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Enzyme Catalysis

Enantiocomplementary Enzymes: Classification, Molecular Basis for Their Enantiopreference, and Prospects for Mirror-Image Biotransformations

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One often-cited weakness of biocatalysis is the lack of mirror-image enzymes for the formation of either enantiomer of a product in asymmetric synthesis. Enantiocomplementary enzymes exist as the solution to this problem in nature. These enzyme pairs, which catalyze the same reaction but favor opposite enantiomers, are not mirror-image molecules; however, they contain active sites that are functionally mirror images of one another. To create mirror-image active sites, nature can change the location of the binding site and/or the location of key catalytic groups. In this Minireview, X-ray crystal structures of enantiocomplementary enzymes are surveyed and classified into four groups according to how the mirror-image active sites are formed.

1. Introduction

Synthetic routes to pure enantiomers involve either resolution or asymmetric synthesis. Both enantiomers are obtained by the resolution of a racemic mixture, albeit in only 50% theoretical yield. Asymmetric syntheses yield one enantiomer with a theoretical yield of 100%. This higher theoretical yield of asymmetric syntheses can lower costs and minimize environmental impact.

A weakness of asymmetric synthesis is the 50% chance that the product enantiomer is the wrong enantiomer. If chemical reagents or catalysts are involved, then the solution is simple: the reagent or catalyst must be used in the appropriate enantiomeric form to yield the desired enantiomer. In the case of biocatalysis, this solution is not possible: enantiomeric biocatalysts do not exist in nature. Researchers often cite this limitation of biocatalysis as a major disadvantage.

Although enantiomeric biocatalysts do not exist in nature, we show herein that enantiocomplementary biocatalysts are surprisingly common. Enantiocomplementary biocatalysts contain active sites that are functionally mirror images of one another. They catalyze the same reaction to yield opposite enantiomers and thus overcome the limitation derived from the lack of true mirror-image biocatalysts.

Another reason to study enantiocomplementary enzymes is to identify the molecular basis of catalysis. Enantiocomplementary enzymes catalyze the same reaction, but may have different protein structures and/or different functional groups within the active site. The identification of common elements in both enzymes may reveal elements essential to catalysis.

Herein, we survey and classify 14 naturally occurring pairs of enantiocomplementary enzymes (Table 1). The focus is on synthetically useful enzymes for which an X-ray crystal structure is available for at least one of the enzymes in the pair. We also describe the discovery or engineering of new enantiocomplementary enzyme pairs. On the basis of a classification system, we suggest pairwise mutagenesis in the active site as a promising route to new enantiocomplementary enzymes.

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Supporting information for this article (five additional figures, in which the active sites of enantiocomplementary enzymes are compared, and three-dimensional pymol models of all structures) is available on the WWW under http://www.angewandte.org or from the author.

2. Proposed Classification

A classification of enantiocomplementary enzyme pairs is proposed in Figure 1. Previously, enantiocomplementary enzymes have been compared case by case; until now, no systematic classification of the strategies of nature has been attempted. In all cases, the active sites of enantiocomplementary enzyme pairs function as mirror images, as the substrates are mirror images of one another. However, if one uses the protein fold or a cofactor as a reference, then one can identify the relationship between these mirror-image active sites in terms of the active-site positions that are exchanged to create the enantiocomplementary active site. The molecular basis for the reversed enantiopreference is classified by defining which positions are exchanged. The Supporting Information includes a flow chart for the classification of enantiocomplementary enzymes.

Group 1 (Figure 1) contains cases of the recreation of mirror-image active sites in different protein folds.[1] As the protein structures differ, there is no reference point for comparison, and one can not identify the specific differences in the two active sites. Examples include true mirror images created by the chemical synthesis of enzymes from D-amino acids (e.g., HIV protease, *ent*-HIV protease) and naturally occurring functional-mirror-image active sites in which the amino acid residues may differ, although their chemical roles remain similar (e.g., *R*- and *S*-selective hydroxynitrile lyases). If the enzymes contain a cofactor, then the active sites are on opposites sides of the cofactor in the two enzymes so that the active sites in combination with the cofactor are mirror images.

Group 2 also contains examples in which the protein folds differ; however, a cofactor serves as a reference point (e.g., α-hydroxy acid dehydrogenases with nicotinamide as a reference point). As the active sites are on the same side of the cofactor, the active sites in combination with the cofactor are not mirror images; instead, the active sites without the cofactor are mirror images. As the reacting substituents must face the cofactor in both enzymes, the active site must exchange two nonreacting substituents to create a mirror image. The domains that position the cofactor are often related even when the active-site domains are not. In these cases, a new active site has been added to an existing cofactor-binding domain.

The last two groups, groups 3 and 4, comprise enzymes with the same protein fold, which serves as a reference that can be used to determine how these proteins diverged to create functionally enantiomeric active sites. In group 3, the binding sites for substituents not directly involved in catalysis are exchanged (e.g., in amino acid oxidases), as described for enzymes in group 2. In group 4, a catalytic group changes its location (e.g., a carboxylate residue in vanillyl-alcohol oxidase/*p*-cresol methylhydroxylase).

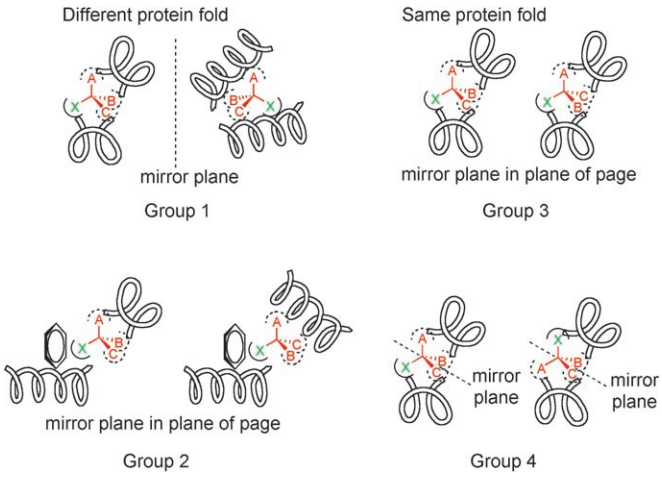

Figure 1. Classification of enantiocomplementary enzyme pairs according to the relationship between the mirror-image active sites. X represents a substituent directly involved in bond making and bond breaking; A, B, and C represent nonreacting substituents. The benzene ring in the schematic illustration for group 2 represents a cofactor, such as flavin, pyridoxal, or nicotinamide.

3. Examples

3.1. Group 1: Different Protein Folds Recreate Mirror-Image Active Sites

The most evident enantiocomplementary enzyme is the true enantiomer of an enzyme. True mirror-image enzymes can not be created from D-amino acids through ribosome-mediated biosynthesis, but they can be produced by chemical

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Photo: Tim Rummelhoff

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Table 1: Some structurally characterized naturally occurring enantiocomplementary enzyme pairs.

