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Site-Saturation Mutagenesis of Tryptophan 116 of *Saccharomyces pastorianus* Old Yellow Enzyme Uncovers Stereocomplementary Variants

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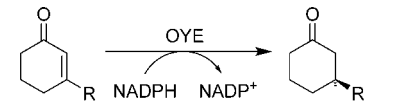
Abstract: Site-saturation mutagenesis was used to generate all possible replacements for Trp 116 of *Saccharomyces pastorianus* (formerly *Saccharomyces carlsbergensis*) old yellow enzyme (OYE). Our original hypothesis—that smaller amino acids at position 116 would allow better acceptance of bulky 3-alkyl-substituted 2-cyclohexenones—proved incorrect. Instead, Phe and Ile replacements favored the binding of some substrates in an opposite orientation, which yielded reversed stereochemical outcomes compared to that of the wild-type OYE. For example, W116I OYE reduced (*R*)- and (*S*)-carvone to enantiomeric products, rather than the diastereomers produced by the wild-type OYE. Deuterium labeling revealed that (*S*)-carvone reduction by the W116I OYE occurred by the same pathway as that by the wild type (net *trans*-addition of H_2), proving that different substrate binding orientations were responsible for the divergent products. Trp 116 mutants also afforded different stereochemical outcomes for reductions of (*R*)-perillaldehyde and neral. Preliminary studies of an OYE family member whose native sequence contains Ile at position 116 (*Pichia stipitis* OYE 2.6) revealed that this enzyme's stereoselectivity matched that of the wild-type *S. pastorianus* OYE, showing that the identity of the residue at position 116 does not solely determine the substrate binding orientation. Computational docking studies using an induced fit methodology successfully reproduced the majority of the experimental outcomes. These computational tools will allow preliminary in silico screening of additional residues to identify those most likely to control the substrate binding orientation and provide some guidance to future experimental studies.

Introduction

The increasing demand for optically pure building blocks has fueled interest in identifying and creating new stereoselective catalysts. Asymmetric alkene reductions are an attractive reaction in this regard since two adjacent sp^3 centers are formed in a single step. In 1995, Massey reported that *Saccharomyces pastorianus*[1] old yellow enzyme (OYE) reduced activated alkenes such as α,β-unsaturated aldehydes and ketones as well as nitroolefins.[2] Müller et al. recently disclosed that this enzyme also reduces an activated alkyne.[3] We[4−6] and others[7−11] have explored the substrate selectivity and stereoselectivity of the *S. pastorianus* OYE and other OYE family members for synthetic applications. Despite many successes in creating chiral building blocks using OYEs, however, two key problems remained. First, catalytic efficiency generally declined as the substrate size increased. This phenomenon was particularly apparent in our studies of 3-alkyl-substituted 2-cyclohexenones,[4] and it limits the enzyme's practical utility (Scheme 1). Second, OYE family members possessed identical stereoselectivities for nearly all the substrates examined.[12] During our efforts to address the first problem of low rates for larger substrates, we unexpectedly uncovered a solution to the lack of stereochemical diversity in these enzymes. Specifically, we found that conservative changes to a single residue (Trp 116) inverted the stereoselectivity of *S. pastorianus* OYE. This opens many possibilities for using this enzyme and related biocatalysts in asymmetric synthesis.

Scheme 1



1a R = H
b R = Me
c R = Et

2a >98% conversion
b 76% conversion
c 25% converion

(1) Formerly known as *Saccharomyces carlsbergensis*.
(2) Vaz, A. D. N.; Chakraborty, S.; Massey, V. *Biochemistry* **1995**, *34*, 4246–4256.
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(4) Swiderska, M. A.; Stewart, J. D. *J. Mol. Catal. B* **2006**, *42*, 52–54.
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10.1021/ja8081389 CCC: $40.75

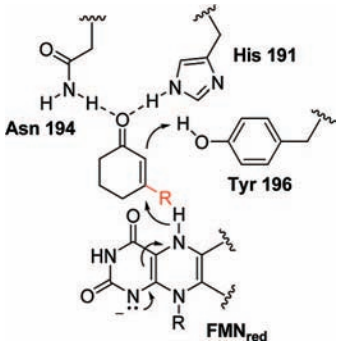

Figure 1. Catalytic mechanism of *S. pastorianus* OYE. Net *trans*-addition of H_2 occurs by hydride attack on the β-carbon with concomitant α-protonation by Tyr 196.

Both the structure and mechanism of the prototype *S. pastorianus* OYE have been well-characterized since its initial discovery in 1932.[13] Net *trans*-addition of H_2 proceeds by initial reduction of the noncovalently bound FMN cofactor at N_5 by the *pro*-(*R*)-hydride of NADPH (Figure 1). $NADP^+$ dissociation precedes binding of the alkene substrate. In the case of α,β-unsaturated aldehydes and ketones, hydrogen bonds contributed by the side chains of His 191 and Asn 194 further polarize the alkene and help orient the substrate within the active site.[14] Hydride is delivered from N_5 to the alkene β-carbon on the side facing the reduced FMN with concomitant α-carbon protonation by the side chain of Tyr 196 on the opposite alkene side to complete the reaction. Several other amino acids also participate in the catalytic mechanism, and mutations of Thr 37, Gln 114, Arg 243, and Tyr 375 all diminished catalytic activity.[15,16] The lack of stereochemical diversity in OYE-mediated alkene reductions is a natural consequence of the multipoint binding of the alkene substrate and FMN along with the fixed location of the highly conserved Tyr 196 side chain.

Results and Discussion

We suspected that diminished rates for OYE-mediated reductions of substrates such as 3-ethyl-2-cyclohexenone (**1c**) were a consequence of unfavorable steric interactions with active site residues, and we hoped that increasing the active site volume would alleviate these adverse contacts. To guide these efforts, we modeled a possible Michaelis complex of **1c** (Figure 2) and focused attention on residues within 5 Å of the bound substrate as candidates for replacement. After elimination of those known to be important for catalytic activity,[17] four candidates remained (Trp 116, Phe 250, Pro 295, and Phe 296). From these, we selected Trp 116 since its side chain is closest to the β-substituents of 2-cyclohexenones and changes at this position would be expected to have the largest effect on increasing the active site volume.

We chose a site-saturation mutagenesis/screening approach since it was not clear which residue(s) would be the optimal replacements for Trp 116. The 5′- and 3′-portions of the *S. pastorianus OYE1* gene were amplified separately by polymerase chain reaction (PCR) using degenerate primers so that all possible codons were included at position 116 (Figure 3). The degenerate primers also created 44 bp homologous regions on the two *OYE1* gene fragments centered on position 116. The other two PCR primers introduced homologous flanking regions (40 bp) that matched sequences in the yeast expression plasmid pYES2. Equimolar quantities of gel-purified PCR fragments were mixed with pYES2 that had previously been digested with *Hin*dIII and *Xba*I, and then the mixture was transformed into *S. cerevisiae* cells.[18] This organism is highly proficient at homologous recombination, and three separate events occurred in vivo to assemble circular plasmids (pSKP3-W116X) that were

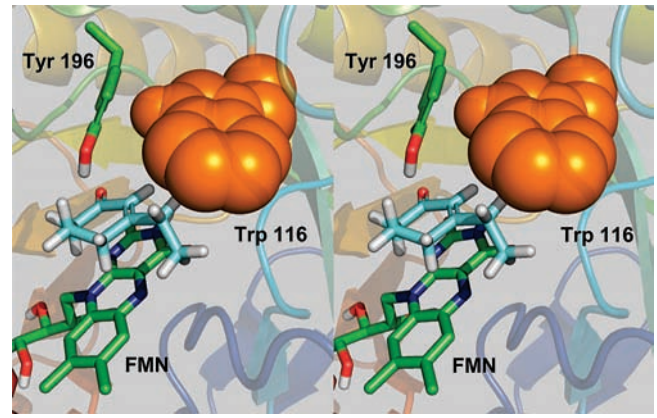

Figure 2. Modeled Michaelis complex of 3-ethyl-2-cyclohexenone (**1c**) within the active site of *S. pastorianus* OYE. The model is based on coordinates from the complex with 4-hydroxybenzaldehyde. A low-energy conformation of the substrate (shown in stick form with light blue carbon atoms) was overlaid with the aromatic ring of the inhibitor, matching the enone carbonyl with the phenolic oxygen. FMN and the side chain of Tyr 196 are depicted in stick form with green carbon atoms, and the side chain of Trp 116 is shown in space-filling form in orange. Steric interference between the Trp 116 side chain and the substrate's β-substituent is readily apparent.[55]

