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Designing New Baeyer−Villiger Monooxygenases Using Restricted CASTing

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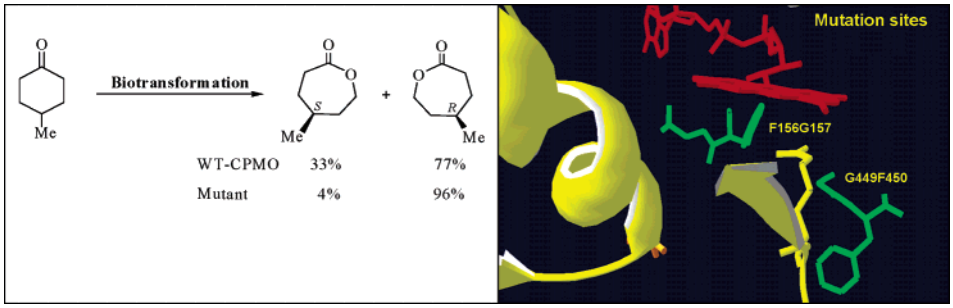

This paper outlines the design and execution of the first mini-evolution of cyclopentanone monooxygenase (CPMO). The methodology described is a relatively inexpensive and rapid way to obtain mutant enzymes with the desired characteristics. Several successful mutants with enhanced enantioselectivities were identified. For example, mutant-catalyzed oxidation of 4-methoxycyclohexanone gave the corresponding lactone with 92% entantiometric excess (ee) compared to the 46% ee achieved with wild-type cyclohexanone monoxygenase (WT-CHMO). The original design of the mini-evolution and the following evaluation of mutants can provide valuable insights into the active site's construction and dynamics and can suggest other catalytically profitable mutations within the putative active site.

Introduction

Several decades ago, Stanley Dagley[1] suggested that the microbial world harbors some strains that can degrade any of the countless compounds biosynthesized by living matter. Over the years, chemists demonstrated that many of these bacteria are also capable of degrading and transforming large numbers of man-made compounds. The principal enzymes involved in these processes are oxidizing enzymes, monooxygenases, and dioxygenases, many of which cleave carbon−carbon bonds by oxygen insertion. These enzymes, vital to microbes, are also of particular interest to chemists, since they can perform enantioselective oxidations on a broad spectrum of substrates. Cyclohexanone monooxygenase (CHMO; EC 1.14.13) from *Acinetobacter* NCIB 9871, first purified by Trudgill and co-workers,[2] belongs to a class of bacterial flavoprotein monooxygenases which carry out oxygen insertion via a Baeyer−Villiger rearrangement mechanism. CHMO, by far the most extensively studied, proved to be a formidable reagent for the Baeyer−Villiger oxidation of innumerable ketones.[3] Careful mechanistic studies by Walsh,[4] and later continued by Ballou and Massey,[5] demonstrated that the flavin C4a-peroxide (shown in Figure 1)

