DOI: 10.1002/cbic.201000464

Towards Practical Baeyer–Villiger-Monooxygenases: Design of Cyclohexanone Monooxygenase Mutants with Enhanced Oxidative Stability

Diederik J. Opperman [a, b] and Manfred T. Reetz*[a]

Baeyer–Villiger monooxygenases (BVMOs) catalyze the conversion of ketones and cyclic ketones into esters and lactones, respectively. Cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 is known to show an impressive substrate scope as well as exquisite chemo-, regio-, and enantioselectivity in many cases. Large-scale synthetic applications of CHMO are hampered, however, by the instability of the enzyme. Oxidation of cysteine and methionine residues contributes to this instability. Designed mutations of all the methionine and cysteine residues in the CHMO wild type (WT) showed that the amino acids labile towards oxidation are mostly either surface-exposed or located within the active site, whereas the two methionine residues identified for thermostabilization are buried within the folded protein. Combinatorial mutations gave rise to two stabilized mutants with either oxidative or thermal stability, without compromising the activity or stereoselectivity of the enzyme. The most oxidatively stabilized mutant retained nearly 40% of its activity after incubation with H_2O_2 (0.2 M), whereas the wild-type enzyme's activity was completely abolished at concentrations as low as 5 mM H_2O_2. We propose that oxidation-stable mutants might well be a "prerequisite" for thermostabilization, because laboratory-evolved thermostability in CHMO might be masked by a high degree of oxidation instability.

Introduction

First reported more than 110 years ago, Baeyer–Villiger (BV) reactions of ketones with the formation of esters or lactones have become a fundamental and useful transformation in synthetic organic chemistry.[1] This C–C bond-cleaving reaction is mediated by peracids, hydrogen peroxide, or alkylhydroperoxides, with acids, bases, or transition metals functioning as catalysts. Attempts at asymmetric catalysis with use of chiral transition metal catalysts or organocatalysts have met with limited success thus far.[2]

Baeyer–Villiger monooxygenases (BVMOs) constitute the biological counterpart and therefore offer an alternative.[1c,3,4] The first enzyme of this type to be isolated and studied mechanistically was the BVMO from *Acinetobacter* sp. NCIMB 9871 (CHMO; EC 1.14.13.22).[5] Subsequently it was used in asymmetric transformations involving desymmetrization of prochiral ketones and oxidative kinetic resolution of racemic substrates.[3,6] In parallel with this development, the list of BVMOs has steadily grown, as has our knowledge of these enzymes' mechanisms, substrate scope, and enantioselectivity.[3,4]

Mechanistically, dioxygen (from air) reacts with enzyme-bound flavin adenine dinucleotide (FAD) to form an intermediate alkylhydroperoxide, which adds nucleophilically to the carbonyl function with formation of the short-lived Criegee intermediate. The usual rearrangement/cleavage follows, delivering the desired product in addition to water and oxidized FAD, which is then regenerated by NADPH-mediated reduction.[4] Most BVMOs are too sensitive to be handled in isolated form, possibly in combination with an NADPH regeneration system, and so biotechnologists generally use whole cells. However, organic chemists are not trained to handle whole cells, so there is a need to make BVMOs more robust. Higher stability is also a desirable feature in whole-cell processes, because it lengthens the lifetime of the catalytic system, be it in BV reactions or in the selective oxidation of thioethers,[7a,b] amines,[7c,d] and olefins.[7e] For this reason the discovery of the first thermostable BVMO—phenylacetone monooxygenase (PAMO)—by Fraaije, Janssen, and co-workers constitutes a landmark,[8a] and its X-ray structure determination by Malito, Mattevi, and co-workers has provided researchers with a means to interpret the details of a BVMO's mechanism.[8b] Although PAMO displays the robustness desired for a biocatalyst, its catalytic profile is limited to phenylacetone and similar linear phenyl-substituted ketones.[8,9] In order to solve this problem, we have previously applied rational design[10] and various strategies based on directed evolution.[11] These approaches led to notable broadening of PAMO's substrate scope with concomitant high enantioselectivity, but the activity still needs to be improved for industrial applications.