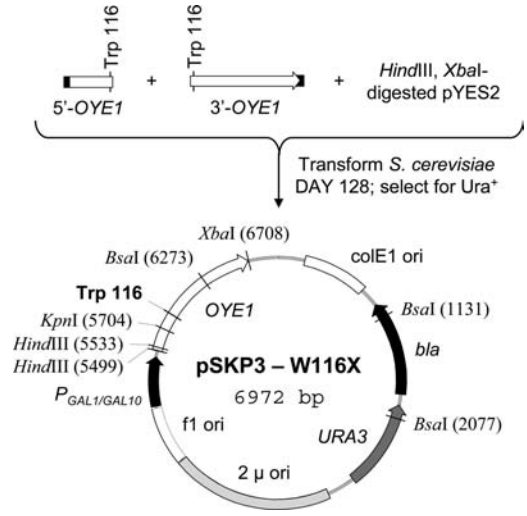

Figure 3. Assembly of plasmids encoding a library of random replacements for Trp 116 by homologous recombination. PCR primers used to amplify the upstream portion of the *OYE1* gene incorporated a 5′-region (40 bp) homologous to a portion of pYES2 and all possible codons at position 116. Analogous primers were used to amplify the downstream portion of the *OYE1* gene. Because of the overlapping sequences on the three linear fragments, they were recombined in vivo after transformation into *S. cerevisiae* to yield circular plasmids whose sequences were identical, except for codon 116. In these plasmids (designated pSKP3-W116X), the *OYE1* gene is under control of the *S. cerevisiae GAL1/GAL10* promoter.

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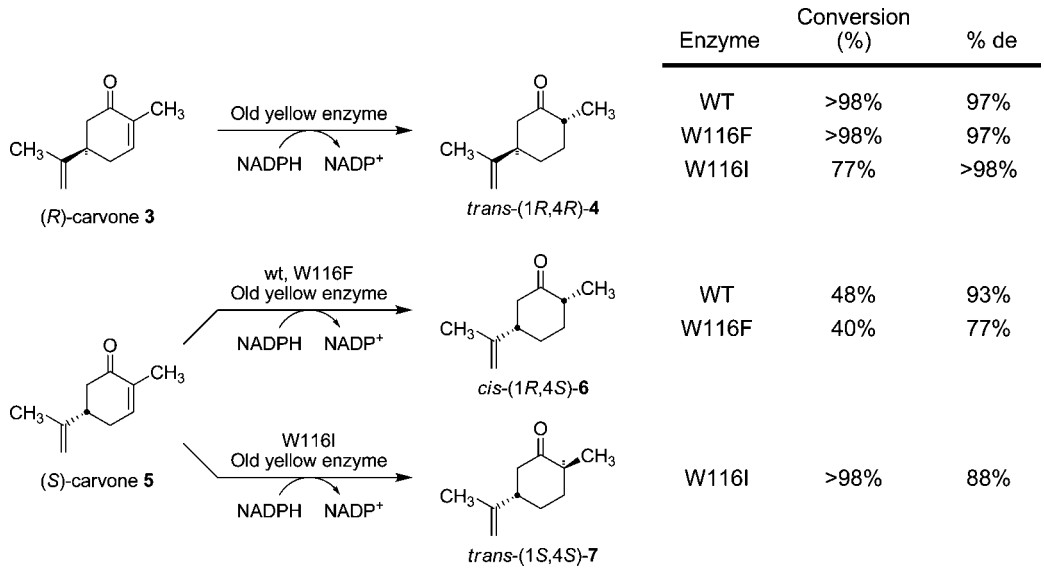
(17) Thr 37, Gln 114, His 191, Asn 194, Tyr 196, Arg 243, and Tyr 375.

Scheme 2



Enzyme	Conversion (%)	% de
WT	>98%	97%
W116F	>98%	97%
W116I	77%	>98%
WT	48%	93%
W116F	40%	77%
W116I	>98%	88%

selected by yeast cell growth in the absence of uracil. The host strain (DAY 128)[19] was a double knockout unable to produce either native OYE[20] so that all alkene reductase activity was due to the cloned *S. pastorianus* OYE variants. The primary library contained 312 colonies, a population sufficient to encompass all possible amino acids at position 116 of OYE.[21] PCR analysis of 10 randomly selected yeast colonies revealed that all 10 possessed an intact *OYE1* gene.

A total of 200 randomly chosen colonies from the library of Trp 116 replacements were screened for catalytic activity toward 3-methyl-2-cyclohexenone (**1b**) in a liquid medium. The 19 colonies that provided at least half the level of product afforded by the wild-type (wt) OYE control were selected for further study. DNA sequencing revealed that 6 encoded Trp at position 116, 6 encoded Phe, 3 encoded Ile (1 with an extra mutation), and there were single examples of Leu, Met, and Tyr.[22] Because Phe and Ile were found most commonly, these variants were chosen for further characterization. *Escherichia coli* overexpression plasmids were constructed in which the *S. pastorianus OYE1* gene was fused at its N-terminus to the glutathione *S*-transferase (GST) gene to facilitate protein purification. The wild-type, W116F, and W116I OYE proteins were expressed and purified as GST-fusion proteins as described previously.[23]

We expected that OYE variants with smaller residues at position 116 would have higher reduction rates for β-substituted 2-cyclohexenones; however, this was not observed. In fact, both the Phe and Ile OYE mutants had lower specific activities for both **1b** and **1c** than the wild-type values, although the enantioselectivities were identical (>98% ee favoring the (*S*)-products). We also screened whole yeast cells expressing the W116L, W116 M, and W116Y OYE variants against **1c**, but none displayed higher catalytic efficiency than the wild-type control. We did not attempt to measure steady-state kinetic parameters since the substrates are only partially soluble under the reaction conditions and values of K_M and k_{cat} would therefore be significantly impacted by incomplete partitioning into the aqueous phase.

Since our initial hypothesis relating improved reaction rates with the identity of the residue at position 116 proved incorrect, we tested purified wild-type, W116F, and W116I OYE proteins against a panel of representative enones and enals to see whether these mutations had other effects on OYE properties. The enantiomers of carvone provided a surprising result: while (*R*)-carvone (**3**) was reduced by all three proteins to the same *trans*-product **4** with very high diastereoselectivity, the fate of the (*S*)-antipode **5** depended on the identity of the residue at position 116 (Scheme 2). The wild-type and W116F reduced (*S*)-**5** to *cis*-**6**, the product expected from the arrangement shown in Figure 1. By contrast, the W116I mutant afforded *trans*-**7** from (*S*)-**5**, the enantiomer of the product that this protein made from (*R*)-**3**.