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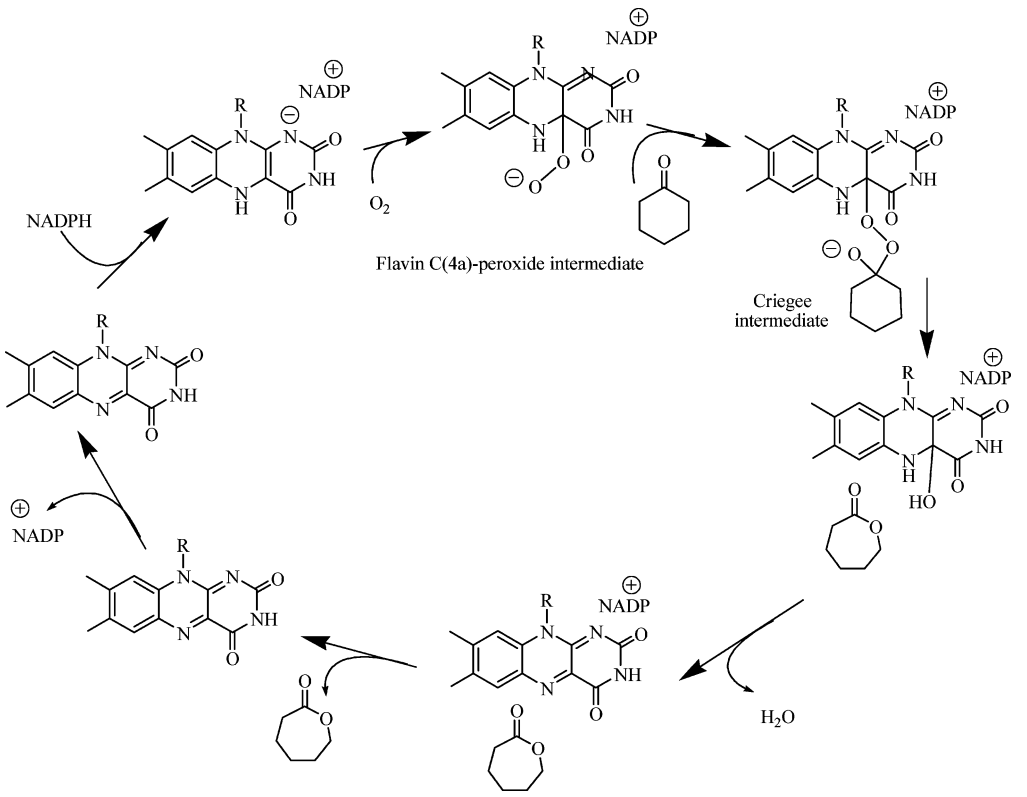

FIGURE 1. Proposed mechanism for cyclohexanone monooxygenase-catalyzed Baeyer−Villiger oxidation of cyclohexanone (adapted from ref 5).

is the key intermediate during the catalysis process involving the four-electron reduction of O_2 at the expense of a two-electron oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) and a two-electron oxidation of cyclohexanone to ϵ-caprolactone. It was also shown that a CHMO-catalyzed Baeyer−Villiger oxidation involves a tetrahedral Criegee intermediate[6] and, in general, resembles "chemical" oxidation.[4,5] Schwab and co-workers elegantly proved that fragmentation of the Criegee intermediate occurs with retention of configuration at the migrating carbon.[7]

During the catalytic cycle, the oxidized flavin adenine dinucleotide cofactor (FAD) that is tightly bound to the enzyme is first reduced by hydride transfer from NADPH. It then reacts with oxygen to form flavin C4a-peroxide, which rapidly oxidizes the substrate. In the absence of a substrate, a relatively stable C(4a)-hydroperoxyflavin is formed; the latter is transformed back into peroxide when a substrate becomes available. After hydride transfer, NADP remains attached to the enzyme and stabilizes the normally labile C4a-oxygenated flavin. However, it is the deprotonated flavin C4a-peroxide that participates in Baeyer−Villiger oxygen insertion;[5] in other words, the flavin peroxide is reactive only in the presence of a substrate, and therefore, the enzyme's turnover is substrate-controlled. The lactone product is released, followed slowly by NADP. Thus, the catalytic turnover of the most reactive substrates may actually be limited by the release of NADP. As NADP dissociates, the C4a-hydroxyflavin rapidly dehydrates to give oxidized FAD.[5] The key steps in the catalytic cycle are shown in Figure 1.

Early investigators recognized CHMO's potential as a synthetic reagent;[4,8] however, it was the work of Taschner and co-workers[9] that conclusively revealed this enzyme's exceptional enantioselectivity and remarkably broad substrate base. In successive years, several groups employed CHMO as the isolated enzyme, as the whole-cell *Acinetobacter*, or as overexpression systems in yeast and *Escherichia coli*, to prepare a multitude of lactones with high optical purities.[3a,b] The related NADPH-dependent cyclopentanone monooxygenase (CPMO), first purified and investigated by Griffin and Trudgill,[10] was in the shadow of its more famous cousin, probably because the initial studies indicated that it might be less enantioselective than CHMO.[11,12] Later, it was shown to be quite versatile[13] and, in some cases, highly enantioselective.[14,15]

Since Baeyer−Villiger oxidation steps are found in the metabolic pathways of many fungi and microorganisms, several monooxygenases, in addition to CHMO and CPMO, have been isolated and purified over the last 30 years.[3b,c] However, once the potential of flavin monooxygenases as reagents for enan-

