HPLC grade) and analyzed by HPLC on a Chiralcel OD-H column (Daicel) eluted with hexane:isopropanol (96:04) at 1.5 ml/min. Benzaldehyde eluted at 4.0 min, and (*S*)- and (*R*)-mandelonitrile eluted at 13.8 and 14.9 min, respectively, which confirmed using commercial (*R*)-mandelonitrile. The peak areas correspond to 1.0 μmol of mandelonitrile, but no enantioselectivity. Wild-type SABP2 showed no product; however, one blank experiment showed formation of mandelonitrile. To confirm this, we repeated the above experiment (due to lack of enzyme we had to skip the SABP2-G12T-M239K experiment) which did not show any mandelonitrile formation in both the control experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.06.013.

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