The origin of the altered stereoselectivity displayed by the W116I OYE variant was probed by using (4*R*)-NADPD as the cofactor for reducing (*S*)-**5**. MS analysis of the *trans*-product revealed incorporation of a single deuterium atom, as expected (76% of the total product). The ^{1}H NMR spectrum showed that the C_6 axial proton (H_{6ax}) of **7** had been deuterated (Figure 4). This assignment was supported by careful analysis of the coupling patterns. In nondeuterated **7**, H_{6ax} appears at 1.38 ppm as a dddd; three J values are coincidentally 13 Hz ($J_{6ax}J_{6eq}$, $J_{6ax}J_{1ax}$, and $J_{6ax}J_{5ax}$), while the fourth is smaller (3.8 Hz, $J_{6ax}J_{5eq}$). When H_{6ax} was replaced by deuterium, the intensity of the 1.38 ppm signal was significantly depressed and the loss of all four couplings was apparent in the expected signals. Taken together, these data indicate that the W116I mutant carries out net *trans*-addition of H_2 across the double bond of (*S*)-**5**; however, the substrate binds in a "flipped" orientation compared to that of (*R*)-**3** (Figure 5). To the best of our knowledge, this is the first time that an alternative substrate binding mode has been created by protein engineering for a member of the OYE family of alkene reductases. This has obvious utility for obtaining otherwise inaccessible alkene reduction products.

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(20) Encoded by the *S. cerevisiae OYE2* and *OYE3*genes.
(21) While the plasmids could also be assembled in vivo in *Escherichia coli* cells (see ref 37), the lower homologous recombination efficiency of this organism yielded library sizes too small to encompass all possible Trp 116 substitutions.
(22) One clone contained a mixed population of *OYE1* genes, so it was eliminated from further consideration.
(23) Kaluzna, I. A.; Matsuda, T.; Sewell, A. K.; Stewart, J. D. *J. Am. Chem. Soc.* **2004**, *126*, 12827–12832.

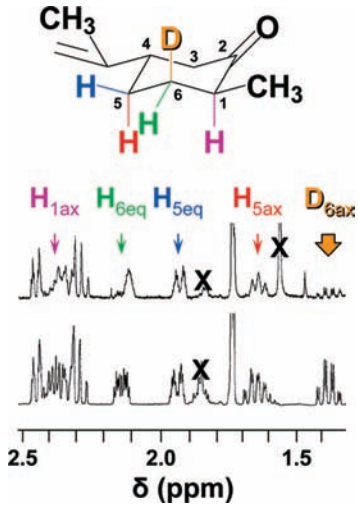

Figure 4. NMR analysis of (*S*)-carvone reduction products. Data from the top spectrum were obtained using (4*R*)-NADPD as the cofactor and the W116I mutant OYE. The bottom spectrum was obtained from commercially available *trans*-(1*R*,4*R*)-**4**. From these data, it is clear that H_{6ax} is replaced by deuterium when a labeled cofactor is used. Signals marked with "X" are due to an impurity.

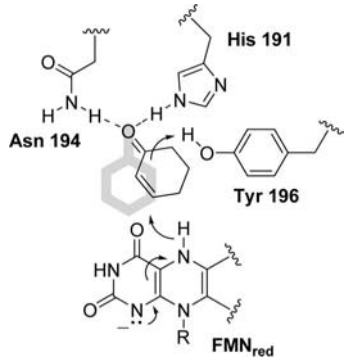

Figure 5. Alternate, "flipped" substrate binding mode allowed by Trp 116 substitutions. The 2-cyclohexenone backbone can be flipped from the orientation shown in Figure 1 while maintaining an identical relative position of the β-carbon and a similar orientation of the carbonyl oxygen. The gray outline represents the "normal" substrate binding mode as depicted in Figure 1.

We examined three carvone analogues to determine which structural feature(s) lead to the flipped substrate binding mode observed for (*S*)-**5** (Table 1). 2-Methyl-2-cyclohexenone (**8**), which lacks the isopropylidene side chain, was reduced by the wild-type OYE and both mutant OYEs to the same (*R*)-product in >98% ee. Likewise, all three proteins reduced (4*R*)-**10** to the same *trans*-product, consistent with their behavior toward (*R*)-carvone.[24] These results indicate that all three enzymes bind both **8** and **10** in the "normal" orientation. Likewise, the wild-type and W116F OYEs reduced (4*S*)-**12** to *cis*-**13**, which arose from normal substrate binding. The W116I variant, on the other hand, bound (4*S*)-**12** in the flipped orientation to produce the *trans*-product **14**. Taken together, these data indicate that a C_4 substituent in the (*S*)-configuration (carvone numbering) is necessary for a cyclohexenone to bind in the flipped orientation to W116I OYE, but some flexibility in the substituent's structure is allowable.

(24) GC peak assignments for products derived from **10** and **12** (*trans* followed by *cis*) were made by analogy to those from (*R*)- and (*S*)-carvone.

Two additional substrates showed different binding orientations between the OYE variants (Table 2). While (*S*)-perillaldehyde (**15**) was reduced by all enzymes to the expected *trans*-product, the (*R*)-antipode **17** was converted to the *cis*- and *trans*-products **18** and **16** by the wild-type and W116I OYEs, respectively. In this case, however, the conversions and stereoselectivities were only modest. The geometric isomers geranial and neral also showed different reduction behavior. The wild-type and W116F OYEs reduced geranial (**19**) with very high stereoselectivities to the same product, (*R*)-citronellal (**20**). On the other hand, these two enzymes reduced neral (**21**) predominantly to opposite enantiomers **20** and **22**, respectively.

We also probed the preference for normal and flipped substrate binding modes computationally, taking advantage of the increasingly accurate algorithms[25,26] that have proven effective in other systems.[27−31] The X-ray crystal structure of wild-type OYE with bound *p*-hydroxybenzaldehyde[32] was used as the starting point for all docking studies. Initial analysis of (*R*)- and (*S*)-carvone binding with a rigid protein yielded calculated binding modes inconsistent with the experimental data. All subsequent studies therefore utilized an induced fit methodology. This involves in silico mutation of selected amino acids to Ala followed by initial ligand docking, in silico replacement of Ala residues with the native side chains, and energy minimization to yield candidate complexes. Since Phe 296 and Tyr 375 had the highest crystallographic *B* factors of residues within 5 Å of *p*-hydroxybenzaldehyde, these two residues were chosen for temporary in silico mutation to Ala. Calculations were carried out for both (*R*)- and (*S*)-carvone. Candidate complexes were accepted only if they met three criteria: (1) hydrogen bonds were observed between the substrate carbonyl oxygen and the side chains of both His 191 and Asn 194, (2) the distance between the FMN N_5 atom and the β-carbon of the enone was $\leq$4.1 Å, and (3) the angle of hydride attack was between 86° and 111.8°. The first constraint was based on mutagenesis studies of His 191 and Asn 194, while the second and third constraints were derived from Fraaije and Mattevi's analysis of flavoprotein crystal structures[33] and our own examination of OYE crystal structures that included a cocrystallized ligand. Analogous calculations were carried out for the W116F and W116I mutant OYEs (Table 3). For reasons that remain unclear, no calculated substrate complexes of the W116F OYE met the three criteria outlined above and no predictions could be made for this protein's behavior toward (*R*)- and (*S*)-carvone.