Enzymes	Reaction(s) catalyzed	Same protein fold?[a]	Group
R- and *S*-selective hydroxynitrile lyases; pdb 1ju2 and 1yb6	addition of hydrogen cyanide to aldehydes and ketones (mandelonitrile, $E=200$ (*R*); 2-cyclohexyl-2-hydroxyethanenitrile, $E=200$ (*S*))	no; oxidoreductase fold with flavin versus α/β hydrolase fold	1
methionine sulfoxide reductases A and B; pdb 1fvg and 1l1d	reduction of methionine sulfoxides to methionine; MsrA: $E\approx10$ (*S*), MsrB favors *R*, no quantitative data available	no; rolled mixed β sheet flanked by three α helices versus antiparallel β strands organized in two sheets that face each other to form a barrellike core	1
lipase and subtilisin; pdb 1lpm and 1cse	hydrolysis of esters of secondary alcohols; acylation of secondary alcohols in non-aqueous media (e.g., hydrolysis of menthyl acetate with CRL, $E>100$ (1*R*); acylation of 1-phenylethanol with subtilisin Calsberg in dioxane, $E=25$ (*S*))	no; α/β hydrolase fold versus subtilisin fold	1
D-amino acid oxidase and flavocytochrome b_2; pdb 1c0p and 1fcb	flavin-dependent oxidation of D-alanine to the imine and oxidation of L-lactate to pyruvate, *E* high	no; *p*-hydroxybenzoate hydroxylase topology versus $(\alpha/\beta)_8$ barrel topology for FCB	1
L-aspartate aminotransferase and D-amino acid aminotransferase; pdb 1ajs and 3daa	pyridoxal-dependent transfer of ammonia between amino acids and the corresponding 2-keto acids; L-AAAT: L-aspartate, $E\approx10^6$; D-AAAT: *E* high	no; aminotransferase fold I versus fold IV	1
hydratase 1 (crotonase) and hydratase 2; pdb 1mj3 and 1pn4	addition of water to *trans*-2-enoyl-CoA; crotonyl-CoA: $E>20$ (L); *trans*-2-decanoyl-CoA: *E* high (D)	no; crotonase fold for hydratase 1 and hotdog fold for hydratase 2	1
R- and *S*-α-hydroxyacid reductases; pdb 1dxy and 1hyh	nicotinamide-dependent reduction of 2-ketoisocaproic acid to 2-hydroxyisocaproic acid, $E>200$ (*R*), *E* high (*S*)	no; limited similarity only in the nicotinamide-binding domain (7% sequence identity, Z score 6.1 for 120 of 330 aa)	2
L- and D-lactate dehydrogenases; pdb 1ez4 and 2dld	nicotinamide-dependent reduction of pyruvate to lactate, $E=46$ (D), *E* high (L)	no; limited similarity only in the nicotinamide-binding domain (13% sequence identity, Z score 6.0 for 119 of 333 aa)	2
D-lactate dehydrogenase and flavocytochrome b_2; pdb 1f0x and 1fcb	flavin-dependent oxidation of lactate to pyruvate, *E* high	no, seven-stranded β sheet surrounded by seven α helices versus $(\alpha/\beta)_8$ barrel topology for FCB	2
D- and L-amino acid oxidases; pdb 1c0p and 2iid	flavin-dependent oxidation of amino acids to imines, *E* high	no; D-amino acid oxidase domain versus amine oxidase domain; limited similarity only in the flavin-binding domain (13% sequence identity, Z score 13 for 247 of 484 aa)	2
naphthalene dioxygenase and toluene dioxygenase; pdb 1o7p	non-heme iron-catalyzed dihydroxylation of 1,2-dihydronaphthalene; sulfoxidation of alkyl aryl sulfides; benzylic hydroxylation of indan-2-ol, $E>50$ (1*R*,2*S*) for NDO and $E>50$ (1*S*,2*R*) for TDO	very likely; 35% sequence identity, but no structure solved for TDO	3
aminotransferases for branched-chain L- and D-amino acids; pdb 1kt8 and 3dda	pyridoxal-dependent transfer of ammonia between amino acids and the corresponding 2-keto acids, $E>100$ for isoleucine	yes; both fold IV (19% sequence identity, Z score 27 for 269 of 365 residues, 2.1 Å mean deviation of C^α positions)	3
D- and L-hydantoinases; pdb 1k1d and 1gkr	hydrolysis of 5-monosubstituted hydantoins to *N*-carbamoyl α-amino acids, $E>50$ (D) for the phenylalanine precursor and $E>50$ (L) for the tryptophan precursor	yes; both contain a TIM barrel and urease-subunit C domain (34% sequence identity, Z score 52 for 435 of 460 aa, 2.1 Å mean deviation of C^α positions)	3
vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase; pdb 2vao and 1diq	flavin-dependent benzylic hydroxylation of 4-ethylphenol, $E=32$ (*R*), $E\approx2$ (*S*)	yes; 33% sequence identity, Z score 48 for 513 of 555 aa; 1.5 Å mean deviation of C^α positions	4

[a] The structures were compared by using DALI Lite: http://www.ebi.ac.uk/DaliLite/.

synthesis. In the 1990s, the D forms of HIV protease (99 amino acids)[2] and oxalocrotonate tautomerase (62 amino acids)[3] were synthesized. The enantiomeric HIV proteases catalyzed the hydrolysis of enantiomeric peptides, and the enantiomeric oxalocrotonate tautomerases catalyzed allylic 1,3-proton shifts in D_2O, with opposite enantiopreference. Similarly, Seelig et al.[4] synthesized the non-natural L form of a 49-mer ribozyme; the synthetic polynucleotide catalyzed a Diels–Alder cycloaddition with opposite enantioselectivity to that of the natural D form. The folding of these enzymes is enantiomeric (e.g., left- versus right-handed helices), so the protein folds differ. The chemical synthesis of enantiomeric enzymes is currently too expensive and too slow to be a practical solution for synthesis. In the future, the creation of an enantiocomplementary ribosome could provide a practical synthetic route to enzymes from D-amino acids.

The naturally occurring proteins in group 1 are not true mirror-image proteins, but are different protein structures that have converged to create active sites that are functionally mirror images (Table 1). For example, enantiocomplementary hydroxynitrile lyases catalyze the addition of hydrogen cyanide to aldehydes and ketones to form enantiomeric cyanohydrins.[5] The *R*-selective hydroxynitrile lyases come from the *Rosaceaea* family (almond, cherry, plum, etc.), are distantly related to the glucose–methanol–choline (GMC) oxidoreductases, and contain the redox cofactor flavin adenine dinucleotide (FAD). The flavin cofactor plays only a structural role and seems to be an evolutionary remnant. The *S*-selective hydroxynitrile lyases come from *Hevea brasiliensis* (rubber tree), *Manihot esculenta* (cassava), *Sorghum bicolor* (millet), and *Linum usitatissimum* (flax). They lack FAD and adopt the α/β-hydrolase fold. The X-ray crystal structures of the *Hevea*[6] and *Prunus*[7,8] hydroxynitrile lyases show that both catalyze cyanohydrin formation by general acid–base catalysis and that they position the carbonyl compound similarly; however, the cyanide nucleophile attacks the opposite face of the carbonyl group in each enzyme to generate enantiomeric cyanohydrins (Figure 2).

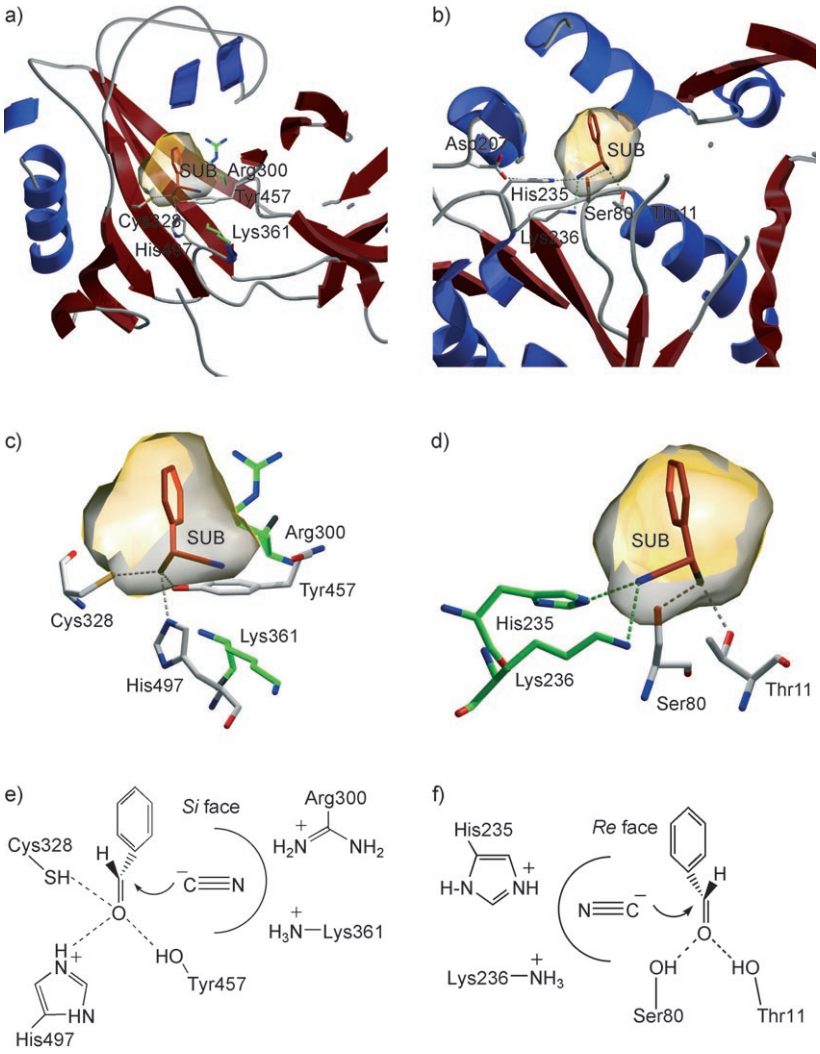

Figure 2. a,b) Active-site environment of the *R*-selective hydroxynitrile lyase (HNL) from the almond tree (PDB entry: 1ju2; a) and the *S*-selective hydroxynitrile lyase from the rubber tree (PDB entry: 1yb6; b) with mandelonitrile in the active site. c,d) Close-up view of the substrate binding of *R*- and *S*-selective hydroxynitrile lyases. Different sets of hydrogen bonds (Cys328, His497, and Tyr457 in *R*-HNL/Ser80; Thr11 in *S*-HNL) position the mandelonitrile hydroxy group (or the benzaldehyde carbonyl oxygen atom for the reverse reaction). A hydrophobic binding site (yellow, back) positions the phenyl group of the substrate (SUB). The positively charged pockets for the cyanide nucleophile lie on opposite sides of the mirror-image active sites. In *S*-HNL, this pocket involves direct hydrogen bonds to Lys236 and His235; however, for *R*-HNL, the positively charged residues, Arg300 and Lys361, are farther away and do not make hydrogen bonds to the cyanide group. e,f) Schematic illustration of the coordination sphere of *R*- and *S*-HNL: The nucleophilic attack of cyanide occurs from opposite sides of the prochiral substrate, although the substrate is positioned in the same way in both enzymes.