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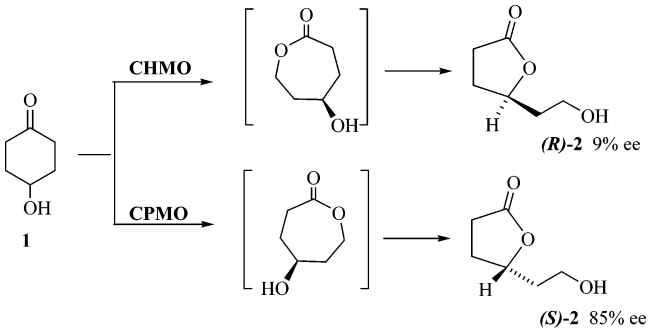
tioselective Baeyer−Villiger oxidations was recognized, an effort to expand the diversity of this family of enzymes began in earnest. One strategy concentrated on finding new organisms harboring Baeyer−Villiger monooxygenases (BVMOs). In a very short time, a number of these proteins were identified, the corresponding genes were cloned, and genetically engineered strains overproducing these proteins became available.[16−21] The dramatic increase in available BVMOs opened the door to extensive screenings that should eventually lead to the creation of a large library of profiles.[16,22] A search of such a library will reveal if an enzyme suitable for a desired transformation exists.

An alternative route to diversification of enzymes focuses on "designing" mutants with the desired characteristics, including substrate acceptance and enatioselectivity. Perhaps the most direct route to modification of an enzyme's performance, in the absence of information on the active site, is directed evolution.[23] The application of directed evolution to the control of *enantioselectivity* was first demonstrated in 1997, using a lipase as the enzyme.[24] Since then, the method has emerged as a fundamentally new approach to asymmetric catalysis, as manifested by a recent comprehensive review.[25] At the onset of this project, no X-ray crystal structure existed for any of the BVMOs, and directed evolution was the best strategy to improve the enantioselectivity of CHMO as well as to provide important structural and mechanistic insights.

SCHEME 1



We were particularly interested in 4-hydroxycyclohexanone (**1**) as a substrate because of an intriguing divergence between the two BVMOs that we were investigating. While both CHMO and CPMO rapidly and completely oxidized the substrate, CPMO gave the (*S*) enantiomer (85% ee) while CHMO gave (*R*), but with a very low enantioselectivity (9% ee) (Scheme 1).

In our original work,[26] several libraries of mutant CHMO genes were produced, and the subsequent screening of 10000 mutants led to the identification of about a dozen mutants with enhanced (*R*) selectivity in addition to several mutants with high (*S*) selectivity. Some of the mutants have been tested successfully in the enantioselective Baeyer−Villiger reaction of a fairly wide range of cyclic and bicyclic ketones.[27,28] The sequencing of several mutants displaying altered enantioselectivity identified the positions in the amino acid sequence of the wild-type (WT) CHMO that seemed to be particularly important in the selectivity control of Baeyer−Villiger reactions.[26a] The two mutations Leu143Phe, resulting in enhanced (*R*) selectivity in the reaction of **1**, and Phe432Ser, causing a switch from a low (*R*) to a high (*S*) selectivity, defined important hot spots. The latter mutation was particularly interesting because the change of phenylalanine to serine resulted in a dramatic switch to high (*S*) selectivity, the same (*S*) selectivity that was observed in the wild-type CPMO that bears a serine residue in an almost identical position (Figure 2).

In this communication, we describe a rapid and inexpensive approach, which can be used to improve the selectivity or extend the substrate acceptability of BVMOs. This semirational approach that we refer to as a mini-evolution can be used when the information about the residues in the close proximity of an active site is available. The "new" enzymes identified in the process give significantly improved enantioselectivity, without loss of activity, for two-probe substrates.

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CPMO-WT_        PNLLFGYGPQSPAGFCNGPSS
CHMO-WT_        PNMFMVLGPNGP--FTNLPPS
CHMO-MU         PNMFMVLGPNGP--STNLPPS
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FIGURE 2. Sequence alignment[29] of the wild-type CHMO and CPMO showing that, in the CHMO mutant with a newly acquired (*S*) selectivity, the serine residue is introduced in a position closely corresponding to the position of serine in the (*S*)-selective wild-type CPMO.