In the case of (*R*)-carvone (**3**), the substrate was predicted to bind to the wild-type and W116I mutant OYE in the normal

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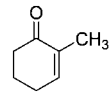
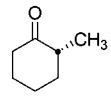
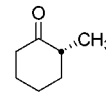
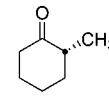
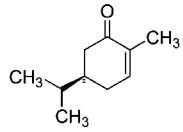
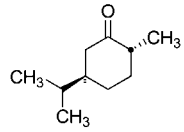
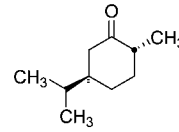
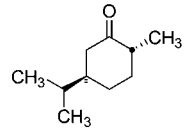
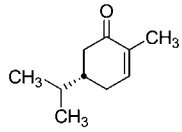
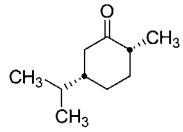
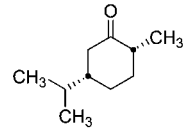
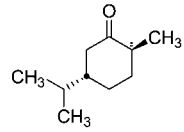
Table 1. Reductions of Carvone Analogues by Wild-Type and Mutant Old Yellow Enzymes

Entry	Substrate	Enzyme		
		WT	**W116F**	**W116I**
1				
	8	(*R*)-**9**	(*R*)-**9**	(*R*)-**9**
	Conversion (4 h)	>98%	>98%	>98%
	% ee	>98%	>98%	>98%
2				
	(4*R*)-**10**	*trans*-(1*R*,4*R*)-**11**	*trans*-(1*R*,4*R*)-**11**	*trans*-(1*R*,4*R*)-**11**
	Conversion (24 h)	75%	91%	84%
	% de	>98%	>98%	57%
3				
	(4*S*)-**12**	*cis*-(1*R*,4*S*)-**13**	*cis*-(1*R*,4*S*)-**13**	*trans*-(1*S*,4*S*)-**14**
	Conversion (24 h)	59%	49%	>98%
	% de	>98%	89%	>98%

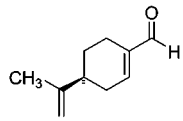
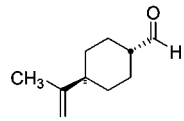
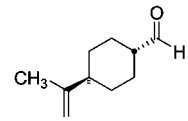
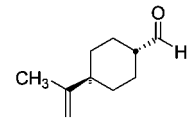
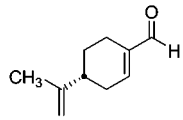
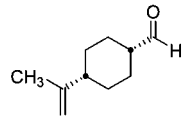
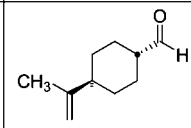
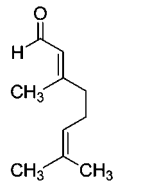
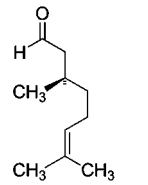
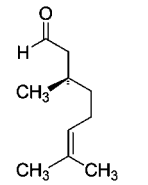
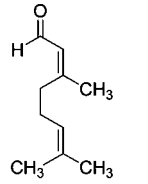
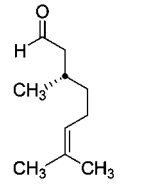
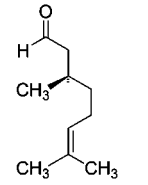
mode, e.g., aligned most closely with *p*-hydroxybenzaldehyde, in accord with the observed stereochemical outcomes of these reductions (Table 3). For (*S*)-carvone (**5**), the docking calculations were consistent with opposite substrate binding modes for the wild-type OYE versus the W116I OYE. The reason for this can be seen in Figure 6A, where replacement of Trp 116 with Ile has opened additional active site space that allows the isopropenyl substituent of **5** to extend into the area previously occupied by the indole ring of Trp 116. Simple cyclohexenone **8** lacks a bulky side chain. As observed experimentally, the best calculated Michaelis complexes for both the wild-type and W116I OYEs predicted a normal substrate binding mode. The calculated results were equivocal for W116F OYE in this case, with good complexes suggested in both the normal and flipped binding modes. Experimentally, (*S*)-perillaldehyde was reduced by all three enzymes from a normal substrate binding mode. This was predicted correctly for the wild-type enzyme; however, no consensus or an incorrect prediction was obtained for the W116F and W116I OYEs, respectively. We have not uncovered a suitable explanation for these failures. By contrast, the divergent binding behavior of (*R*)-perillaldehyde by the wild-type and W116I OYEs was reproduced successfully by our calculations. Taken together, these results suggest that in silico studies may provide at least some useful guidance in designing new OYE variants with altered stereoselectivities that accept even larger substrates. It should also be noted that the calculated substrate complexes support our initial hypothesis that replacing Trp 116 with smaller amino acids would expand the size of the OYE binding pocket in a useful fashion.

Our data clearly show that Trp 116 plays a critical role in the stereochemistry of OYE-mediated alkene reductions by influencing the substrate orientation within the active site. This residue is highly conserved in OYE family members, and of the 100 closest matches for the amino acid sequence of *S. pastorianus* OYE (as determined by BLAST analysis), 91 retain Trp at this position. Of the nine that do not, three contain Phe and three have Ile. Given the influence of Phe and Ile at position 116 of *S. pastorianus* OYE, we asked whether OYE homologues whose native sequences contained Phe or Ile at this location would show the same stereoselectivities as those of the corresponding *S. pastorianus* OYE mutants. We chose two representative OYEs from the recently sequenced genome of the xylose-fermenting yeast *Pichia stipitis* CBS 6054 (ATCC 58785).[34] One (OYE 3.3)[35] contained Phe at position 116 (*S. pastorianus* OYE numbering), while the second (OYE 2.6)[36] had Ile at this location. The genes were amplified from *P. stipitis* genomic DNA and cloned by homologous recombination[37] into an *E. coli* expression plasmid that introduced an N-terminal GST affinity tag. Unfortunately, only one of the two fusion proteins (OYE 2.6) was produced at a high level. The OYE 2.6 protein was expressed and purified as described previously, and the isolated enzyme was used to reduce **3**, **5**, **15**, and **17**. In all cases, the products of these reactions were identical to those produced by the wild-type *S. pastorianus* OYE. This demonstrated clearly that Trp 116 is not the sole determinant of stereoselectivity in OYEs and further suggests that it may be possible to alter stereoselectivities further by focusing on other residues that differ between OYE 2.6 and the *S. pastorianus* OYE.

We have recently disclosed one strategy for obtaining stereocomplementary alkene reduction products by employing

(34) Jeffries, T. W.; Grigoriev, I. V.; Grimwood, J.; Laplaza, J. M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; H., S.; Jin, Y.-S.; Passoth, V.; Richardson, P. M. *Nat. Biotechnol.* **2007**, *25*, 319–326.
(35) Locus EAZ64015.
(36) Locus XP_001384055.
(37) Parrish, J. R.; Limjindaporn, T.; Hines, J. A.; Liu, J. Y.; Liu, G. Z.; Finley, R. L. *J. Proteome Res.* **2004**, *3*, 582–586.

Table 2. Other Alkene Reductions by Wild-Type and Mutant Old Yellow Enzymes[c]

Entry	Substrate	Enzyme		
		WT	W116F	W116I
1				
	(*S*)-perillaldehyde **15**	*trans*-**16**	*trans*-**16**	*trans*-**16**
	Conversion (26 h)	>98%[a]	>98%[a]	19%
	% de	71%	96%	92%
2			---[b]	
	(*R*)-perillaldehyde **17**	*cis*-**18**		*trans*-**16**
	Conversion (26 h)	42%	---	20%
	% de	79%	---	52%
3				---
	geranial **19**	(*R*)-citronellal **20**	(*R*)-citronellal **20**	
	Conversion (24 h)	>98%	>98%	---
	% ee	90%	90%	---
4				---
	neral **21**	(*S*)-citronellal **22**	(*R*)-citronellal **20**	
	Conversion (24 h)	38%	97%	---
	% ee	19%	65%	---

[a] The over-reduction product (primary alcohol) was the major product. [b] Substrate conversion of <10% was observed. [c] The major product is indicated.