Enantiocomplementary methionine sulfoxide reductases (MsrA and MsrB) reduce methionine sulfoxides formed by air oxidation in proteins. This reduction restores the original catalytic activity and conformation of the protein. MsrAs reduce methionine sulfoxides with the *S* configuration at the sulfur center ((*S*)-MetO), whereas MsrBs reduce methionine sulfoxides with the *R* configuration at the sulfur center ((*R*)-MetO).[9] The two Msr types differ in their protein folding but have mirror-image active sites[10] (see Figure S1 in the Supporting Information). Both active sites contain a tryptophan residue to bind the methyl substituent on the sulfoxide, hydrogen-bond donors to bind the sulfoxide oxygen atom, and a catalytic cysteine residue that attacks the sulfoxide sulfur atom. In most cases, MsrA and MsrB are separate enzymes; however, in *Neisseria gonorrhoeae*, one protein contains both active sites in separate domains.

Lipases and subtilisins are examples of enantiocomplementary enzymes with non-natural substrates. Both are serine hydrolases, contain a Ser-His-Asp catalytic triad and an oxyanion hole, and react by a similar mechanism involving an acyl enzyme intermediate. Both enzymes catalyze the hydrolysis of non-natural substrates, such as esters of secondary alcohols, but with opposite enantiopreference.[11] This enan-

tiocomplementary behavior of lipases and subtilisins has been used to synthesize opposite enantiomers of secondary alcohols.[12] When dynamic kinetic resolution was used in a method equivalent to asymmetric synthesis, one enantiomer was obtained in high yield and high enantiomeric purity.[13]

Although lipases and subtilisins have a different protein structure, the three-dimensional arrangements of their catalytic triads are mirror images of one another[14] (see Figure S2 in the Supporting Information). Both active sites have limited space for one of the substituents of the secondary alcohol, but the mirror-image orientation of the catalytic centers favors opposite enantiomers. (Figure S2 in the Supporting Information shows catalytically productive orientations for the *S* enantiomer of an ester of a spirocyclic secondary alcohol in the active site of subtilisin Carlsberg[15] and a (1*R*)-menthyl ester in the active site of *Candida rugosa* lipase.[16])

Two examples of cofactor-containing enantiocomplementary enzyme pairs in group 1 are amino acid aminotransferases (AAATs) with the cofactor pyridoxal and D-amino acid oxidase/flavocytochrome b2 with the cofactor flavin. L-Aspartate aminotransferase[17] and *Bacillus* D-amino acid aminotransferase[18] have different protein folds and mirror-image active sites (see Figure S3 in the Supporting Information). The substrate and catalytic lysine residue lie on opposite faces of the pyridoxal cofactor in the two aminotransferases, so that the combinations of active site and cofactor are mirror images. (Another pair of enantiocomplementary AAATs belong to group 2; see Section 3.2.) D-Amino acid oxidase (D-AAO)[19] and flavocytochrome b2 (FCB)[20,21] catalyze mechanistically similar reactions: the oxidation of alanine (D-AAO) and lactate (FCB), whereby an NH_2-CH and an OH-CH group are oxidized. The X-ray crystal structures reveal active sites that are functionally mirror images for alanine in D-AAO and for pyruvate in FCB (see Figure S4 in the Supporting Information).

Hydratases 1 and 2 catalyze a Michael addition of water to opposite faces of *trans*-2-enoyl-coenzyme A.[22] The protein folds are unrelated and the active sites are functionally mirror images. (There is a good picture that compares the two in reference [23].)

3.2. Group 2: Different Protein Folds with Exchanged Locations of Binding Sites

Group 2 also comprises cases involving different protein folds, but with the active site on the same face of the cofactor (*Re* or *Si*) in the enantiocomplementary enzymes. The catalytic groups of the proteins are approximate mirror images; however, the entire active sites are not approximate mirror images, because the orientation of the cofactor has not changed accordingly. The mirror plane that relates the two active sites (in the plane of the paper in Figure 1) does not apply to the cofactor.

S- and *R*-α-hydroxyacid dehydrogenases, like 2-hydroxyisocaproate dehydrogenases (2-HicDHs), catalyze the enantiocomplementary reduction of a broad range of α-ketocarboxylic acids,[24] but have different protein structures. (*R*)-2-HicDH belongs to the family of tetrameric L-lactate-dehydrogenases,[25] whereas (*S*)-2-HicDH belongs to the family of D-specific NAD^+-dependent 2-hydroxycarboxylate dehydrogenases.[26] The two HicDHs bind the nicotinamide cofactor similarly and position the α-carbonyl moiety of the substrate through hydrogen bonds (to Asn143 in (*S*)-HicDH, to Arg234 in (*R*)-HicDH; Figure 3). A large hydrophobic pocket positions the hydrophobic side chain of the substrate, and the carboxylate group of the substrate is bound by either a salt bridge (to Arg174 in (*S*)-HicDH) or an array of hydrogen bonds (involving Gly78, Asn76, and Tyr100 in (*R*)-HicDH). By exchanging the locations of the carboxylate-docking groups and the hydrophobic pocket, the substrate is turned around so that the opposite face of the carbonyl group faces the nicotinamide. In both cases, the dihydronicotinamide transfers the pro-*R* hydrogen atom to the carbonyl group. A similar exchange of carboxylate- and substituent-binding sites accounts for the enantiocomplementarity of NAD-dependant lactate dehydrogenases,[27] which catalyze the same reaction but favor smaller substituents, such as R = methyl.

In group 1, we paired yeast D-AAO with flavocytochrome b2 as an example of mirror-image active sites in different protein folds. Yeast D-AAO and snake venom L-AAO also form an enantiocomplementary pair, which serves of an example for group 2. The amino acid sequences and domain folds of D-AAO and L-AAO differ. In the X-ray crystal structures of yeast D-AAO and snake venom L-AAO,[28] the substrate is bound on the same side of the flavin cofactor. The α C atom of the amino acid substrate sits above the *Re* face of N5 for direct hydride transfer (see Figure S5a in the Supporting Information). To accommodate the enantiomeric configuration of the substrate, the substrate-binding groups—arginine and the hydrophobic region—have exchanged locations.

The other flavin oxidase from group 1, flavocytochrome b2, also forms an enantiocomplementary pair with membrane-bound D-lactate dehydrogenase (D-LDH) from *E. coli*.[29] The lactate-binding site is on the same face of the flavin cofactor in both enzymes, so this example probably also belongs in group 2.

3.3. Group 3: Same Protein Fold with Exchanged Locations of Binding Sites

Naphthalene dioxygenase (NDO) and toluene dioxygenase (TDO) both catalyze the dihydroxylation of unsaturated compounds; however, in the dihydroxylation of 1,2-dihydronaphthalene they yield opposite enantiomers with excellent enantioselectivity ($E > 100$, Figure 4a).[30] Presumably, the favored substrate orientation differs in the two dioxygenases to expose opposite faces of the substrate to the catalytically active iron center. No X-ray crystal-structure analysis of TDO has been reported; however, the two enzymes show 35% sequence identity and thus probably adopt the same three-dimensional structure. Eight amino acid residues in the substrate-binding site differ in NDO and TDO (Figure 4c), and these differences most likely cause opposite substrate orientations. NDO and TDO also show an opposite enantio-