Results and Discussion

An illuminating event in the development of Baeyer−Villiger monooxygenases was the crystallization and X-ray analysis of phenylacetone monooxygenase (PAMO) by Mattevi and coworkers.[30] Although several monooxygenases have been purified, PAMO, isolated from the moderate thermophilic bacterium *Thermobifida fusca*, has been the only one thus far that produced crystals suitable for X-ray analysis. PAMO was crystallized with a bound FAD cofactor, and although the researchers were not successful in crystallizing the enzyme with both FAD and NADPH in place, the NADP binding site could be inferred from the comparison with the NADPH complexes of flavoenzymes with similar folding topology.[30] A strictly conserved, among Baeyer−Villiger monooxygenases, Arg337 residue was shown to be a part of the active site. Located on the re-face of the flavin, the Arg residue is essential in the catalytic step of oxygen insertion, suggesting that its role is to stabilize the negatively-charged flavin−peroxide intermediate. Since the side chain of the Arg residue can swing in and out, it is possible that it is stabilizing the flavin−peroxide intermediate in an *in-position* and swings into an *out-position* as a substrate takes its place. These results delineated the position of the active site and refined a model for catalysis by Baeyer−Villiger monooxygenases.[30] Shortly thereafter, a homology model of CHMO, based on the crystal structure of PAMO, was proposed, which appeared to form the basis for interpreting the enantioselectivity of WT-CHMO and its mutants.[31] However, because NADPH was not in the PAMO crystal, detailed interpretations are not straightforward.

Sequence alignment of CHMO and CPMO with PAMO shows very high homology (39% and 40%, respectively) and suggests that the structure of all three monooxygenases should be highly conserved. Indeed, in the present study, structural models constructed using CPHmodel 2.0[32] and refined using Moloc, a molecular mechanics program that relaxes the structures,[33] confirm this notion since the cores of the three proteins are almost identical (Figure 3). The homology model also reveals that the two hot-spot mutations identified in the directed evolution of CHMO (L143F and F432S) flank the position where the Criegee intermediate must be located (Figure 4). In CPMO, these two positions correspond to the Phe156 and Phe450; the serine residue (Ser432) in the CHMO mutant is in close proximity (Figure 4). Indeed, it has been suggested recently that Arg337 may stabilize the Criegee intermediate.[30,31]

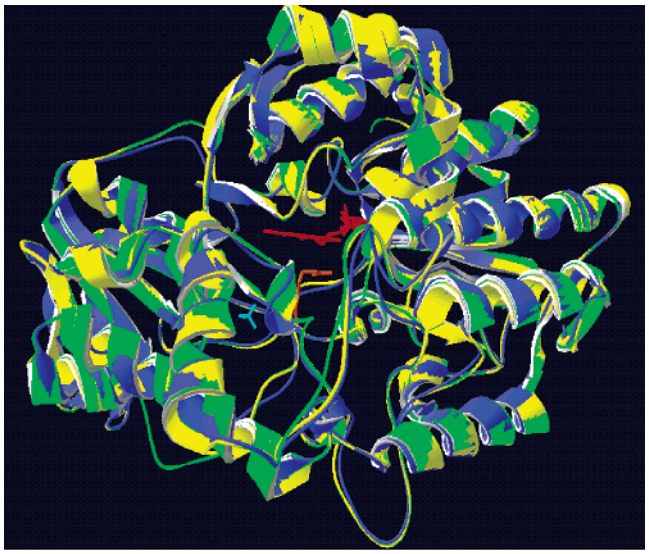

FIGURE 3. Homology models, constructed using CPHmodel 2.0[32] and the molecular mechanics program Moloc,[33] for CHMO (blue) and CPMO (green), shown in ribbon form, indicate that these proteins are almost identical with PAMO's X-ray crystal structure (yellow). The bound FAD is shown in red. The position of the phosphate of NADPH is simulated by a sulfate ion (turquoise), and the catalytically essential Arg337 residue is shown in orange.