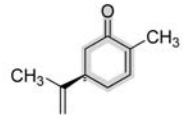
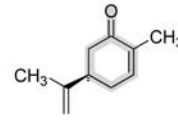
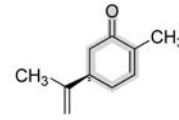
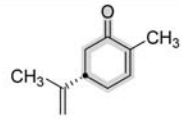
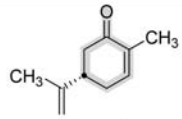
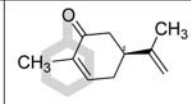
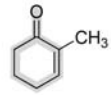
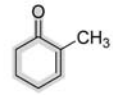
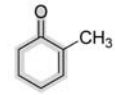
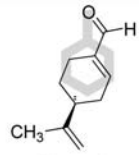
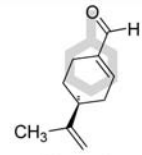
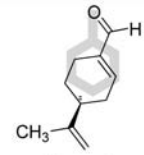
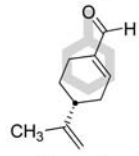
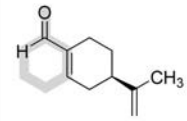
a single enzyme capable of following two alternate mechanistic pathways (net *cis*- versus net *trans*-reductions).[6] While this stereochemical divergence was highly interesting from an enzyme mechanism standpoint, the behavior was limited to a single pair of substrate enantiomers ((*R*)- and (*S*)-perillaldehyde), which severely limited its practical utility. Faber has recently shown that wild-type alkene reductases can bind at least one alkene substrate (2-methyl-2-cyclopentenone) in an opposite orientation compared to other substrates,[10,11] although the generality of this observation remains to be demonstrated. By contrast, identifying pairs of naturally occurring alkene reductases with complementary stereoselectivities offers a more general route to obtaining both product enantiomers. One example involving native OYE family members has been reported by Faber and co-workers for a nitroalkene reduction.[11,38] The protein engineering strategy described here is another approach that may be even more general in opening the door to engineered variants with diverse stereoselectivities with applications in organic synthesis. Similar approaches have proven very effective in other enzymes (for recent reviews, see refs 39 and 40). These studies also underscore the role of other residues in addition to that at position 116 in controlling the orientation of substrate binding. Combining sequences from several OYE family members may lead to variants with even more useful properties.

Experimental Section

General Procedures. Restriction endonucleases, *Taq* DNA polymerase, and DNA ligase were purchased from New England Biolabs. Primers were obtained from Integrated DNA Technologies. Plasmid pET3b-OYE[41] was provided by Professor Vincent Massey's laboratory. *S. cerevisiae* strain DAY128 (*ura3*-52, *leu2*Δ1, *his3*Δ200, *trp1*Δ63, *oye2*-Δ2::*TRP1*, *oye3*-Δ2::*HIS3*) was a gener-

(38) Stueckler, C.; Hall, M.; Ehammer, H.; Pointner, E.; Kroutil, W.; Macheroux, P.; Faber, K. *Org. Lett.* **2007**, *9*, 5409–5411.
(39) Toscano, M. D.; Woycechowsky, K. J.; Hilvert, D. *Angew. Chem., Int. Ed.* **2007**, *46*, 3212–3236.
(40) Mugford, P. F.; Wagner, U. G.; Jiang, Y.; Faber, K.; Kazlauskas, R. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8782–8793.
(41) Saito, K.; Thiele, D. J.; Davio, M.; Lockridge, O.; Massey, V. *J. Biol. Chem.* **1991**, *266*, 20720–20724.

Table 3. Calculated Substrate Binding Modes Using Induced Fit Methodology[c]

Entry	Substrate	Enzyme		
		WT	**W116F**	**W116I**
1	(*R*)-carvone **3**			
	Experimental	Normal	Normal	Normal
	Predicted	Normal	No acceptable poses[a]	Normal
2	(*S*)-carvone **5**			
	Experimental	Normal	Normal	Flipped
	Predicted	Normal	No acceptable poses	Flipped
3	2-methyl-2-cyclohexenone **8**			
	Experimental	Normal	Normal	Normal
	Predicted	No suitable poses	No prediction[b]	Normal
4	(*S*)-perillaldehyde **15**			
	Experimental	Normal	Normal	Normal
	Predicted	Normal	No prediction[b]	Flipped
5	(*R*)-perillaldehyde **17**		---	
	Experimental	Normal	No reaction	Flipped
	Predicted	Normal	---	Flipped

[a] No calculated complex successfully met all three criteria for a productive Michaelis complex. [b] Nearly isoenergetic calculated complexes predicted both normal and flipped binding orientations; no consensus prediction could be made in such cases. [c] The experimentally observed orientation of the bound substrate is shown. The gray outline corresponds to the position of *p*-hydroxybenzaldehyde.

ous gift from Professor David Amberg. *E. coli* strains TOP 10, BL21(DE3), and KC8 were obtained from Invitrogen, Novagen, and Clontech, respectively. Labeled *i*-PrOH-d_8 contained 99+ atom % D. YPD medium contained 10 g/L Bacto-yeast extract, 20 g/L Bacto-peptone, and 20 g/L Bacto-dextrose (added after separate autoclave sterilization). SC medium contained 6.7 g/L yeast nitrogen base and 20 g/L glucose (added after separate autoclave sterilization). YM medium contained 3 g/L Bacto-yeast extract, 3 g/L Bacto-malt extract, 5 g/L Bacto-peptone, and 10 g/L Bacto-dextrose. LB medium contained 5 g/L Bacto-yeast extract, 10 g/L Bacto-tryptone, and 10 g/L NaCl. All other reagents were obtained from commercial suppliers and used as received. GC and GC/MS analyses were carried out with a DB-17 column (0.25 mm × 30 m).

Trp 116 Replacement Library Creation. A 20 μg sample of plasmid pYES2 (Invitrogen) was digested with *Hin*dIII and *Xba*I overnight at 37 °C. The large fragment (5851 bp) was isolated by low-melt agarose gel electrophoresis and further cut with *Eco*RI to eliminate incompletely digested vector.

The upstream region of the *S. pastorianus OYE1* gene[42] was amplified from pET3b-OYE with primers pYES2-OYE1F (AGC AGC TGT AAT ACG ACT CAC TAT AGG GAA TAT TAA GCT TAT GTC ATTTGT AAA AGA TTT TAA GCC ACA AG; the underline indicates the region corresponding to the *OYE1* gene) and OYE1W116XR (GGG AAA GCA GCC CAA CCC AAA ACN NNT AAC TGA ACC CAA ACG AAC). The downstream region of the OYE1 gene utilized primers OYE1W116XF (GTT CGT TTG GGT TCA GTT ANN NGT TTT GGG TTG GGC TGC TTT CC) and pYES2-OYE1R (TAA GCG TGA CAT AAC TAA TTA CAT GAT GCG GCC CTC TAG ATT ACT TTT TGT CCC AGC CTA ATT TGA GAG C). PCR reactions (100 μL) contained 5× *Pfu* buffer[43] (10 μL), dNTPs (500 μM), template (pET3b-OYE1, 180 ng), a 2:1 mixture of *Taq* and *Vent* DNA polymerases (1 μL), and the appropriate primers (200 ng each). A total of 25 cycles of 94 °C (1 min), 54 °C (2 min), and 72 °C (3 min) were carried out, followed by 72 °C (10 min) and storage at 4 °C. Both amplification products were purified by low-melting agarose gel electrophoresis.

A single colony of DAY128 was used to inoculate 5 mL of 2× YPD medium. The culture was grown overnight at 30 °C and then diluted into 50 mL of the same medium at a density of ca. 5×10^6 cells/mL ($OD_{600} = 0.54$). The culture was shaken at 30 °C until the cells had divided at least twice ($OD_{600} = 2.4$ after 280 min). Cells were collected by centrifugation at 6000*g* for 5 min at 4 °C. The pellet was washed with 25 mL of sterile distilled water, then collected by centrifugation as above, and resuspended in 1.16 mL of sterile distilled water to achieve a density of 2×10^7 cells/mL.

(42) Locus X53597.

(43) Tris−HCl (200 mM), $MgSO_4$ (0.20 mM), KCl (1 mM), $(NH_4)_2SO_4$ (165 mM), Triton X-100 (1%), BSA (10 mg/mL), pH 9.0.