An alternative strategy would be to enhance the robustness of the less stable cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871, discussed above, which

[a] *D. J. Opperman, Prof. M. T. Reetz*
Max-Planck-Institut für Kohlenforschung
Department of Synthetic Organic Chemistry
Kaiser-Wilhelm-Platz 1, 45470 Mülheim an der Ruhr (Germany)
Fax: (+49) 208-306-2985
E-mail: reetz@mpi-muelheim.mpg.de

[b] *D. J. Opperman*
University of the Free State, Department of Biotechnology
Bloemfontein 9300 (South Africa)

shows an impressive substrate scope as well as high chemo-, regio-, and enantioselectivity in numerous cases.[3a,5,6] It has also been the subject of several directed evolution studies to broaden its substrate scope even further and to increase its enantioselectivity with various substrates.[12] Large-scale synthetic applications of CHMO are hampered, however, not only by the need for cofactor regeneration, but also by the relative instability of the enzyme.[13]

Through the ongoing elucidation of crystal structures of thermostable proteins, our increased knowledge of the molecular mechanisms governing the thermostability of proteins has allowed protein engineering through rational design,[14] computational strategies,[15] and random mutagenesis through directed evolution.[16] A combination of these approaches is the B-FIT method, according to which saturation mutagenesis at sites displaying high B-factors is performed iteratively, the overall process being guided by the computer aid B-FITTER.[17] The physical basis of this highly efficient method is the idea that sites showing high B-factors are those that are characterized by maximum flexibility, and that appropriate mutations at these can lead to rigidification and therefore enhanced thermostability. This has been demonstrated experimentally[17] and corroborated by a theoretical study.[18] However, this approach does not consider oxidative stress leading to undesired chemical reactions at cysteine and methionine, which can likewise reduce the robustness of proteins.[19,20] Indeed, preliminary B-FIT-based experiments directed towards stabilizing CHMO proved to be problematic, due to the fact that its crystal structure has to this day not been determined and that homology models or computational means are uncertain guides in this context.

We therefore turned our attention to the stabilization of CHMO towards oxidation through rational design. Hydrogen peroxide can form in CHMO as the result of the slow decay of an unproductive peroxyflavin. Cysteine and methionine residues are the only two amino acids containing sulfur centers, which are often major sites for the oxidation of proteins, leading to loss of activity.[19] This inactivation is not limited to oxidation of Cys and Met residues at the active sites of enzymes, but can also occur at distal parts of the enzymes through remote effects, leading to catalytically compromised enzymes.[20] Because these oxidations also occur more readily at elevated temperatures, the corresponding amino acid substitutions could enhance both oxidative *and* thermal stability. In this study we therefore set out to investigate the effect of amino acid substitutions at the Met and Cys residues in CHMO, primarily for oxidative stability in the presence of an added oxidant (H_2O_2), while also checking possible effects on thermostability. We surmised that if the observed degree of thermostabilization should prove to be meager or non-existent, then the achieved increase in oxidative stability might provide a basis for a subsequent random mutagenesis study specifically focusing on thermostabilization alone.

Results and Discussion

Site-directed mutagenesis

In order to make rational decisions relating to site-specific mutagenesis, we first considered the mechanism of the enzyme under study. CHMO catalyzes a typical Baeyer–Villiger oxidation through the formation of a C4a-peroxyflavin that can perform a nucleophilic attack on the carbonyl group of the substrate. In the absence of substrate, however, the unproductive peroxyflavin will slowly decay to form hydrogen peroxide. The sulfur-containing amino acids—cysteine and methionine—are by far the most susceptible to various reactive oxygen species (ROS), including oxidation by hydrogen peroxide. These two amino acids are also considered thermolabile because their oxidation occurs more readily at higher temperatures. CHMO contains 12 methionine residues (excluding the start codon methionine) and five cysteine residues, roughly double the amounts found in the homologues PAMO and the newly crystallized CHMO from *Rhodococcus*.[21]