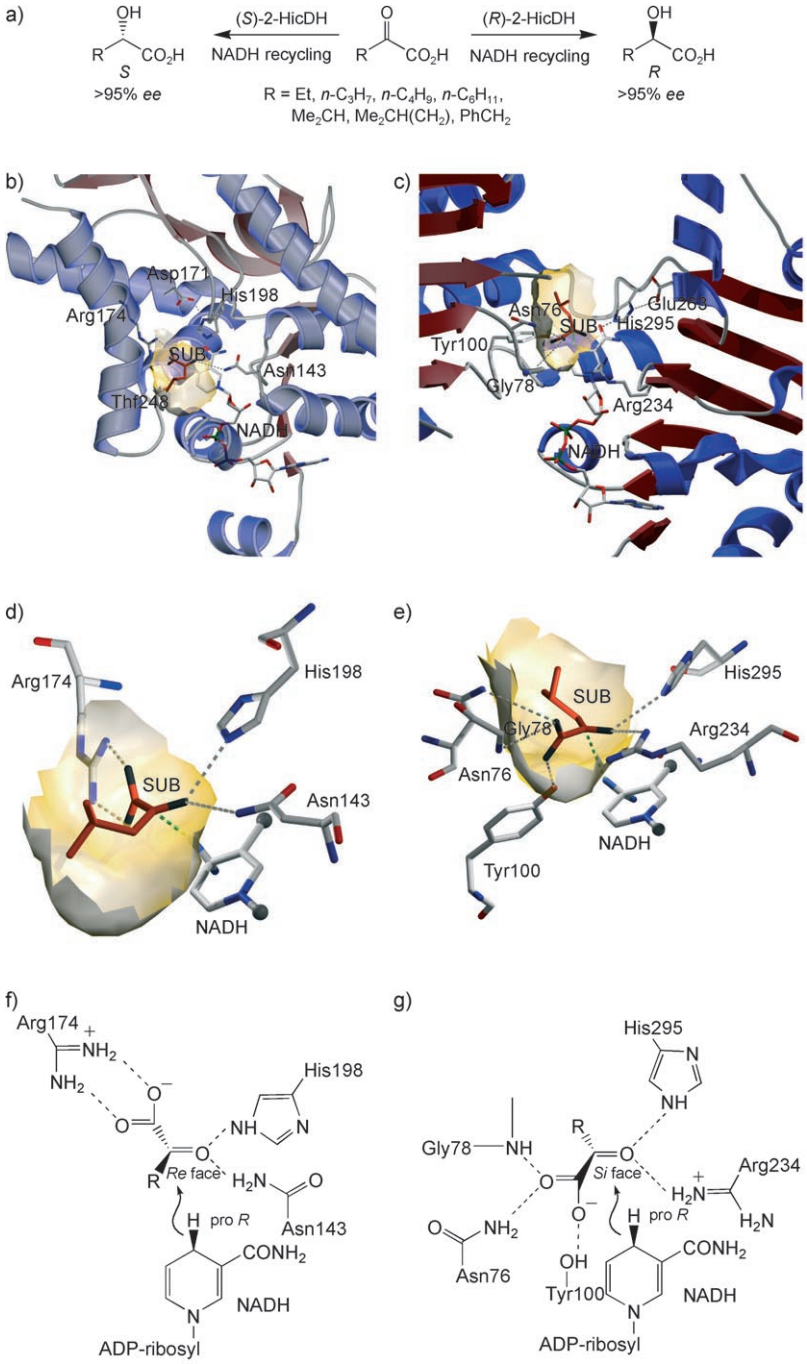

Figure 3. a) Enantiocomplementary reduction of α-ketocarboxylic acids with (*R*)- and (*S*)-2-hydroxyisocaproate dehydrogenase. Active-site environment of b) (*S*)-2-hydroxyisocaproate dehydrogenase containing the modeled substrate (PDB entry: 1hyh) and c) (*R*)-2-hydroxyisocaproate dehydrogenase (PDB entry: 1dxy), in which the substrate 2-oxoisocaproate occupies the opposite orientation. d,e) Focus on the active sites. The α-carbonyl moiety is positioned by H bonds (Asn143 and His198 in (*S*)-2-HicDH; Arg234 and His295 in (*R*)-2-HicDH) in superimposable positions. The large side chain is accommodated in a lipophilic pocket (yellow) in opposite orientations in the two enzymes (towards the front and towards the back, respectively), whereas the carboxylate terminus is fixed in opposite orientations by a salt bridge or a tight network of H bonds (Arg174 in (*S*)-2-HicDH; Gly78, Asn76, and Tyr100 in (*R*)-2-HicDH). The attack of the pro-*R* hydride from NADH (the reduced form of nicotinamide adenine dinucleotide) onto C^{α} occurs in both enzymes from the bottom (indicated in green). The substituents of NADH are simplified by spheres. f,g) Schematic illustration of the coordination sphere of an α-ketocarboxylic acid bound to (*S*)- and (*R*)-2-hydroxyisocaproate dehydrogenase.

preference in the oxidation of alkyl aryl sulfides to sulfoxides[31] and in the benzylic hydroxylation of indan-2-ol.[32]

Consistent with this notion of different substrate orientations, site-directed mutagenesis of NDO reversed its enantiopreference in the 3,4-dihydroxylation of biphenyl. Wild-type NDO catalyzes the highly enantioselective dihydroxylation of biphenyl at both the 2,3-position (87%, major product, not shown) and the 3,4-position (13%, minor product, $E > 100$; Figure 4b). The replacement of Phe352 with valine in NDO altered the regioselectivity, so that the 3,4-dihydroxylated product was formed as the major product (96%), and reversed the enantioselectivity (albeit with just $E = 7.7$).[33] This reversal in the enantioselectivity presumably results from an altered substrate orientation, as Phe352 forms part of the substrate-binding site (Figure 4b).[34]

Bacillus D-amino acid aminotransferase (see group 1, Section 3.1) also forms an enantiocomplementary pair with branched chain L-amino acid aminotransferase.[35] Both enzymes have the same protein fold (type IV) and position the substrate on the same face of pyridoxal phosphate (PLP; *Re* face; see Figure S5b in the Supporting Information). An exchange of the binding sites for the α carboxylate and the side chain reverses the enantiopreference.

Recently, Andexer et al. reported an *R*-selective hydroxynitrile lyase with an amino acid sequence—and presumably a 3D structure—similar to that of the *S*-selective hydroxynitrile lyase from *Hevea brasiliensis*.[36] They suggested that the reversal in enantioselectivity stems from a switch in the binding location of the hydrogen atom and the aromatic side chain of the substrate (e.g., benzaldehyde).

Hydantoinases adopt the TIM (triosephosphate isomerase) barrel fold and catalyze the enantioselective hydrolysis of 5-monosubstituted hydantoins to yield *N*-carbamoyl derivatives of α-amino acids.[37] Most hydantoinases are D selective; however, the hydantoinase from *Arthobacter aurescens* favors the L enantiomer of 5-(3′-indoylmethyl)hydantoin, a precursor of L-tryptophan. Modeling based on the X-ray crystal structures suggests that the hydantoin side chain binds at different locations in the two enzymes.[38]

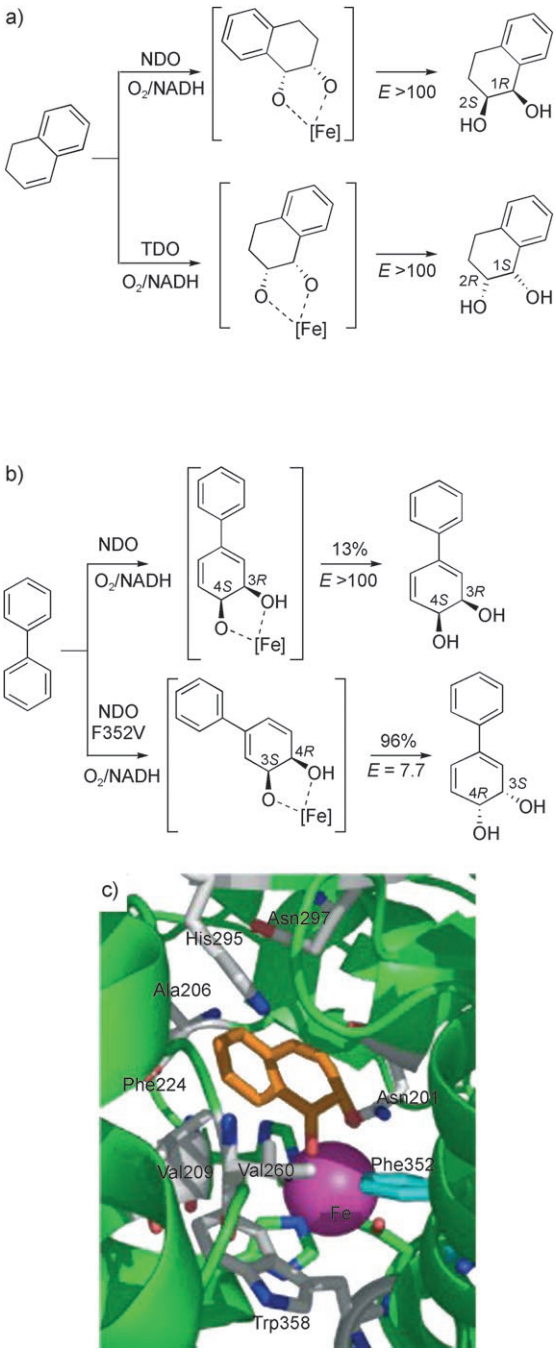

Figure 4. Enantiocomplementary oxidation reactions catalyzed by naphthalene dioxygenase (NDO) and toluene dioxygenase (TDO). a) NDO catalyzes the dihydroxylation of dihydronaphthalene with high enantioselectivity for the 1*R*,2*S* enantiomer, whereas the related enzyme toluene dioxygenase (TDO) favors the 1*S*,2*R* enantiomer. The two different product orientations shown in brackets present opposite faces of the substrate to the catalytic iron center and probably account for the opposite enantiopreference. b) NDO catalyzes the 3,4-dihydroxylation of biphenyl to give the minor product (shown) with high stereoselectivity ($E > 100$; 3*R*,4*S*). The Phe352Val mutation reverses the enantiopreference ($E = 7.7$; 3*S*,4*R*). c) X-ray structure of a naphthalene dioxygenase (green) complexed with a dihydroxylated product (1,2-dihydronaphthalene-1,2-diol, orange carbon atoms) at the active-site iron atom (magenta sphere; PDB entry: 1o7p). The substrate-binding site of toluene dioxygenase differs from that of NDO at eight amino acid positions, seven of which are shown with gray bonds (sticks). (One such residue, Phe224, lies outside this view; see the 3D computer model in the Supporting Information). Phe352 (cyan) is the mutation that reverses the enantioselectivity of NDO for the 3,4-dihydroxylation of biphenyl.