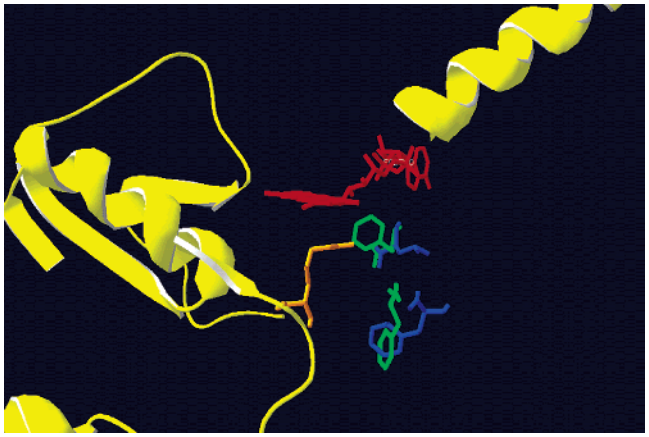

FIGURE 4. Positions of residues 143 and 432 CHMO (blue) and 156 and 450 CPMO (green) shown relative to FAD (red) and the catalytically essential Arg337 (orange).

Mini-evolution of CPMO. Equipped with the active site information gained in the directed evolution of CHMO and the results of homology modeling, we decided to determine if mutations in the active site's "hot-spots" could be a shortcut to an improvement in enantioselectivity in the often unselective CPMO. The combination of rational design and random mutagenesis at the predefined positions gives rise to focused libraries[23,34] that have been created to improve the catalytic performance of enzymes, including enhanced enantioselectivity.[24b,c,35,36] A particularly efficient approach based on the use of focused libraries is the recently developed Complete Active Site Saturation Test (CAST).[34,36] CASTing involves the systematic design and screening of focused libraries around the

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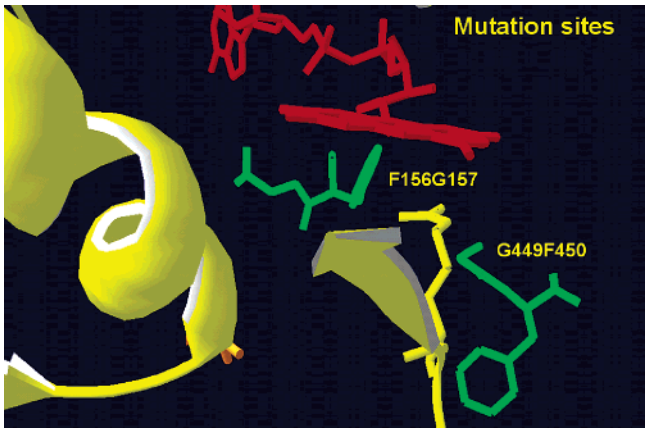

FIGURE 5. Phe156 and Phe450 are located on the loops, and the nearest amino acids with side chains pointing in the direction of the active site are the next-door neighbors Gly157 and Gly449, respectively.

complete binding pocket. Based on the 3D structure of an enzyme (X-ray or homology model), two or three amino acids whose side chains reside next to the binding pocket are identified and the respective positions at these sites are randomized with the creation of libraries of mutants.[34] To exert evolutionary pressure in those cases in which the initial libraries provide mutants that require further optimization, iterative CASTing[36] or combining mutations of two different variants produced by initial CASTing[37] can be applied. These approaches have led to lipases showing dramatically enlarged substrate scope[34,37] and epoxide hydrolases with enhanced enantioselectivity.[36]

In the present study, we decided to apply a variation of CASTing by replacing the usual NNK degeneracy, which encodes all 20 proteinogenic amino acids, by NDT degeneracy utilizing only 12 amino acids, namely, Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly. The advantage of using fewer "building blocks" has to do with the fact that the number of clones needed to be screened for 95% coverage of a given library is considerably lower,[38] which means that the screening effort is dramatically reduced. For example, when randomizing two positions simultaneously using NNK degeneracy, about 3000 clones need to be evaluated for 95% coverage, while the use of NDT degeneracy requires only about 430 clones. Of course, such high coverage may not be mandatory. To test this approach (restricted CASTing), we focused on sequence positions 156 and 450 of CPMO corresponding to positions 143 and 432 in CHMO, which were identified previously as hot spots (Figure 4). Since the key residues Phe156 and Phe450 are located on the loops, the nearest amino acids with side chains pointing in the direction of the active site are the next-door neighbors (Figure 5). Hence, the amino acid pairs Phe156/Gly157 and Gly449/Phe450 were used to create Libraries A and B, respectively. Upon screening only 150 clones, about 65% coverage of the protein sequence space defined by the CAST positions can be expected when using NDT degeneracy.