Standard site-directed mutagenesis was therefore undertaken at the positions harboring Met and Cys, with screening of the corresponding mutants for robustness by a simple process. After expression in *E. coli*, cells were harvested and broken with a freeze/thaw cycle, and the supernatants were transferred either into deep-well plates for oxidative stability checks (various concentrations of H_2O_2) or into 96-well PCR plates for thermostability checks (incubation at 37 °C for 1 h). In both sets of parallel experiments, the BV reaction of cyclohexanone was used as the reference transformation (Experimental Section). Structural alignments of a homology model of CHMO from *Acinetobacter* both with PAMO (40% sequence identity) and with the CHMO from *Rhodococcus* (55% sequence identity) were used to determine the replacing amino acid where possible. This was done in order to check whether there were any absolutely conserved Cys or Met residues and, more importantly, to provide a guide with regard to appropriate amino acids to be introduced at the chosen positions. None of the Cys and Met residues was conserved between CHMO and both the PAMO and the $CHMO_{rhod}$ enzymes, and only Cys64 was conserved with respect only to PAMO. Four of the Met and two of the Cys residues were conserved in the $CHMO_{rhod}$ enzyme. All the Met and Cys residues were replaced with amino acids with small hydrophobic side-chains, seen in the corresponding homologous structures. Met5, Met291, Met424, and Cys64 were mutated to Ile, Leu, Leu, and Ala, respectively, potentially to avoid any charge or steric hindrance from the corresponding amino acids found in the homologue structures.

All the single-amino-acid mutants had activity towards cyclohexanone comparable to that of the WT CHMO, with the exception of the Cys330Leu variant, which showed reduced activity (Figure 1). Crude cell extracts of the Cys330Leu mutant proved to be markedly less yellow in color, suggesting either lower expression levels or a reduced ability to bind the FAD co-factor, because Cys330 is present within the active site of CHMO (Figure 2B). SDS-PAGE analysis revealed the expression

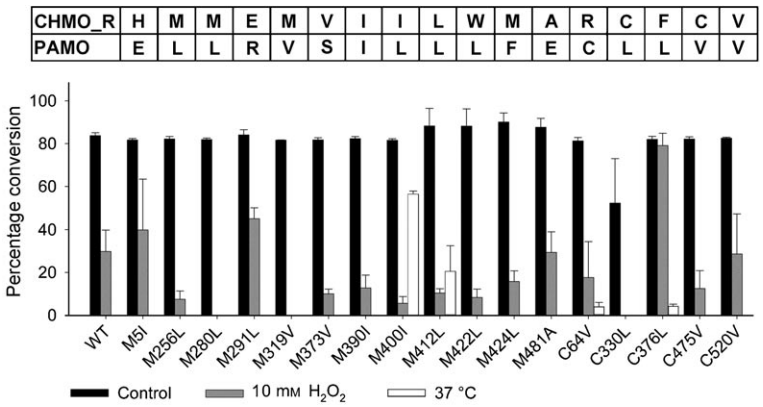


Figure 1. Effects of single-site mutations on the stability of CHMO towards hydrogen peroxide and temperature.

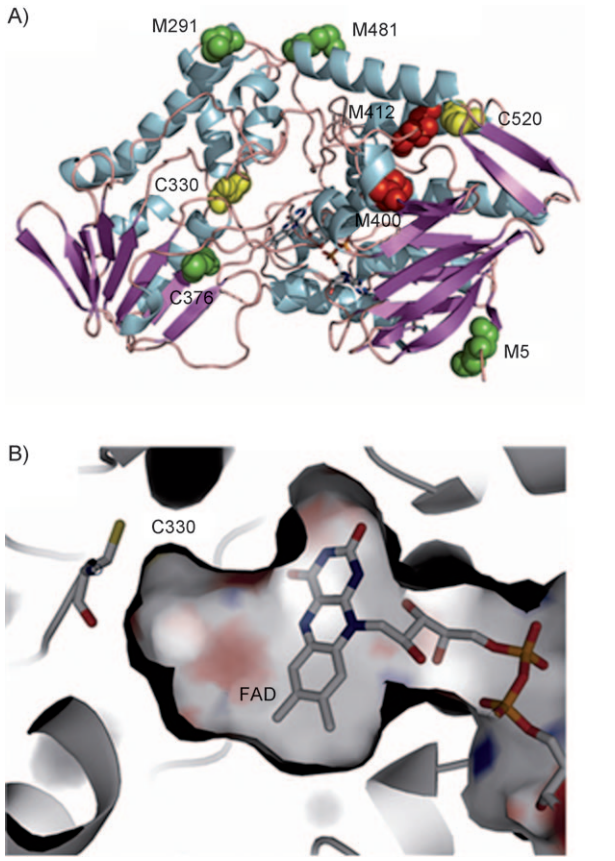


Figure 2. A) Ribbon diagram of CHMO with the amino acids used in the combinatorial mutants shown as space-filled atoms. Methionines implicated in thermostability are shown in red, whereas the cysteine and methionine residues considered for oxidative stability are shown in yellow and green, respectively. B) Cys330 is located within the active site of CHMO in close proximity to the FAD co-factor.