3.4. Group 4: Same Protein Fold with Different Locations of a Catalytic Group

Vanillyl-alcohol oxidase (VAO) catalyzes the enantioselective oxidation of 4-ethylphenol to (*R*)-1-(4′-hydroxyphenyl)ethanol ($E = 32$),[39] whereas structurally related *p*-cresol methylhydroxylase (PCMH) forms the *S* enantiomer ($E \approx 2$; Figure 5).[40,41] The two enzymes share 32 % sequence identity and X-ray crystal structures show similar active sites and suggest similar reaction mechanisms.[42] Both enzymes contain a flavin cofactor, which oxidizes the phenol to a quinone methide intermediate. Next, water adds to one face of the quinone methide to form the product alcohol. The X-ray crystal structures revealed an aspartate residue in VAO near one face of the intermediate and a glutamate residue in PCMH near the opposite face. It is thought that these (catalytically equivalent) carboxylate groups position a water molecule appropriately to form the *R* alcohol in VAO and the *S* alcohol in PCMH. The location of these carboxylate groups on opposite sides of the substrate suggests that the opposite enantiopreference stems from this reversed arrangement of catalytic amino acid residues. This hypothesis was tested by van den Heuvel et al. by reversing the enantiopreference of VAO by site-directed mutagenesis.[43] They prepared a double mutant of VAO (Asp170Ser/Thr457Glu), whereby the aspartate residue was removed from one side of the intermediate, and a glutamate residue was introduced on the other side. As predicted, this double mutant showed opposite enantioselectivity and formed (*S*)-1-(4′-hydroxyphenyl)ethanol ($E = 9$; Figure 5).

All four classifications are based on the assumption that the enantiocomplementary active site is created by exchanging the locations in the active site of the binding sites for two groups at the stereocenter formed in the enzymatic reaction, as this hypothesis is compatible with the available examples. It may possible to create an enantiocomplementary enzyme by changing the location of only one binding site; however, no structurally characterized examples currently exist. The Supporting Information includes a discussion of this hypothesis.

4. Discovery and Creation of New Enantiocomplementary Enzymes

4.1. Why Enantiocomplementary Enzymes Exist

Nature may create enantiocomplementary enzymes to confer an evolutionary advantage, but may also create them accidentally. One evolutionary advantage would be the ability to use both enantiomers of a carbon or nitrogen source by using enantiocomplementary amino acid oxidases or aminotransferases. Similarly, the ability to activate both epimers of proteins containing methionine sulfoxides is probably also an evolutionary advantage. Enantiocomplementary epoxide hydrolases may be involved in the detoxification of chiral xenobiotics.[44] A nonenantioselective enzyme would confer similar advantages; however, nature rarely uses this ap-

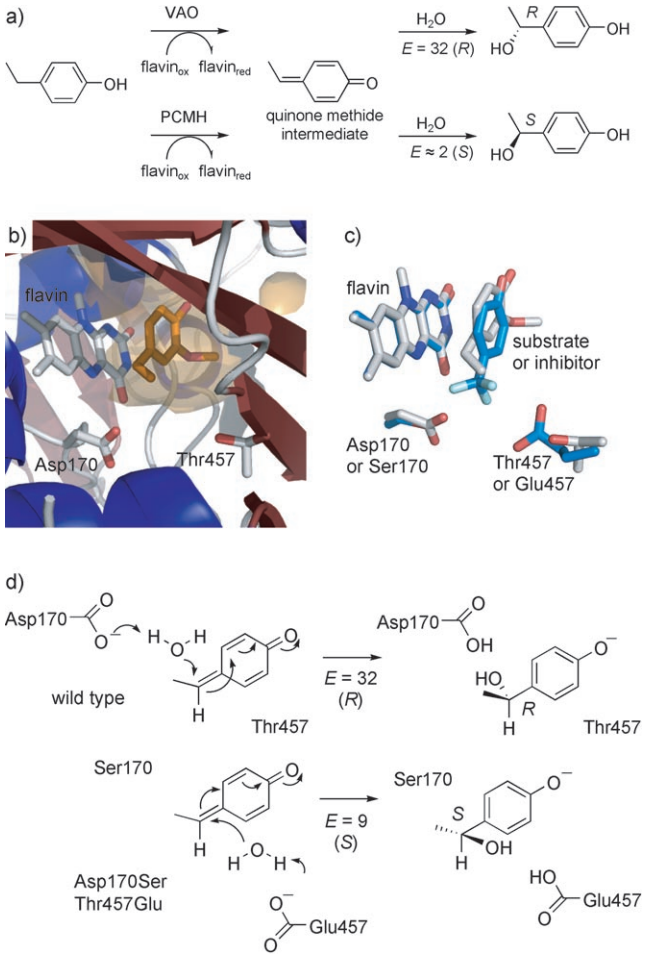

Figure 5. The flavin-containing enzymes vanillyl-alcohol oxidase (VAO) and *p*-cresol methylhydroxylase (PCMH) both catalyze the oxidation of 4-ethylphenol, but yield enantiomeric alcohols. a) In this transformation, the oxidation of 4-ethylphenol to a quinone methide is followed by the addition of water to form the alcohol. b) The X-ray structure of wild-type VAO (PDB entry: 2vao) shows the flavin cofactor (gray sticks), the substrate analogue 2-methoxy-4-vinylphenol (orange sticks), and two key catalytic residues, Asp170 and Thr457 (gray sticks). c) Site-directed mutagenesis of a catalytic group inverts the enantiopreference. The overlaying of the X-ray crystal structures of wild-type VAO (gray sticks) and the Asp170Ser/Thr457Glu double mutant (blue sticks; PDB entry: 1e0y) shows the carboxylate groups on opposite faces of the substrate analogues. d) The reaction mechanism involves the addition, catalyzed by the Asp170 carboxylate group, of a water molecule to the *Re* face (back) of the carbon–carbon double bond to form the *R* alcohol. Site-directed mutagenesis moved the carboxylate group to the opposite face of the quinone methide intermediate and reversed the enantiopreference.

proach. Perhaps nonenantioselective enzymes would be poor catalysts as a result of imprecise substrate positioning.[45]

One case in which enantiocomplementarity offers no evident evolutionary advantage (or disadvantage) is when the synthesis of either enantiomer achieves the metabolic goal. For example, the catabolism of aromatic compounds by naphthalene or toluene dioxygenases or the oxidation of vanillyl alcohol by vanillyl-alcohol oxidase or *p*-cresol methylhydroxylase proceeds to achiral catabolites regardless of which enantiomer is formed in the initial oxidation. Similarly, hydratases produce chiral intermediates for the synthesis and oxidation of fatty acids, the configuration of which is lost in subsequent reaction steps. Indeed, Filppula et al. replaced the yeast enzyme that catalyzes the second and third reactions of the β oxidation of fatty acids via D-3-hydroxyacyl-CoA intermediates with the corresponding rat enzyme, which catalyzes the same reactions via L-3-hydroxy intermediates.[46] Both stereochemical alternatives enabled the yeast cells to grow on oleic acid. The defensive release of cyanide from hydroxynitriles is similarly effective from *R* or *S* hydroxynitriles; the metabolic purpose of lactate dehydrogenase in glycolysis is the regeneration of NAD^+. Lactate is a waste product, so either enantiomer of the substrate is suitable.

Reactions involving non-natural substrates are another case in which enantiocomplementarity offers no obvious evolutionary advantage (or disadvantage), as evolutionary pressures do not play a role. For example, the natural role of lipases is lipid hydrolysis, and that of subtilisins is peptide hydrolysis; their enantiocomplementary behavior toward secondary alcohols is irrelevant to their natural function and simply fortuitous. Similarly, the natural function of hydantoinases ("dihydropyrimidinases") is probably the degradation of purine and pyrimidines (achiral substrates); therefore, the enantioselectivity of these enzymes toward hydantoins is irrelevant in nature.

4.2. Discovery of New Enantiocomplementary Enzyme Pairs in Nature

Besides the structurally characterized enantiocomplementary enzymes discussed in previous sections, there are many examples for which structural details are unknown. Enantiocomplementary terpene cyclases produce either enantiomer of α-pinene[47] or bornyl pyrophosphate.[48] Baker's yeast[49] and other yeasts[50] contain both L-selective and D-selective reductases, which reduce α- and β-ketoesters, often with high enantioselectivity. Different bacterial strains contain enantiocomplementary Baeyer–Villiger monooxygenases.[51]

This surprisingly common occurrence of enantiocomplementary enzyme pairs suggests that many more natural examples exist and that the screening of environmental samples is likely to yield new examples. Recent genome mining for nitrilases identified 137 new *R*- and *S*-selective nitrilases. The grouping of these nitrilases into clades showed that most members of a clade tended to have the same enantiopreference.[52]

4.3. Engineering Enzymes with Reversed Enantiopreference

It has been possible to reverse the enantiopreference of synthetically useful enzymes by protein engineering, either by exchanging the location of binding sites or by changing the location of a catalytic group (Table 2). For example, Raushel and co-workers reversed the enantioselectivity of organophosphorus hydrolase (OPH) by exchanging the binding sites (Figure 6). OPH shows moderate enantioselectivity ($E = 21$)

Table 2: Examples of the use of protein engineering or directed evolution to reverse enantiopreference ($E > 5$).