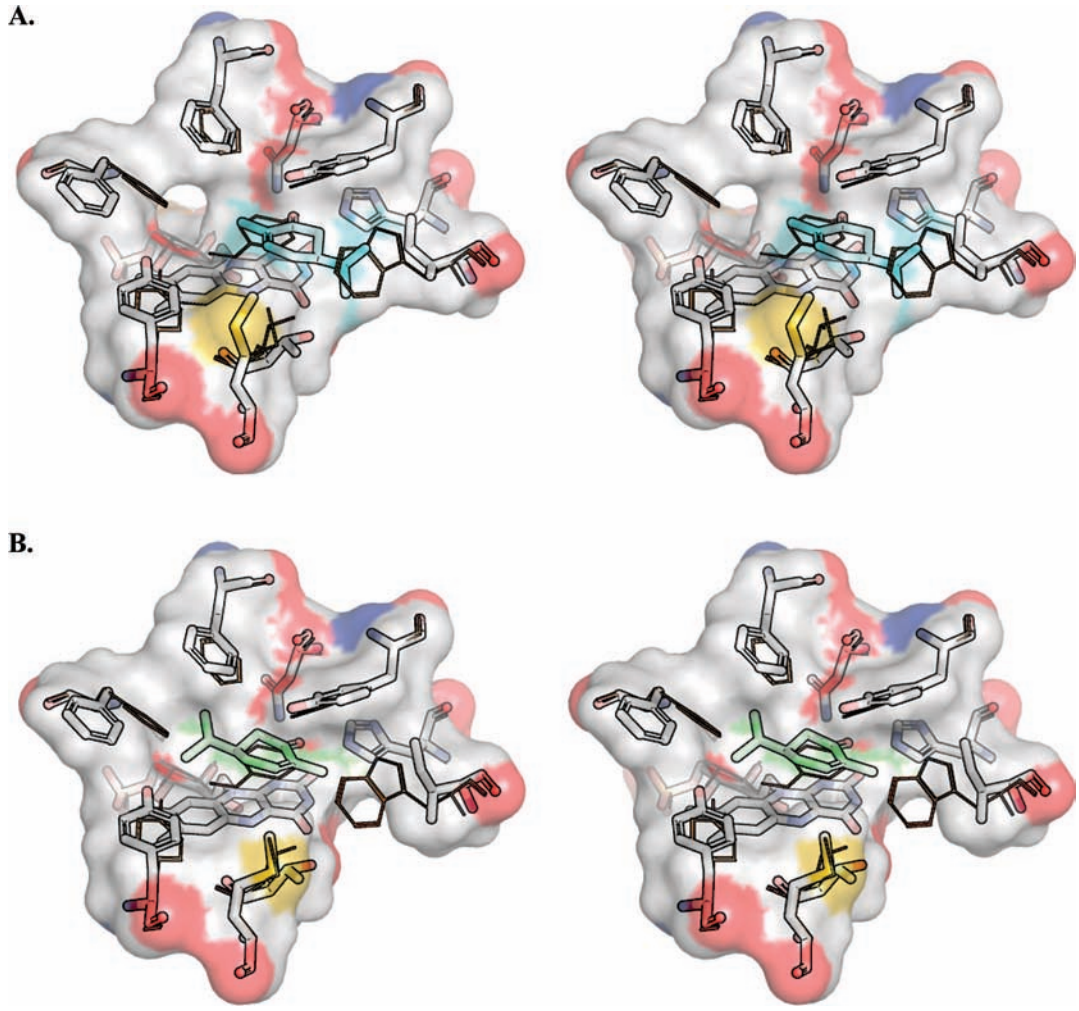


Figure 6. Induced fit docking of (*S*)- and (*R*)-carvone with the W116I mutant OYE. (A) Selected residues of the W116I OYE are depicted in ball-and-stick form along with the most favorable complex calculated for (*S*)-carvone (light blue). The experimental structure of wt OYE with bound *p*-hydroxybenzaldehyde (depicted in black lines) is overlaid for reference. (B) Same as part A, except that (*R*)-carvone is depicted (light green).

Aliquots (100 μL) were transferred to five sterile microcentrifuge tubes. Cells were collected by brief centrifugation at 4 °C. Homologous recombination was carried out by adding PEG 3350 (250 μL of a 50%, w/v, solution in water), ss carrier DNA (50 μL, boiled for 5 min and cooled prior to use),[44] 1.0 M LiOAc (36 μL), and sterile distilled water (33 μL) along with approximately equal amounts of the 5′- and 3′-portions of the *OYE1* gene and digested pYES2 DNAs. Cells were resuspended in the transformation mixture by vigorous vortex mixing, incubated at 42 °C for 45 min, and then collected by centrifuging at 12 000 rpm for 30 s. The supernatant was discarded, and the pellet was suspended in sterile water (1 mL) by vortex mixing. Aliquots were plated onto solid SC medium supplemented with leucine (30 μg/mL) and grown at 30 °C for 3−4 days. Colony PCR was carried out on 10 randomly selected clones using primers pYES2-OYE1F and pYES2-OYE1R. All 10 showed the expected 1.2 kb product corresponding to the intact *OYE1* gene.

Library Screening. A total of 200 aliquots (3 mL) of liquid SC medium supplemented with leucine (100 μg/mL) in 10 mL glass culture tubes were inoculated with individual library colonies and incubated with shaking at 30 °C for 16 h. OYE production was induced by adding 300 μL of sterile galactose solution (20%, w/v, in water) and shaking for an additional 3 h. Enone **1b** (1 M stock solution in EtOH) was added to a final concentration of 5 mM, and then the tubes were shaken at 30 °C for 48 h. Reaction mixtures (1 mL) were extracted with an equal volume of EtOAc by vortex mixing (30 s) followed by centrifugation (2 min) in a microcentrifuge. The organic layer was transferred to a GC sample vial and supplemented with 20 μL of 10 mM ethyl benzoate (internal standard, dissolved in EtOAc). Samples were analyzed by GC. Control reactions with wild-type OYE (positive) and no colony (negative) were carried out in parallel with the screening reactions. Under the screening conditions, wild-type OYE afforded 7−26% conversion.

The library of Trp 116 mutants was screened against (*S*)-carvone using the same conditions as described above. On a DB-17 GC column, enantiomers *trans*-**4** and *trans*-**7** eluted before *cis*-**6**.

Sequence Analysis and Subcloning in an *E. coli* Overexpression Vector. Total DNA was isolated individually from all 19 yeast colonies identified as positives from the library screening against **1b**. Cells from overnight cultures (5 mL) in SC medium supplemented with leucine (100 μg/mL) were collected by centrifugation at 6000*g* at 4 °C, resuspended in 200 μL of lysis solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris−Cl, 1 mM EDTA, pH 8.0), and then transferred to a 1.5 mL microcentrifuge tube. To this were added 200 μL of phenol−chloroform and ca. 300 μL of glass beads, and then the mixture was vortex mixed vigorously for 4 min. A 200 μL portion of TE buffer (10 mM Tris−Cl, 1 mM EDTA, pH 8.0) was added, and the mixture was centrifuged in a microcentrifuge at maximum speed for 5 min. The aqueous layer was transferred to a microcentrifuge tube and mixed with 2.5 volumes of HPLC grade 2-propanol before

(44) Gietz, R. D.; Schiestl, R. H. *Nat. Protoc.* **2007**, *2*, 31–34.

incubation at room temperature (rt) for 2 h. A crude DNA pellet was obtained by centrifuging in a microcentrifuge at maximum speed for 10 min. This was washed with 200 μL of 70% EtOH, dried for 1 min (Speed-Vac), and resuspended in 30 μL of sterile water. A portion was used to transform *E. coli* TOP10 cells by electroporation. After selection of transformants by plating on LB supplemented with ampicillin (120 μg/mL), a single colony was used for large-scale plasmid DNA isolation followed by purification by CsCl ultracentrifugation and automated DNA sequencing.

Individual *OYE1* variants were subcloned into *E. coli* expression vector pDJB5 by digesting the appropriate yeast shuttle vector (designated pSKP3-W116X, where X is the one-letter code for the amino acid at position 116) with *Bsa*I and *Kpn*I sequentially and then isolating the 569 bp *OYE1* fragment containing codon 116 using low-melt agarose gel electrophoresis. Similar treatment of plasmid pDJB5 yielded a 7.15 kb fragment that was ligated to the 569 bp fragments to create a full-length *OYE1* gene fused at its N-terminus to GST. Ligation mixtures were used to transform *E. coli* TOP10 cells with selection on solid LB medium supplemented with kanamycin (40 μg/mL). Plasmids were designated pSKP4-W116X.