levels to be highly reduced only for this mutant. Saturation mutagenesis was therefore performed at residue Cys330 to obtain a library consisting of all the possible amino acids. Screening of 96 clones showed that CHMO could still function to some degree with various different amino acids at position 330, even with bulkier amino acids such as His and Tyr. However, the incorporation of serine resulted in the most comparable activity relative to WT CHMO. Because it is also the closest structural match, it was chosen for use in the combinatorial mutants. This single amino acid change also conferred great thermal and oxidative stability on the enzyme. Other single mutations that also contribute to an increase in oxidative stability include Met5Ile, Met291Leu, Met481Ala, Cys376Leu, and Cys520Val. In particular, the Cys376Leu mutant exhibits minimal loss in activity after incubation with low concentrations of hydrogen peroxide. Cys376 is situated close to the predicted NADPH binding site, and its oxidation could possibly have a direct effect on enzyme activity.

Increased thermal stability was achieved through the mutation of Met400 and Met412 to Ile and Leu, respectively, but no increased oxidative stability was observed. Like the Cys330Leu mutation, mutations Met280Leu and Met319Val had a deleterious effect on CHMO stability. The amino acids implicated in oxidation stability are located either in a solvent-exposed surface region or within the active site of CHMO, whereas the two amino acids Met400 and Met412 are either buried within the structure or are only partially accessible from the surface (Figure 2 A).

Combinatorial mutants

The identified mutations were systematically combined into combinatorial mutants, thereby allowing their cumulative effect on oxidative stability to be determined. On this basis we also hoped to identify the most thermostable variant. Combining mutations Met400Ile and Met412Leu, with the formation of the double mutant Met400Ile/Met412Leu, resulted in notably increased thermostability. The mutant showed almost no loss in activity even after one hour incubation at 38 °C, in contrast to the wild type's activity, which was completely abolished under identical conditions. Combining the mutations Met5Ile, Met291Ile, Met481Ala, Cys376Leu, and Cys520Val showed a positive cumulative effect on oxidative stability, resulting in a mutant (MUT18) almost completely stable towards 100 mM H_2O_2 (Figure 3); moreover, a small degree of thermostabilization was likewise achieved. The further introduction of the Cys330Ser mutation (MUT19) stabilized CHMO completely under the reaction conditions, and also conferred an improvement with regard to thermal inactivation (Figure 3). The introduction either of Met400Ile or of Met412Leu into MUT19 (to give MUT20 and MUT16, respectively) did confer additional thermostability, but at the expense of stability towards oxidation. Especially when the two mutations were introduced together (MUT15), a great loss of oxidative stability was seen; however, this combination resulted in the most thermostable

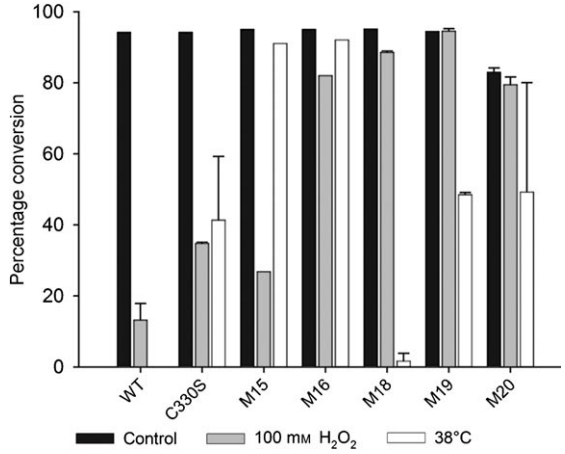


Figure 3. Effects of combinatorial mutations on the stability of CHMO towards hydrogen peroxide and temperature: MUT15: CHMO_M5I_M291I_C330S_C376L_M400I_M412L_M481A_C520V; MUT16: CHMO_M5I_M291I_C330S_C376L_M412L_M481A_C520V; MUT18: CHMO_M5I_M291I_C376L_M481A_C520V; MUT19: CHMO_M5I_M291I_C330S_C376L_M481A_C520V; MUT20: CHMO_M5I_M291I_C330S_C376L_M400I_M481A_C520V.

mutant. Although there seems to be some correlation between the mutations conferring both oxidative stability and thermostability, especially when considered cumulatively, a clear branching exists for the evolution either of oxidative stability or of thermostability. The two CHMO mutants MUT15 and MUT16 were therefore chosen for characterization with regard to thermostability, oxidative stability, substrate scope, and stereoselectivity.