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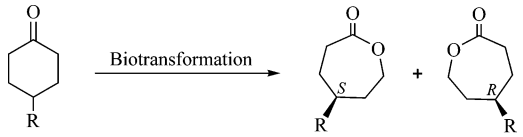
SCHEME 2



3a R = Me
3b R = *t*-Bu
3c R = OAc

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4b R = *t*-Bu
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The resulting plasmids were used to transform *E. coli* BL21-(DE3), and the integrity of the coding region was verified by DNA sequencing. The mutants grown on agar plates produced a large number of bacterial colonies, 150 of which were harvested from each of the two libraries. The colonies were placed in the deep, round wells of microtiter plates containing a rich broth. Following the growth of bacteria, the expression of enzymes was induced using isopropyl-*β*-D-thiogalactopyranoside (IPTG), and then the substrates **3a**−**c** were added to the reaction mixture. The reactions were monitored by chiral phase GC.

Evaluation of Mutants. Three compounds were selected as probe substrates for screening Libraries A and B. 4-Methylcyclohexanone (**3a**) and 4-acetoxycyclohexanone (**3c**) are rapidly oxidized by WT-CPMO, but with low enantioselectivities, whereas 4-*tert*-butylcyclohexanone (**3b**) is a poor substrate for the isolated WT-CHMO[3] and is not transformed by whole-cell WT-CPMO under the screening conditions (Scheme 2). No mutants capable of transforming the latter compound were discovered in this search. There were, however, numerous mutants that showed enhanced enantioselectivity for one or both of the other two substrates. The six most robust and versatile mutants (three from each library) were selected for further study and were sequenced. The results are shown in Table 1. The absolute configuration of lactone **4c** was established by hydrolysis of the acetate group and comparison of the resulting rearranged lactone with (*S*)-**2** from CPMO oxidation, as shown in Scheme 3.

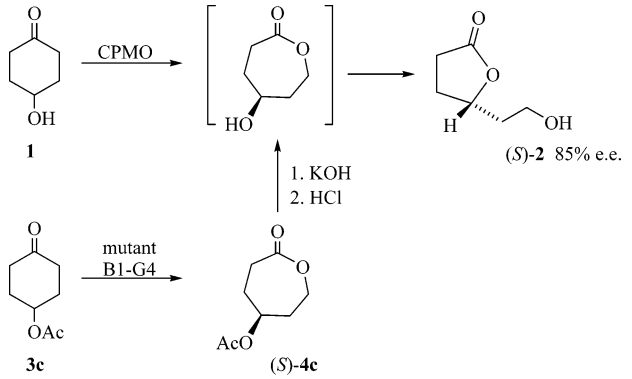
The results listed in Table 1 show that, for 4-methylcyclohexanone (**3a**), the most profitable mutation in Library A (giving the highest increase in (*R*) enantioselectivity) involves the mutation of Phe to Ile, i.e., an amino acid with a long hydrophobic side chain, suggesting an enhanced hydrophobic interaction with the methyl substituent. Interestingly, in Library B, the size of the residue was more important than the polarity and in the three cases with increased enantioselectivity (Table 1, R = methyl), the small Gly residue was replaced with something bulkier. This suggests that the residues in the 156/157 position are not in direct contact with the methyl group of the substrate and that the increased size of the amino acid residue results in a "push/shove" effect that creates a smaller, more stabilizing hydrophobic pocket for the methyl substituent. In contrast, the amino acid switch that accompanied the most dramatic change in the enantioselectivity of the Baeyer−Villiger oxidation of 4-acetoxycyclohexanone (**3c**) (5% ee (*S*) to 90% ee (*R*)) is observed in the reaction catalyzed by mutant B1-G4, in which the two hydrophobic residues phenylalanine (Phe156) and glycine (Gly157) are replaced with hydrophilic asparagine and tyrosine, respectively. This may imply that these residues are in contact with the polar OAc group of the substrate. The consequence of this analysis may be that the 4-substituted substrate can assume two positions (or two conformations) within the active site: one where the 4-substituent is in contact with residues 449/450, leading to (*S*) selectivity, and the other