Enzyme	Substrate	Product	Mutation(s)	Enantioselectivity reversal	Group
organophosphorus hydrolase	ethyl phenyl 4-nitrophenyl phosphate	ethyl phenyl diphosphate (achiral)	His257Tyr Phe132Gly Ser308Gly Ile106Gly	$E = 21$ (S_P)[a] to $E > 100$ (R_P)	3, changes substrate orientation
naphthalene dioxygenase	biphenyl (achiral)	*cis*-3,4-dihydroxy-3,4-dihydro-biphenyl	Phe352Val	$E > 100$ (3*R*,4*S*) to $E = 7.7$ (3*S*,4*R*)	3, changes substrate orientation
horseradish peroxidase	thioanisole	methyl phenyl sulfoxide	Leu41His His42A	$E = 66$ (*S*)[b] to $E > 100$ (*R*)	3, changes substrate orientation
lipase from *Burkholderia cepacia*	1,4-dihydropyridine diester	1,4-dihydropyridinecarboxylic acid monoester	Phe221Leu Val266Leu Leu287Ile	$E = 6.5$ (*R*) to $E > 100$ (*S*)	3, changes substrate orientation
lipase from *Burkholderia cepacia*	ethyl 3-phenylbutyrate	3-phenylbutyrate	Leu17Phe[c] Phe119Leu Leu167Gly Leu266Val	$E = 33$ (*S*) to $E = 38$ (*R*)	3, changes substrate orientation
esterase from *Burkholderia gladioli*	methyl 2-hydroxy-2-methyl-propanoate	2-hydroxy-2-methylpropanoic acid	Leu135Phe Ile152Asn Val351Ser His253Phe	$E = 6.1$ (*S*) to $E = 29$ (*R*)	3, changes substrate orientation
esterase from *Bacillus subtilis*	1,1,1-trifluoro-2-phenyl-but-3-yn-1-yl acetate	1,1,1-trifluoro-2-phenylbut-3-yn-1-ol	Asp188Trp Met193Cys[d]	$E > 100$ (*R*) to $E = 64$ (*S*)	3, changes substrate orientation
lipase from *Pseudomonas aeruginosa*	*p*-nitrophenyl 2-methyl-decanoate	2-methyldecanoate	17 substitutions[e]	$E = 51$ (*S*) to $E = 30$ (*R*)	3, changes substrate orientation
lipase B from *Candida antarctica*	1-phenylethanol	1-phenylethyl butyrate	Trp104Ala	$E \gg 200$ (*R*) to $E = 13$ (*S*)	3, changes substrate orientation
vanillyl-alcohol oxidase	4-ethylphenol (achiral)	4-(1′-hydroxyethyl)phenol	Asp170Ser Thr457Glu	$E = 32$ (*R*) to $E = 9$ (*S*)	4, moves key catalytic group
arylmalonate decarboxylase	α-methyl-α-(2-thienyl)-malonic acid	α-(2-thienyl)propionic acid	Gly74Cys Cys188Ser	$E > 100$ (*S*) to $E = 32$ (*R*)	4, moves key catalytic group

[a] Raushel and co-workers also increased the S_P selectivity of the wild-type enzyme to more than 100 with a Gly60Ala mutation, which decreased the size of the small subsite. [b] The *S*-selective enzyme is the Phe41Leu mutant. Wild-type horseradish peroxidase shows a lower enantioselectivity of $E = 6$. [c] Both variants with reversed enantioselectivity contained the four mutations listed. One contained an additional Thr251Ala mutation, whereas the other contained an additional Asp21Asn mutation. The authors suggest that these additional mutations have little effect on enantioselectivity. [d] The wild-type enzyme has Glu188, not Asp188, but the Asp188 variant shows higher enantioselectivity for the *R*-configured substrate: $E > 100$ versus $E = 42$ for the wild type. [e] The wild-type enzyme showed almost no enantioselectivity ($E = 1.1$). Of the amino acid residues present in the wild-type enzyme, 11 were substituted to form the *R*-selective mutant, and a different set of six were substituted to form the *S*-selective enzyme. None of the mutations involved active-site residues.

toward phosphate triesters, such as ethyl phenyl 4-nitrophenyl phosphate.[53] The hydrolysis favors the S_P enantiomer of the substrate, but yields an achiral product, a phosphorus diester. The substrate-binding pocket contains both a small and a large subsite.[54] To reverse the enantiopreference, Raushel and co-workers first increased the size of the small subsite through the substitution of three amino acid residues, whereby the enantioselectivity was eliminated. Next, they decreased the size of the large subsite through the substitution of a histidine residue for a tyrosine residue, whereby the enantioselectivity was increased to $E > 100$ in favor of the R_P enantiomer. A similar mutant showed a reversed enantiopreference toward methyl phenyl phosphinates.[55]

Such pairwise mutagenesis in the active site may be the best strategy for reversing enantiopreference, as reversal involves cooperativity, and in this way the locations of two substituents of the chiral substrate can be changed. Indeed, multiple mutations have reversed the enantioselectivity of horseradish peroxidase,[56] the lipase from *Burkholderia cepacia*,[57,58] and esterases from *Burkholderia gladioli*[59] and *Bacillus subtilis*.[60] Of the nine examples in Table 2, only two involved a single mutation: A Phe352Val mutation in the

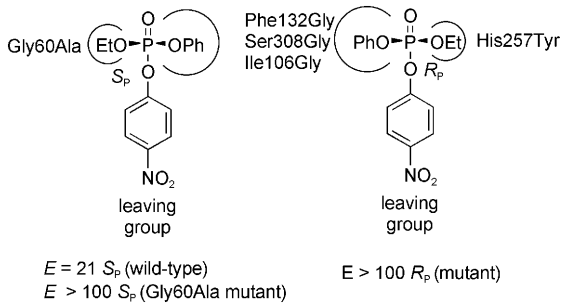

Figure 6. Organophosphorus hydrolase catalyzes the enantioselective hydrolysis of phosphate triesters. The wild-type enzyme favors the S_P enantiomer of ethyl phenyl 4-nitrophenyl phosphate ($E = 21$). The enantioselectivity was increased by decreasing the size of the small subsite through a Gly60Ala mutation. In contrast, when the sizes of the subsites were reversed through the four mutations shown, the enantiopreference was reversed.

active site of naphthalene dioxygenase reversed its enantiopreference in the hydroxylation of biphenyl,[33] and a Trp104Ala mutation in the active site of lipase B from *Candida antarctica* reversed its enantiopreference in the acylation of 1-phenylethanol.[61] In both cases, the enantioselectivity decreased from a high enantioselectivity ($E > 100$) to a moderate enantioselectivity for the other enantiomer (7.7 and 6.6, respectively). Additional mutations on the other side of the active site might increase the selectivity for the new enantiomer. Some attempts to reverse enantioselectivity by exchanging the positions of the binding sites were only partially successful or led to low enantioselectivities ($E < 5$).[62–65] These examples are not included in Table 2; nor are numerous examples that involve diastereomers, even if mutations reversed the enantioselectivity with respect to one center, as the presence of additional stereocenters complicates the interpretation. A change of solvent can also reverse the enantioselectivity (see, for example, reference [66]), an effect most likely due to solvation changes in the solvent-exposed portions of the substrate. This approach affects only substituents exposed to the solvent and has been less effective than protein engineering.

Directed evolution has also been used to reverse the enantioselectivity of enzymes; however, multiple mutations were required, possibly because of the difficulty in discovering cooperative mutations. Reetz and co-workers dramatically altered the enantiopreference of lipase from *Pseudomonas aeruginosa*.[67] The wild-type lipase was nonselective toward a 2-methyldecanoate ester ($E = 1.1$). Repeated random mutagenesis by different strategies combined with screening yielded two variants with good selectivity for opposite enantiomers. The *R*-selective lipase ($E = 30$) differed from the wild type at eleven amino acid positions, whereas the *S*-selective lipase ($E = 51$) differed in six other substitutions.

The repositioning of catalytic groups is another effective strategy for the reversal of enantiopreference. The reversal of the enantiopreference of vanillyl-alcohol oxidase by a double mutation was mentioned in Section 3.4. In another example, Ohta and co-workers reversed the enantiopreference of an arylmalonate decarboxylase by moving the cysteine residue to the other side of the active site.[68]

5. Summary and Outlook

Although some researchers have viewed enantioselectivity as a fundamental property of biological catalysis that is difficult to alter, these examples show that a few changes in amino acid residues can reverse the enantiopreference of an enzyme. Given that screening for a naturally occurring enantiocomplementary enzyme is also possible, it is no longer correct to cite the lack of readily available mirror-image enzymes as a disadvantage of biocatalysis.