Further characterization of thermostability and oxidative stability

Because the native *E. coli* enzymes present in the crude extracts offer a notable degree of protection of the CHMO toward hydrogen peroxide, WT CHMO and the MUT15 and MUT16 variants were purified to homogeneity (Figure 4) by metal-affinity and size-exclusion chromatography and again subjected to thermostability and oxidative stability studies.

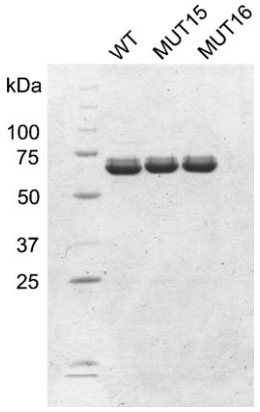


Figure 4. SDS-PAGE analysis of the purified C-terminally His-tagged WT CHMO and the MUT15 and MUT16 variants.

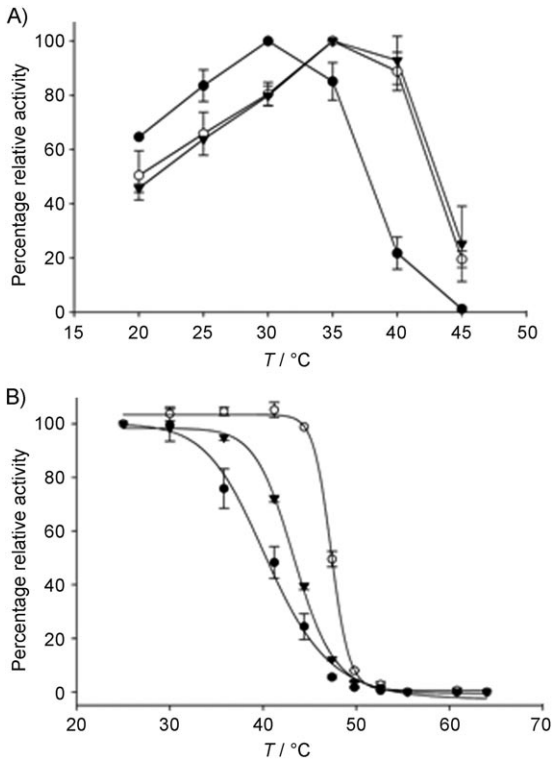


Figure 5. A) Optimum enzyme activity temperatures, and B) thermostability profiles (T_{50}^{10}) of WT CHMO (●) and the MUT15 (○) and MUT16 (▼) mutants.

Interestingly, both MUT15 and MUT16 showed increases of 5 °C in their optimum temperatures, and even retained approximately 20% of their activities at 45 °C (Figure 5A). The T_{50}^{10} values for MUT15 and MUT16 were increased from 40.5 °C (WT CHMO) to 47.3 °C and 43.4 °C, respectively (Figure 5B). The MUT15 variant was significantly stabilized over the temperature range of 35–45 °C, with the activity sharply decreasing only when approaching 50 °C, in contrast with the gradual decrease in the residual activity of WT CHMO at temperatures of just 30 °C. Both mutants also showed significantly increased stability toward hydrogen peroxide (Figure 6). Whereas the wild-type enzyme's activity is completely lost after incubation