TABLE 1. Bio-Baeyer−Villiger Oxidations of 4-Substituted Cyclohexanones to the Corresponding Lactones Catalyzed by WT-CPMO and Six Mutants[a]

	Library A [positions 449/450]			
	lactonic products **4a−c** [% conv (% ee)]			
substrates **3a−c**	WT-CPMO	A1−A3 [GlyPhe → SerTyr]	A1−A10 [GlyPhe → GlyIle]	A1−F12 [GlyPhe → GlyCys]
R = methyl	100 (46*R*)	27 (65*R*)	74 (92*R*)	37 (68*R*)
R = OAc	81 (5*S*)	20 (59*R*)	100 (8*R*)	89 (13*S*)
	Library B [positions 156/157]			
	lactonic products **4a−c** [% conv (% ee)]			
substrates **3a−c**	WT-CPMO	B1−A10 [PheGly → LeuPhe]	B1−G4 [PheGly → AsnTyr]	B1−H7 [PheGly → HisLeu]
R = methyl	100 (46*R*)	89 (91*R*)	67 (88*R*)	69 (80*R*)
R = OAc	81 (5*S*)	10 (ND)[b]	19 (90*R*)	55 (74*R*)

[a] Note: Under the screening conditions (growing whole cells), *tert*-butyl, the third probe compound, was not converted either by WT-CPMO or by any of the mutants. [b] ND = not determined.

SCHEME 3



where the substituent is stabilized by residues 156/157, leading to (*R*) selectivity. How well does this stabilization work? According to the enantioselectivity enhancement presented here, the arginine/tyrosine couple is more effective (90% ee) than the histidine/leucine couple in the same position (74% ee).

Conclusion

The selective mutation of CPMO, based on restricted CASTing using NDT degeneracy followed by modest library screening, was achieved quickly. Nonetheless, this rapid mini-evolution of CPMO succeeded in identifying mutants with greatly enhanced enantioselectivity for two kinds of 4-substituted cyclohexanones: nonpolar and polar. We were not successful in finding mutants capable of accepting bulky 4-*tert*-butylcyclohexanone. The analysis of a model-derived active site, in combination with the screening results, offers insight into the mechanism of enantioselection and provides guidelines for future modifications of CPMO that are tailored to specific substrates and desired enantioselectivities.

Experimental Section

Generation of Mutants. The isolated supercoiled double-stranded DNA (dsDNA) pET22b(+) vector[39] containing the desired insert (expressing CPMO) was used as a template for Quik-Change polymerase chain reaction (PCR) to generate the desired mutants. Primers were designed to incorporate the desired mutational changes within a given region, namely, the 156/157 and 449/450 regions of the CPMO gene. PCR reactions were performed using the established Quik-Change Kit protocol with HotStart KOD-Polymerase using the following primers:

Library A 449/450 position:
5′-GTCATCGCCAACCTTGGCNDTNDTGCCAGCCCCAGCACG-3′;
and reverse complementary.

Library B 156/157 position:
5′-GGACCTCAATCGCCTGCGNDTNDTTGCAACGGTCCGTCGAGC-3′;
and reverse complementary.

The resulting plasmids were transformed directly into *E. coli* BL21(DE3). The integrity of the coding region was verified by DNA sequencing.

Characterization of New Substrates and Products: 4-Acetoxycyclohexanone (3c). A mixture of 4-hydroxycyclohexanone (0.5 g, 4.4 mmol), acetic anhydride (3 mL), and pyridine (0.5 mL) was stirred at rt for 12 h. The solution was vacuum distilled to half volume and showed 93% conversion. The concentrated solution was added to ethyl acetate and acidified to pH ∼5 with concentrated HCl. The layers were separated, and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporation. The crude product was purified by chromatography on silica gel using hexane/ethyl acetate (2:1) to give the title compound as a pale yellow oil (0.45 g, 65% yield). IR (in $CHCl_3$) ν_{max}: 2958 (m), 2873 (m), 1733 (vs), 1437 (m), 1376 (m), 1239 (s), 1127 (m), 1041 (m), 956 (m) cm^{-1}. ^1H NMR δ: 3.84 (2H, m), 2.64 (1H, dd, J_1 = 15 Hz, J_2 = 6 Hz), 2.61 (1H, dd, J_1 = 15 Hz, J_2 = 6 Hz), 2.22 (1H, s), 2.07 (2H, m), 1.98 (1H, dd, J_1 = 13 Hz, J_2 = 6 Hz), 1.95 (1H, dd, J_1 = 13 Hz, J_2 = 6 Hz), 1.45 (1H, s). ^{13}C NMR δ: 209.7, 170.3, 68.6, 37.2, 30.4, 21.2.