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[1] Protein structures were compared by using the program DALI: L. Holm, J. Park, *Bioinformatics* **2000**, *16*, 566–567.
[2] R. C. L. Milton, S. C. F. Milton, S. B. H. Kent, *Science* **1992**, *256*, 1445–1448; commentary: G. Jung, *Angew. Chem.* **1992**, *104*, 1484–1486; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1457–1459.
[3] M. C. Fitzgerald, I. Chernushevich, K. G. Standing, S. B. H. Kent, C. P. Whitman, *J. Am. Chem. Soc.* **1995**, *117*, 11075–11080.
[4] B. Seelig, S. Keiper, F. Stuhlmann, A. Jäschke, *Angew. Chem.* **2000**, *112*, 4764–4768; *Angew. Chem. Int. Ed.* **2000**, *39*, 4576–4579.
[5] For a review, see: H. Griengl, A. Hickel, D. V. Johnson, M. Schmidt, C. Kratky, H. Schwab, *Chem. Commun.* **1997**, 1933–1940; H. Griengl, H. Schwab, M. Fechter, *Trends Biotechnol.* **2000**, *18*, 252–256.
[6] K. Gruber, G. Gartler, B. Krammer, H. Schwab, C. Kratky, *J. Biol. Chem.* **2004**, *279*, 20501–20510.
[7] I. Dreveny, K. Gruber, A. Glieder, A. Thompson, C. Kratky, *Structure* **2001**, *9*, 803–815.
[8] I. Dreveny, C. Kratky, K. Gruber, *Protein Sci.* **2002**, *11*, 292–300.
[9] The methionine sulfoxides are epimers, not enantiomers, because they have the same configuration at C^{α}, but different configurations at the sulfoxide group. The Msrs show enantiocomplementary behavior with respect to reaction at the sulfoxide stereocenter. For a review, see: B. Kauffmann, A. Aubry, F. Favier, *Biochim. Biophys. Acta Proteins Proteomics* **2005**, *1703*, 249–260; for reports of enantioselectivity, see: V. S. Sharov, D. A. Ferrington, T. C. Squier, C. Schoneich, *FEBS Lett.* **1999**, *455*, 247–250; R. Grimaud, B. Ezraty, J. K. Mitchell, D. Lafitte, C. Briand, P. J. Derrick, F. Barras, *J. Biol. Chem.* **2001**, *276*, 48915–48920.
[10] W. T. Lowther, H. Weissbach, F. Etienne, N. Brot, B. W. Matthews, *Nat. Struct. Biol.* **2002**, *9*, 348–352.
[11] For reviews, see: R. J. Kazlauskas, A. N. E. Weissfloch, *J. Mol. Catal. B* **1997**, *3*, 65–72; T. Ema, K. Yamaguchi, Y. Wakasa, A. Yabe, R. Okada, M. Fukumoto, F. Yano, T. Korenaga, M. Utaka, T. Sakai, *J. Mol. Catal. B* **2003**, *22*, 181–192; for the example in Table 1, see: S. Vorlova, U. T. Bornscheuer, I. Gatfield, J.-M. Hilmer, H.-J. Bertram, R. D. Schmid, *Adv. Synth. Catal.* **2002**, *344*, 1152–1155; P. A. Fitzpatrick, A. M. Klibanov, *J. Am. Chem.*