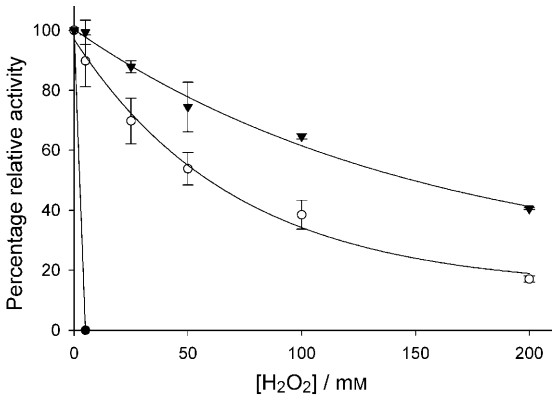


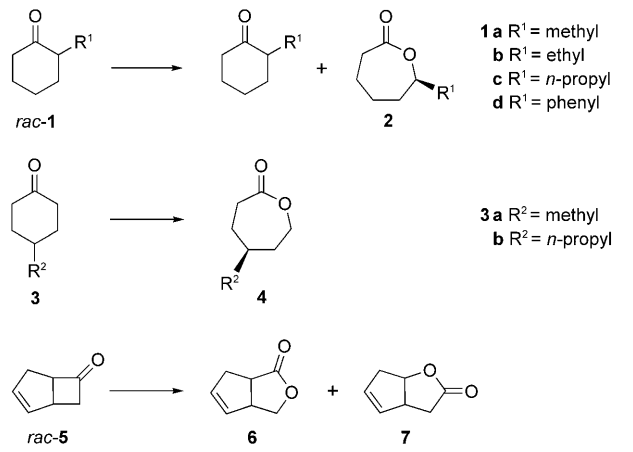
Figure 6. Oxidative stabilities of WT CHMO (●) and of the MUT15 (○) and MUT16 (▼) mutants towards hydrogen peroxide.

with 5 mM H_2O_2, both mutants showed more than 40% residual activity even after 3 h incubation with concentrations 20 times higher. As was seen with the crude extracts, the MUT16 variant proved to be more stable than MUT15, and retained approximately 40% of the residual activity when incubated with 6×10^4 times molar excess of H_2O_2.

Substrate scope and stereoselectivity

CHMO is well known for its ability to accept a variety of substrates, often with high degrees of enantioselectivity, in oxidative kinetic resolution and desymmetrization.[3–6] Because some of the mutations are located within, or close to, substrate or cofactor binding areas (in particular Cys330Ser and Cys376Leu), and also in view of the recent report illustrating an induced allosteric effect for directed evolution of PAMO's activity and selectivity,[11c] the stabilized mutants were compared to the wild type.

CHMO and the MUT15 and MUT16 variants were compared with respect to their levels of conversion of, as well as their selectivities towards, various 2- or 4-substituted cyclohexanones (*rac*-**1** and **3**, respectively; Scheme 1; Table 1) and bicyclo-[3.2.0]hept-2-en-6-one (*rac*-**5**). Gratifyingly, the mutations had no marked effect on the levels of conversion (activity) nor on the selectivities of the mutants relative to the catalytic profile of WT CHMO (Table 1). The ratios of "abnormal" versus "normal" lactones in the biotransformation of *rac*-**5**, as well as the corresponding stereoselectivities, also remained relatively unchanged.



Scheme 1. CHMO-catalyzed BV oxidations of various substrates.

Table 1. Regio- and enantioselectivities of the oxidatively stable and thermostabilized mutants of CHMO.

Substrate	*ee* [%][a]			*E*			Regioselectivity		
	WT	MUT15	MUT16	WT	MUT15	MUT16	WT	MUT15	MUT16
rac-**1a**	72.2	73.2	74	8	9	8			
rac-**1b**	>99	>99	>99	>200	>200	>200			
rac-**1c**	>99	>99	>99	>200	>200	>200			
rac-**1d**	>99	>99	>99	>200	>200	>200			
3a	>99	>99	>99						
3b	95.6	96.0	96.0						
rac-**5a**							1.0: 0.99[b]	1.0: 1.02[b]	1.0: 1.01[b]

[a] Enantiomeric purity of product. [b] "Abnormal (**7**)"/"normal (**6**)" lactone.

Conclusions

This study demonstrates that although oxidative stability and thermal stability are often considered two separate properties, their determinants can in fact be overlapping, as has been noted previously in rare cases. In their study of horse radish peroxidise, for example, Arnold and co-workers reported that the replacement of Asn in the active site by Ser contributes both to thermostability and to robustness towards hydrogen peroxide.[19e] Oh and co-workers discovered in their directed evolution study of *N*-carbamyl-D-amino acid amidohydrolase that four of the six mutations contribute both to oxidative stability and to thermal stability, the remaining two leading only to enhanced oxidative stability.[19f] Oxidation of Cys and/or Met residues can abolish enzyme activity when these residues are located at the active sites of enzymes, as shown by a number of previous studies.[19] Importantly, oxidation of Cys and Met residues at remote sites can lead to altered structural conformations that can influence the enzyme's rate adversely[20a] and/or lead to lower stability due to faster unfolding.[20b,c] In such cases mutational exchanges may lead to higher oxidative and thermal stability. However, the situation may well be more complex than shown here.