Wild-Type and Mutant GST-Fusion Protein Isolation. *E. coli* overexpression strains were created by transforming BL21(DE3) cells with the appropriate plasmid encoding either the wild-type or W116 mutant OYE. A 5 mL overnight preculture of the resulting strain grown in LB medium supplemented with 40 μg/mL kanamycin was diluted 1:100 into 500 mL of the same medium in a 2 L baffled flask. This was shaken at 37 °C until the OD_{600} was between 0.5 and 1.0. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 100 μM, and the culture was shaken for an additional 6 h at 30 °C. Cells were collected by centrifuging at 5000*g* for 15 min at 4 °C. The pellet was washed twice with cold water and then resuspended in 30 mL of loading buffer (50 mM Tris−Cl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10% glycerol, pH 7.5; DTT and PMSF were added immediately before use). All purification steps were carried out at 4 °C. Cells were lysed by two passages through a French pressure cell at 16 000 psi, and then debris was removed by centrifuging the lysate at 15000*g* for 20 min at 4 °C followed by passage through a 0.45 μm filter. The filtrate was thoroughly mixed with 10 mL of glutathione resin (Clontech) in a column, and then the resin was allowed to settle. The protein sample was continuously circulated through the column for 3.5 h using a peristaltic pump (0.5 mL/min), then excess liquid was drained from the column, and the resin was washed twice with 20 mL of loading buffer. GST-fusion proteins were eluted with 40 mL of freshly prepared elution buffer (39.6 mL of loading buffer plus 0.40 mL of 2 M NaOH and 0.31 g of solid reduced glutathione). The eluant was concentrated by ultrafiltration (Amicon), then dialyzed overnight against 1 L of 20 mM Tris−Cl, 4 mM $MgCl_2$, 55 mM NaCl, 2 mM EDTA, 1 mM DTT, 50% glycerol, pH 7.5, and stored in aliquots at −20 °C.

General Procedure for OYE-Mediated Reductions. Biotransformations were typically carried out in 1 mL volumes for 20−24 h at rt. Reactions contained glucose-6-phosphate (14 μmol), glucose-6-phosphate dehydrogenase (5 μg), $NADP^+$ (0.20 μmol), alkene substrate (5 mM, added as a concentrated solution in EtOH), and purified OYE (ca. 50 μg). GC samples were prepared by extracting 100 μL aliquots of the reaction mixture with an equal volume of EtOAc.

Carvone Reductions and NMR Analysis. (4*R*)-NADPD was prepared by stirring 132 mg of $NADP^+$ (176 μmol), 4.8 mL of *i*-PrOH-d_8 and *Thermoanaerobium brockii* alcohol dehydrogenase (4.2 U) in 60 mL of 25 mM Tris−Cl, pH 9.0, in an Erlenmeyer flask at 43 °C.[45] Conversion was monitored by A_{340} measurements (50 μL of reaction mixture diluted to 1.0 mL with 25 mM Tris−Cl, pH 9.0), and the reaction was stopped after 1 h when the A_{340} value began to decrease. Ultrafiltration was used to remove protein, and then the filtrate was lyophilized to afford a white powder that was further purified by anion exchange chromatography (Pharmacia Hi Load 16/10 Q Sepharose).[46] After equilibration of the column with water (total 180 mL, flow rate maintained at 1 mL/min throughout), crude (4*R*)-NADPD (dissolved in 15 mL of water) was loaded. The column was washed with 20 mL of water and then developed with a linear gradient from water to 1 M NH_4HCO_3 (over 60 min). Fractions (5 mL) were collected and analyzed by UV−vis (50 μL samples diluted 20-fold with water); those with $A_{260}/A_{340} < 2.3$ were combined and lyophilized to afford a white powder that was stored at 4 °C. The total yield was calculated from A_{340} to be 89 mg (67%). ^{1}H NMR analysis showed that the deuterium content was >95% and all the label was located at the *pro*-(*R*)-position.[47]

To determine the stereochemical course of its reduction, (*S*)-carvone (**5**) (37 μL, 38 μmol) was mixed with (4*R*)-NADPD (110 mg, 54 μmol) and the W116I OYE enzyme (0.75 mg) in 3.75 mL of 100 mM KP_i, pH 7.0. After incubation in a glass vial at rt for 29 h, the reaction reached 80% conversion according to GC/MS analysis. Additional NADPD (20 mg, 9.8 μmol) and enzyme (0.14 mg) were added. Substrate was consumed completely after a total of 44 h. The reaction mixture was extracted with $CDCl_3$ (2 × 1 mL), and then the combined organics were dried with $MgSO_4$ and used directly for NMR and GC/MS analyses.

Synthesis of (*R*)- and (*S*)-5-Isopropyl-2-methyl-2-cyclohexenones (10 and 12). This conversion was a modification of the procedure of Shipe and Sorensen.[48] (*S*)-Carvone (**5**) (0.40 mL, 2.6 mmol) was dissolved in EtOAc (2 mL) in a 50 mL round-bottom flask, and then PtO_2 (2.5 mg, 11 μmol) and a balloon filled with H_2 were added. After the resulting mixture was stirred at rt for 5 h, GC/MS analysis showed complete consumption of substrate. The reaction mixture was filtered through a short silica gel pad using EtOAc. After drying with $MgSO_4$ and concentration under reduced pressure, GC/MS indicated that the crude product (98% yield) was 98% pure. Spectral data matched those reported previously.[49] (*R*)-Carvone was partially hydrogenated under the same conditions.

Synthesis of Geranial (19) and Neral (21). Using the procedure of Lautens and Maddess,[50] geraniol (12 g, 78 mmol) was dissolved in 300 mL of CH_2Cl_2 and cooled by an ice bath. Solid MnO_2 (75 g, 860 mmol) was added, and the reaction was held at reflux for 4 h before the mixture was filtered through a Celite pad. After the solid was washed with hexanes, the solvents were removed under reduced pressure to yield crude geranial (10 g, 83% yield) that was used without further purification. Neral (**21**) was prepared by an analogous procedure.

Cloning and Overexpression of *P. stipitis* OYE 2.6 and OYE 3.3. Genomic DNA was isolated from *P. stipitis* cells grown overnight at rt in 40 mL of YM medium by centrifuging at 6000*g* for 10 min at 4 °C and resuspending in 3 mL of 900 mM sorbitol, 100 mM EDTA, pH 7.5.[51] To this was added 100 μL of Zymolase (2.5 mg/mL in water), and the mixture was incubated for 1 h at 37 °C before centrifugation as above. The supernatant was discarded, the pellet was resuspended in 5 mL of 50 mM Tris−Cl, 20 mM EDTA, pH 7.5, then 0.50 mL of 10% SDS was added, and the mixture was incubated for 30 min at 65 °C. A 2.5 mL portion of 3 M KOAc, pH 5.2, was added, and then the mixture was kept on ice for 1 h before centrifugation at 15000*g* for 10 min at 4 °C. The

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(46) Esaki, N.; Shimoi, H.; Nakajima, N.; Ohshima, T.; Tanaka, H.; Soda, K. *J. Biol. Chem.* **1989**, *264*, 9750–9752.

(47) The signal for the *pro*-(*R*)-proton of NADPH appears at δ 2.81 ppm, while that for the *pro*-(*S*)-proton is located at δ 2.72 ppm. We observed only a doublet at δ 2.7 ppm in the ^{1}H NMR spectrum, confirming that only the (4*R*)-position had been deuterated.

(48) Shipe, W. D.; Sorensen, E. J. *J. Am. Chem. Soc.* **2006**, *128*, 7025–7035.