Soc. **1991**, *113*, 3166 – 3171; C. K. Savile, R. J. Kazlauskas, *J. Am. Chem. Soc.* **2005**, *127*, 12228 – 12229; see also Table S3 in the Supporting Information.
[12] M.-J. Kim, Y. I. Chung, Y. K. Choi, H. K. Lee, D. Kim, J. Park, *J. Am. Chem. Soc.* **2003**, *125*, 11494 – 11495.
[13] F. F. Huerta, A. B. E. Minidis, J.-E. Bäckvall, *Chem. Soc. Rev.* **2001**, *30*, 321 – 331.
[14] D. L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, M. Harel, S. J. Remington, I. Silman, J. Schrag, J. L. Sussman, K. H. G. Verschueren, A. Goldman, *Protein Eng.* **1992**, *5*, 197 – 211.
[15] P. F. Mugford, S. M. Lait, B. A. Keay, R. J. Kazlauskas, *ChemBioChem* **2004**, *5*, 980 – 987.
[16] M. Cygler, P. Grochulski, R. J. Kazlauskas, J. D. Schrag, F. Bouthillier, B. Rubin, A. N. Serreqi, A. K. Gupta, *J. Am. Chem. Soc.* **1994**, *116*, 3180 – 3186.
[17] S. Rhee, M. M. Silva, C. C. Hyde, P. H. Rogers, C. M. Metzler, D. E. Metzler, A. Arnone, *J. Biol. Chem.* **1997**, *272*, 17293 – 17302; R. A. John, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1995**, *1248*, 81 – 96.
[18] D. Peisach, D. M. Chipman, P. W. Van Ophem, J. M. Manning, D. Ringe, *Biochemistry* **1998**, *37*, 4958 – 4967; S. Sugio, G. A. Petsko, J. M. Manning, K. Soda, D. Ringe, *Biochemistry* **1995**, *34*, 9661 – 9669; K. Yonaha, H. Misono, T. Yamamoto, K. Soda, *J. Biol. Chem.* **1975**, *250*, 6983 – 6989.
[19] S. Umhau, L. Pollegioni, G. Molla, K. Diederichs, W. Welte, M. S. Pilone, S. Ghisla, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12463 – 12468; M. P. Simonetta, M. A. Vanoni, P. Casalin, *Biochim. Biophys. Acta* **1987**, *914*, 136 – 142.
[20] A. Mattevi, M. A. Vanoni, F. Todone, M. Rizzi, A. Teplyakov, A. Coda, M. Bolognesi, B. Curti, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 7496 – 7501; C. G. Mowat, A. Wehenkel, A. J. Green, M. D. Walkinshaw, G. A. Reid, S. K. Chapman, *Biochemistry* **2004**, *43*, 9519 – 9526.
[21] Z. X. Xia, F. S. Mathews, *J. Mol. Biol.* **1990**, *212*, 837 – 863.
[22] T. Fukui, N. Shiomi, Y. Doi, *J. Bacteriol.* **1998**, *180*, 667 – 673; Y.-M. Qin, M. H. Poutanen, H. M. Helander, A.-P. Kvist, K. M. Siivari, W. Schmitz, E. Conzelmann, U. Hellman, J. K. Hiltunen, *Biochem. J.* **1997**, *321*, 21 – 28.
[23] M. K. Koski, A. M. Haapalainen, J. K. Hiltunen, T. Glumoff, *J. Biol. Chem.* **2004**, *279*, 24666 – 24672.
[24] H. K. W. Kallwass, *Enzyme Microb. Technol.* **1992**, *14*, 28 – 35; W. Hummel, H. Schutte, M.-R. Kula, *Appl. Microbiol. Biotechnol.* **1985**, *21*, 7 – 15; H. Schutte, W. Hummel, M. R. Kula, *Appl. Microbiol. Biotechnol.* **1984**, *19*, 167 – 176.
[25] K. Niefind, H.-J. Hecht, D. Schomburg, *J. Mol. Biol.* **1995**, *251*, 256 – 281; U. Dengler, K. Niefind, M. Kiess, D. Schomburg, *J. Mol. Biol.* **1997**, *267*, 640 – 660.
[26] C. Vinals, E. Depiereux, E. Feytmans, *Biochem. Biophys. Res. Commun.* **1993**, *192*, 182 – 188.
[27] V. S. Stoll, M. S. Kimber, E. F. Pai, *Structure* **1996**, *4*, 437 – 447; K. Arai, T. Kamata, H. Uchikoba, S. Fushinobu, H. Matsuzawa, H. Taguchi, *J. Bacteriol.* **2001**, *183*, 397 – 400; T. Bhowmik, J. L. Steele, *Appl. Microbiol. Biotechnol.* **1994**, *41*, 432 – 439.
[28] P. D. Pawelek, J. Cheah, R. Coulombe, P. Macheroux, S. Ghisla, A. Vrielink, *EMBO J.* **2000**, *19*, 4204 – 4215; I. M. Moustafa, S. Foster, A. Y. Lyubimov, A. Vrielink, *J. Mol. Biol.* **2006**, *364*, 991 – 1002; see also: H. Zhang, M. Teng, L. Niu, Y. Wang, Y. Wang, Q. Liu, Q. Huang, Q. Hao, Y. Dong, P. Liu, *Acta Crystallogr. Sect. D* **2004**, *60*, 974 – 977; for biological properties, see: G. Ponnudurai, M. C. M. Chung, N.-H. Tan, *Arch. Biochem. Biophys.* **1994**, *313*, 272 – 378.
[29] O. Dym, E. A. Pratt, C. Ho, D. Eisenberg, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9413 – 9418; M. Futai, *Biochemistry* **1973**, *12*, 2468 – 2474.
[30] D. S. Torok, S. M. Resnick, J. M. Brand, D. L. Cruden, D. T. Gibson, *J. Bacteriol.* **1995**, *177*, 5799 – 5805.
[31] D. R. Boyd, N. D. Sharma, S. A. Haughey, M. A. Kennedy, B. T. McMurray, G. N. Sheldrake, C. C. R. Allen, H. Dalton, K. Sproule, *J. Chem. Soc. Perkin Trans. 1* **1998**, *1*, 1929 – 1934.
[32] N. I. Bowers, D. R. Boyd, N. D. Sharma, P. A. Goodrich, M. R. Groocock, A. J. Blacker, P. Goode, H. Dalton, *J. Chem. Soc. Perkin Trans. 1* **1999**, *1*, 1453 – 1461.
[33] R. E. Parales, S. M. Resnick, C. Yu, D. R. Boyd, N. D. Sharma, D. T. Gibson, *J. Bacteriol.* **2000**, *182*, 5495 – 5504.
[34] A. Karlsson, J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund, S. Ramaswamy, *Science* **2003**, *299*, 1039 – 1042.
[35] N. H. Yennawar, M. E. Conway, H. P. Yennawar, G. K. Farber, S. M. Hutson, *Biochemistry* **2002**, *41*, 11592 – 11601; J. Davoodi, P. M. Drown, R. K. Bledsoe, R. Wallin, G. D. Reinhart, S. M. Hutson, *J. Biol. Chem.* **1998**, *273*, 4982 – 4989.
[36] J. Andexer, J. von Langermann, A. Mell, M. Bocola, U. Kragl, T. Eggert, M. Pohl, *Angew. Chem.* **2007**, *119*, 8833 – 8835; *Angew. Chem. Int. Ed.* **2007**, *46*, 8679 – 8681.
[37] C. Syldatk, O. May, J. Altenbuchner, R. Mattes, M. Siemann, *Appl. Microbiol. Biotechnol.* **1999**, *51*, 293 – 309; O. Keil, M. P. Schneider, J. P. Rasor, *Tetrahedron: Asymmetry* **1995**, *6*, 1257 – 1260.
[38] Y. H. Cheon, H. S. Kim, K. H. Han, J. Abendroth, K. Niefind, D. Schomburg, J. Wang, Y. Kim, *Biochemistry* **2002**, *41*, 9410 – 9417; J. Abendroth, K. Niefind, O. May, M. Siemann, C. Syldatk, D. Schomburg, *Biochemistry* **2002**, *41*, 8589 – 8597.
[39] F. P. Drijfhout, M. W. Fraaije, H. Jongejan, W. J. H. van Berkel, M. C. R. Franssen, *Biotechnol. Bioeng.* **1998**, *59*, 171 – 177.
[40] A. Mattevi, M. W. Fraaije, A. Mozzarelli, L. Olivi, A. Coda, W. J. van Berkel, *Structure* **1997**, *5*, 907 – 920.
[41] W. McIntire, D. J. Hopper, J. C. Craig, E. T. Everhart, R. V. Webster, M. J. Causer, T. P. Singer, *Biochem. J.* **1984**, *224*, 617 – 621.
[42] L. M. Cunane, Z. Chen, N. Shamala, F. S. Mathews, C. N. Cronin, W. S. McIntire, *J. Mol. Biol.* **2000**, *295*, 357 – 374.
[43] R. H. H. van den Heuvel, M. W. Fraaije, M. Ferrer, A. Mattevi, W. J. H. van Berkel, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9455 – 9460.
[44] F. Oesch, *Prog. Clin. Biol. Res.* **1983**, *135*, 81 – 105.
[45] One exception is DL-2-haloacid dehalogenase, which displaces the halogen atom from both enantiomers with water at the same slow rate ($< 1\ U\,mg^{-1}$): V. Nardi-Dei, T. Kurihara, C. Park, N. Esaki, K. Soda, *J. Bacteriol.* **1997**, *179*, 4232 – 4238. Racemases, single enzymes that convert both substrate enantiomers with approximately equal efficiency by using functionally symmetrical active sites, are another exception.
[46] S. A. Filppula, R. T. Sormunen, A. Hartig, W.-H. Kunau, J. K. Hiltunen, *J. Biol. Chem.* **1995**, *270*, 27453 – 27457.
[47] M. A. Phillips, M. R. Wildung, D. C. Williams, D. C. Hyatt, R. Croteau, *Arch. Biochem. Biophys.* **2003**, *411*, 267 – 276.
[48] R. Croteau, D. M. Satterwhite, D. E. Cane, C. C. Chang, *J. Biol. Chem.* **1986**, *261*, 13438 – 13445.
[49] I. A. Kaluzna, T. Matsuda, A. K. Sewell, J. D. Stewart, *J. Am. Chem. Soc.* **2004**, *126*, 12827 – 12832.
[50] D. Zhu, Y. Yang, L. Hua, *J. Org. Chem.* **2006**, *71*, 4202 – 4205; D. Zhu, C. Mukherjee, J. D. Rozzell, S. Kambourakis, L. Hua, *Tetrahedron* **2006**, *62*, 901 – 905.
[51] P. Cernuchova, M. D. Mihovilovic, *Org. Biomol. Chem.* **2007**, *5*, 1715 – 1719.
[52] D. E. Robertson, J. A. Chaplin, G. DeSantis, M. Podar, M. Madden, E. Chi, T. Richardson, A. Milan, M. Miller, D. P. Weiner, K. Wong, J. McQuaid, B. Farwell, L. A. Preston, X. Tan, M. A. Snead, M. Keller, E. Mathur, P. L. Kretz, M. J. Burk, J. M. Short, *Appl. Environ. Microbiol.* **2004**, *70*, 2429 – 2436.
[53] M. Chen-Goodspeed, M. A. Sogorb, F. Wu, F. M. Raushel, *Biochemistry* **2001**, *40*, 1325 – 1331; M. Chen-Goodspeed, M. A. Sogorb, F. Wu, F. M. Raushel, *Biochemistry* **2001**, *40*, 1332 – 1339.

[54] J. L. Vanhooke, M. M. Benning, F. M. Raushel, H. M. Holden, *Biochemistry* **1996**, *35*, 6020 – 6025.
[55] Y. Li, S. D. Aubert, E. G. Maes, F. M. Raushel, *J. Am. Chem. Soc.* **2004**, *126*, 8888 – 8889.
[56] S.-I. Ozaki, P. R. Ortiz de Montellano, *J. Am. Chem. Soc.* **1994**, *116*, 4487 – 4488; M. I. Savenkova, S. L. Newmyer, P. R. Ortiz de Montellano, *J. Biol. Chem.* **1996**, *271*, 24598 – 24603.
[57] Y. Hirose, K. Kariya, Y. Nakanishi, Y. Kurono, K. Achiwa, *Tetrahedron Lett.* **1995**, *36*, 1063 – 1066.
[58] Y. Koga, K. Kato, H. Nakano, T. Yamane, *J. Mol. Biol.* **2003**, *331*, 585 – 592.
[59] M. Ivancic, G. Valinger, K. Gruber, H. Schwab, *J. Biotechnol.* **2007**, *129*, 109 – 122.
[60] S. Bartsch, R. Kourist, U. T. Bornscheuer, *Angew. Chem.* **2008**, *120*, 1531 – 1534; *Angew. Chem. Int. Ed.* **2008**, *47*, 1508 – 1511.
[61] A. O. Magnusson, M. Takwa, A. Hamberg, K. Hult, *Angew. Chem.* **2005**, *117*, 4658 – 4661; *Angew. Chem. Int. Ed.* **2005**, *44*, 4582 – 4585.
[62] R. Sakowicz, M. Gold, J. B. Jones, *J. Am. Chem. Soc.* **1995**, *117*, 2387 – 2394.
[63] O. May, P. T. Nguyen, F. H. Arnold, *Nat. Biotechnol.* **2000**, *18*, 317 – 320.
[64] M. T. Reetz, B. Brunner, T. Schneider, F. Schulz, C. M. Clouthier, M. M. Kayser, *Angew. Chem.* **2004**, *116*, 4167 – 4170; *Angew. Chem. Int. Ed.* **2004**, *43*, 4075 – 4078; M. D. Mihovilovic, F. Rudroff, A. Winninger, T. Schneider, F. Schulz, M. T. Reetz, *Org. Lett.* **2006**, *8*, 1221 – 1224.
[65] S. A. Funke, A. Eipper, M. T. Reetz, N. Otte, W. Thiel, G. Van Pouderoyen, B. W. Dijkstra, K.-E. Jaeger, T. Eggert, *Biocatal. Biotransform.* **2003**, *21*, 67 – 73.
[66] C. K. Savile, R. J. Kazlauskas, *J. Am. Chem. Soc.* **2005**, *127*, 12228 – 12229.
[67] D. Zha, S. Wilensek, M. Hermes, K. E. Jaeger, M. T. Reetz, *Chem. Commun.* **2001**, 2664 – 2665.
[68] Y. Terao, Y. Ijima, K. Miyamoto, H. Ohta, *J. Mol. Catal. B* **2007**, *45*, 15 – 20.