In this study, mutations of all the Cys and Met residues in WT CHMO showed that the amino acids labile to oxidation are mostly either surface-exposed or located within the active site, whereas the two Met residues identified for thermostabilization are buried within the folded protein. Although some notable synergistic effects were observed between the different mutations, a branching in the evolution pathway towards either thermal or oxidative stability seems to occur.

Simultaneous evolution of two different properties is by no means an easy feat, and researchers often opt for an approach in which one function is evolved first, followed by optimization of the second desired trait. Recent investigations in our laboratory into the simultaneous optimization of two different properties during directed evolution have shown that less stringent selection parameters (that is, not always the best mutant for continuing the evolutionary process) should be considered.[22] Moreover, it has also been found by Arnold and co-workers[23] that protein stability promotes evolvability. Although these findings focused on the evolution of new catalytic functions, the same could apply to the evolvability of two stability parameters. Oxidatively stable mutants could be a "prerequisite" for addressing thermostabilization, because laboratory-evolved thermostability in CHMO might be masked by a high degree of oxidation damage. Microheterogeneity caused by the oxidation of Cys or Met residues could also hamper other downstream experiments such as the crystallization of the protein.

We conclude that this study constitutes a step forward in the engineering of practical Baeyer–Villiger monooxygenases (BVMOs) for application in synthetic organic chemistry and biotechnology. A pronounced increase in the oxidative stability of the most commonly applied BVMO—cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO)—has been achieved through rational and combinatorial design, without compromising activity, substrate scope, or stereoselectivity. The results set the stage for further thermostabilization by random or focused mutagenesis. User-friendly processes might then emerge based on the in vitro use of isolated CHMO mutants in combination with a practical NADPH-regeneration system utilizing a robust secondary alcohol dehydrogenase and isopropanol as the reductant.

Experimental Section

Materials: All substrates were obtained from commercial sources (Sigma–Aldrich, Acros, and Fluka) and were used without any further purification. NADPH was purchased from Sigma–Aldrich and hydrogen peroxide from Merck.

Strains, vectors, and growth conditions: The CHMO wild type and mutants were expressed from the pET22b(+) vector (Novagen), which also served as template for site-directed mutagenesis. *E. coli* strains were routinely grown in Luria–Bertani (LB) medium at 37 °C with shaking (200 rpm). Expression of the CHMO wild type and mutants was done in *E. coli* BL21-Gold (DE3) (Stratagene) in ZYP5052 medium[24] at 25 °C (200 rpm).

Site-directed mutagenesis: Site-directed mutagenesis was performed by the QuickChange (Stratagene) PCR method. PCR reaction mixtures (50 μL) consisted of 10× KOD Hot Start Polymerase buffer (5 μL), $MgSO_4$ (1.5 mM), deoxynucleoside triphosphates (0.2 mM each), KOD Hot Start DNA polymerase (1 U), plasmid DNA (20 ng), and both the forward and the reverse primers (Table 2, 0.1 μM). The reaction conditions consisted of an initial denaturing step at 95 °C for 2 min, followed by 18 cycles of denaturing at 95 °C (30 s), annealing at 61 °C (30 s), and elongation at 70 °C (4 min), with a final extension at 70 °C for 5 min. PCR products were digested with DpnI (New England Biolabs, 0.4 U) at 37 °C for 3 h, to ensure removal of the template plasmid DNA. DpnI-digested products were purified with the aid of the QIAquick PCR purification kit (Qiagen) and subsequently transformed into *E. coli* DH5α. Random colonies were selected and grown overnight in LB medium (5 mL) containing carbenicillin (100 μg mL^{-1}). Plasmid extractions were performed by use of the QIAprep Spin Miniprep kit (Qiagen) and mutagenesis was confirmed through sequencing of the CHMO gene (Eurofins MWG Operon, Germany). C330 site-directed saturation mutagenesis was performed as described above, with 96 random colonies inoculated into deep-well plate format. Library quality was assessed through sequencing of a pooled library consisting of more than 100 colonies.