Biotransformations. A. General Procedure for Biotransformations with the *E. coli* CPMO Mutants. All cultures were done using LB medium containing 0.1 g/L of carbenicillin. Initially, 96-deep-well plates containing 200 μL of LB were inoculated with colonies expressing the engineered *E. coli* strain BL21(DE3)-(pET22b(+)) and shaken at 700 rpm and 37 °C for 16 h. For the reactions, 40 μL of the overnight culture was used to inoculate 360 μL of LB medium in the depressions of a 96-deep-well plate. The cultures were shaken at 700 rpm and 37 °C until they reached an OD_{600} between 0.3 and 0.4. IPTG was then added to a final concentration of 0.047 mM (0.2 mg/well) along with a solution of the test substrate (0.1 g/L) in water. In cases where substrate toxicity

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or solubility was a problem (4-methylcyclohexanone and 4-*tert*-butylcyclohexanone), the substrate solution contained β-cyclodextrin (0.4 equiv) to overcome this problem. The reaction cultures were shaken at 900 rpm at rt for 24 h. The products of the biotransformations were extracted with 250 μL of ethyl acetate and analyzed by chiral-phase GC using a BGB-178 column (0.25 mm × 15 m, 0.25 μm film thickness), hydrogen (or nitrogen) as a carrier gas, and flame ionization detection.

B. 5-Methyloxepan-2-one (2e). *E. coli* BL21(DE3)(pET22b-(+))-mediated oxidation of ketone **3a** (110 mg, 0.98 mmol) was performed in the presence of 0.5 g of β-cyclodextrin according to the general procedure. Chromotography on silica gel using petroleum ether/ethyl acetate (4:1) as eluant afforded lactone **4a** (61 mg, 49% yield): 88% ee by chiral phase GC; $[\alpha]_D = +50.313$ (c = 2.3 in CH_2Cl_2). IR (neat) ν_{max}: 2955 (m), 2974 (s), 2873 (m), 1730 (s), 1449 (s), 1338 (s), 1164 (m), 1078 (m) cm^{-1}. ^1H NMR δ: 4.27 (2H, m), 2.65 (2H, m), 1.88 (2H, m), 1.78 (2H, m), 1.52 (2H, m), 1.01 (3H, t, J = 6.7 Hz). ^{13}C NMR δ: 175.2, 68.2, 41.9, 35.0, 33.2, 29.1, 28.5.

C. 5-Acetoxyoxepan-2-one (4c). *E. coli* BL21(DE3)(pET22b-(+))-mediated oxidation of ketone **3c** (100 mg, 0.64 mmol) was performed according to the general procedure. Chromotography on silica gel using hexane/ethyl acetate (9:1) as eluant gave lactone **4c** (21 mg, 19% yield): 90% ee by chiral phase GC; $[\alpha]_D = +4.940$ (c = 2.2, in CH_2Cl_2). IR (neat) ν_{max}: 2955 (m), 1737 (vs), 1441 (w), 1372 (m), 1248 (m), 1151 (m), 1061 (m) cm^{-1}. ^1H NMR δ: 5.15 (1H, m), 4.47 (1H, ddd, J_1 = 13 Hz, J_2 = 8.4 Hz, J_3 = 4.9 Hz), 4.17 (1H, ddd, J_1 = 13 Hz, J_2 = 6.7 Hz, J_3 = 6.1 Hz), 2.94 (1H, m), 2.57 (1H, m), 2.14−2.01 (7H, m). ^{13}C NMR δ: 174.7, 169.5, 69.2, 63.5, 34.0, 28.5, 27.6, 21.2.

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