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supernatant was transferred to a centrifuge tube, and 2 volumes of absolute ethanol was added. After mixing, the solution was kept at rt for 15 min and the DNA was precipitated by centrifugation at 6000 rpm for 15 min. The pellet was dried and mixed overnight with 3 mL of TE, pH 7.5. The mixture was subsequently centrifuged for 15 min at 10 000 rpm, and the pellet was discarded. RNase A (150 μL of a 1 mg/mL solution) was added to the supernatant, and the mixture was incubated at 37 °C for 30 min before addition of 1 volume of 100% 2-propanol. After gentle mixing, a white precipitate formed, which was removed with a glass Pasteur pipet. The precipitate was air-dried and then resuspended in 0.5 mL of TE, pH 7.5.

The *OYE 2.6* and *OYE 3.3* genes were amplified from *P. stipitis* genomic DNA by PCR. The reactions utilized primers pDJB2-OYE2.6F (5′-TCC AAA ATC GGA TCA TCT GGT TCC GCG TCA TAT GCC CAT GTC TTC AGT CAA AAT TTC TCC ATT GAA GG-3′; the underlined portion corresponds to the *P. stipitis* coding region) and pDJB2-OYE2.6R (5′-CAG TGG TGG TGG TGG TGG TGC TCG AGT GCG GCC GCA TCA CAA AGC TTC AAT GGC CGA AGG AAC TCT CTT G-3′) or pDJB2-OYE3.3F (5′-TCC AAA ATC GGA TCA TCT GGT TCC GCG TCA TAT GCC CAT GAC GAA CTA CAA GAC TTC TCT TGA TGA G-3′) and pDJB2-OYE3.3R (5′-CAG TGG TGG TGG TGG TGG TGC TCG AGT GCG GCC GCA TTA CAC CAA TGG AAC ACC CTC TCT GGA CAA CTC C-3′), respectively. Reactions containing 10× *Taq* polymerase buffer 10 μL), the appropriate forward and reverse primers (2 μL of 100 ng/mL stocks), *P. stipitis* genomic DNA (1 μL), *Taq* polymerase (1 μL), and 82 μL of sterile water were amplified for 25 cycles of 94 °C (1 min), 54 °C (2 min), and 72 °C (3 min), followed by 72 °C (10 min) and storage at 4 °C. PCR products were purified by low-melt agarose gel electrophoresis, portions (150 ng) were mixed with an equal quantity of pDJB3 that had previously been digested with *Nco*I and *Hin*dIII, and then the mixture was used to transform *E. coli* KC8 cells with selection for ampicillin resistance. Colony PCR was used to identify clones with the desired inserts (pDJB32 for *OYE 2.6* and pDJB33 for *OYE 3.3*), which were sequenced completely to ensure that no mutations had been introduced. Both plasmids were used to transform *E. coli* BL21(DE3) cells. GST-fusion proteins were expressed and isolated as described above. An attempt to isolate the OYE 3.3 protein using the method described above afforded only a very small amount of enzyme. By contrast, several milligrams of the OYE 2.6 protein was isolated from *E. coli* BL21(DE3)(pDJB32) cells using the same procedure.

Reduction of (*R*)- and (*S*)-Carvone and (*R*)- and (*S*)-Perillaldehyde by *P. stipitis* OYE 2.6. Biotransformations of these substrates were carried out in parallel with purified wt *S. pastorianus* OYE and *P. stipitis* OYE 2.6 using the general procedure outlined above. Samples were analyzed by GC/MS.

Computational Methods. 1. Ligand Preparation. Molecular structures of carvone, perillaldehyde, and 2-methyl-2-cyclohexenone were built using Maestro v. 8.0 (Schrödinger LLC). Geometry cleanup with a universal force field was used to minimize the energy of all structures, which were used as inputs to LigPrep v. 2.1. This used the OPLS_2005 force field to optimize geometries and generate antipodes for carvone and perillaldehyde. Carvone and perillaldehyde conformers with axial C_4 substituents were eliminated from further consideration.

2. Receptor Preparation. The experimental structure of *S. pastorianus* OYE with bound $NADP^+$ and *p*-hydroxybenzaldehyde (PDB code 1OYB)[32] was optimized for docking by the Protein Preparation Wizard. Water molecules were deleted, hydrogen atoms were added, bond orders and formal charges were adjusted, and the phenolic hydroxyl of *p*-hydroxybenzaldehyde was deprotonated. The last ensured formation of two hydrogen bonds (with the side chains of His 191 and Asn 194) during the refinement process. Finally, the hydrogen bonds were optimized and a restrained minimization of the obtained structure was run using the default 0.3 Å rmsd tolerance. Structures of the W116I and W116F OYE variants were obtained by in silico substitution of Trp 116 followed by restrained minimization.

3. Receptor Grid Generation. The Glide application v. 4.5 (Schrödinger LLC) was used to generate grids for the prepared wild-type, W116I, and W116F OYEs. The default Coulomb−van der Waals (vdW) scaling factor (1.0) for receptor atoms with partial atomic charge less than 0.25 was selected. Grids were centered on the aromatic ring of *p*-hydroxybenzaldehyde, and two hydrogen bond constraints were defined (with the side chains of His 191 and Asn 194).

4. Flexible Ligand Docking with a Rigid Receptor. Each substrate ((*R*)- and (*S*)-carvone, (*R*)- and (*S*)-perillaldehyde, and 2-methyl-2-cyclohexenone) was docked individually into wild-type, W116I, and W116F OYE using the Glide application and the standard precision (SP) scoring function. The default Coulomb−vdW scaling factor (0.8) for ligand atoms with partial atomic charge less than 0.25 was selected. For each docking study, the program returned a maximum of 20 poses that satisfied at least 1 of the hydrogen-bonding constraints defined above. Each pose was analyzed manually to determine the substrate binding mode (normal or flipped), the number of intermolecular hydrogen bonds between the ligand and receptor (zero, one, or two),[52] the distance between the flavin N_5 atom and the substrate's β-carbon of the ligand, and the angle defined by the plane of the flavin molecule and the substrate's β-carbon.

5. Induced Fit Docking. The same docking studies were carried out using the induced fit docking protocol (Maesto v. 8.0). The enclosing box for grid generation was defined as before, and at least one H-bond between the substrate carbonyl and the side chain of His 191 was required for a pose to be accepted. During the initial docking phase, the default option Protein Prep constrained refinement was selected and both Phe 296 and Tyr 375 were replaced temporarily with Ala.[53] Ligands were docked with Coulomb−vdW scaling factors of 0.7 and 0.5 for the receptor and ligand, respectively, and a maximum of 20 poses were retained. In the second docking phase, the Prime application reversed the temporary replacements of Phe 296 and Tyr 375 with Ala, refined nearby residues, and optimized side chains. In the final docking phase, the ligand was redocked into all induced fit protein structures that were within 30 kcal/mol of the lowest energy structure using the Glide SP scoring function. A maximum of 20 poses were requested. Each was ranked using the composite induced fit score[54] followed by manual analysis as described above.

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Supporting Information Available: Complete ref 49, maps of plasmids pDJB3, pDJB5, and pSKP4-W116X, specific activities of the wt and W116I and W116F mutants for 2- and 3-substituted 2-cyclohexenones, and details of the computational docking studies. This material is available free of charge via the Internet at http://pubs.acs.org/.

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(52) Using the display H-bond utility of Maestro with default parameters (maximum distance 2.50 Å, minimum donor angle 120.0°, and minimum acceptor angle 90.0°).
(53) The average *B* factor values for the 1OYB structure were obtained from http://swift.cmbi.ru.nl/servers/html/listavb.html.
(54) Glide score plus 5% of the Prime energy, which is an estimation of the protein energy after the refinement step.
(55) Figure rendered in PyMol: Delano, W. L. The PyMOL Molecular Graphics System, 2002, http://www.pymol.org.