Mutant screening: Initial analyses and screening of the mutants were performed in 96-well plate format. CHMO mutants were transformed into *E. coli* BL21-Gold (DE3) and plated on LB plates containing carbenicillin (100 μg mL^{-1}). Single colonies were inoculated into deep-well plates (2.2 mL) containing LB medium (0.8 mL) and carbenicillin (100 μg mL^{-1}). Deep-well plates containing ZYP-5052 autoinduction medium (0.8 mL) were inoculated with overnight-grown cultures of the CHMO mutants. Expression of the recombinant proteins was allowed to proceed for 24 h at 25 °C (800 rpm). Cells were harvested through centrifugation (2700 *g*, 15 min) and pellets were resuspended in lysis buffer [350 μL, Tris-HCl (pH 8, 50 mM), lysozyme (1 g L^{-1}), EDTA-free Protease Inhibitor (Roche) and DNAse (1 h)]. Cells were broken with a single freeze-thaw cycle, after which the cellular debris was removed through centrifugation (2700 *g*, 30 min). Supernatant was transferred either (80 μL) to 96-well PCR plates (for thermal stability studies) or (50 μL) to 1.8 mL deep-well plates containing Tris-HCl buffer (pH 8, 250 μL) and various concentrations of hydrogen peroxide. Thermal stability was assessed through incubation of the crude soluble fractions at 37 °C for 1 h, and oxidative stability was determined by incubating the crude extract with hydrogen peroxide (10–100 mM) overnight at 4 °C. Residual activity was measured by determining the percentage conversion of cyclohexanone (2 mM) at room temperature after 15 min. Reaction mixtures (400 μL) contained equimolar concentrations of NADPH and were stopped/extracted through the addition of equal volumes of ethyl acetate. The reaction mixtures were centrifuged and the organic phases were transferred to 96-well glass plates for GC analysis.

Table 2. Regio- and enantioselectivities of the oxidatively and thermostable mutants of CHMO. Primers used for site-directed and saturation mutagenesis.

CHMO mutation	Primer (5′→3′)[a]
M256L	CACAGTGCCAGCATTGAGCGTATCAG
M280L	CGGTTTCCGTTTCTTGTTTGAAACTTTCG
M373V	CGTTGAATTAGACGTGCTGATATGTGC
M390I	GCAACTATGTGCGCATAGACATTCAAGG
M400L	GCTTGGCCTTGAAAGACTACTGGAAAG
M412L	GAAGGTCCGTCGAGCTATTTGGGTGTC
M422L	CGTAAATAACTATCCAAACTTGTTCATGGTGC
M481A	CCAATATTGCGGAAGCGACCTTATTCC
C475V	GGACTCAAACTGTGGCCAATATTGCG
C520V	GTGCGCTAGCCAACGTCAAAAACCATG
M5I	GTCACAAAAACTGGATTTTGATGCTATCG
M319V	CCATTGCACAGAAGCTTGTGCCACAG
M291L	GGTGATATTGCCACCAATTTGGAAGC
C376L	CGTTGAATTAGACATGCTGATATTAGCCACAG
C64V	CAGAAACCCACCTCTACGTCTATTCTTGG
M424L	TGTTCTTGGTGCTTGGACCGAATGG
C330L	CAAAACGTCCGTTGTTAGACAGTGGTTACTAC
C330NNK	CAAAACGTCCGTTGNNKGACAGTGGTTACTAC
C330S	CAAAACGTCCGTTGTCTGACAGTGGTTACTAC

[a] Only forward primers are listed, with the reverse primers being the reverse compliments of the tabulated primers.

Biotransformations: The CHMO wild type and the mutants MUT15 and MUT16 were expressed as described above. Crude soluble fractions, obtained after cell lysis and centrifugation, were incubated overnight at 4 °C with excess FAD. Samples were desalted and unbound FAD was removed through passage of the extracts through a PD-10 desalting column (GE Healthcare). Activity was determined as the percentage conversion after 2 h by crude extract (50 μL) in a reaction volume of 500 μL containing substrate (2 mM) and NADPH (3 mM) at room temperature [Tris-HCl (pH 8, 50 mM)]. Reaction mixtures were again extracted with equal amounts of ethyl acetate. The organic phases were transferred to GC vials for analysis. The selectivities of CHMO and its MUT15 and MUT16 mutants were compared by determination of enantiomeric excesses either after complete conversion of the 4-substituted cyclohexanones or after approximately 30% conversion of the 2-substituted cyclohexanones. These reaction mixtures were adjusted by increasing the substrate concentrations to 4 mM and limiting the reaction time and the NADPH concentration (to 1.5 mM).

Histidine-tagged variants: C-terminally His_6-tagged variants of the CHMO WT and the MUT15 and MUT16 mutants were prepared by deletion of the stop codon and DNA preceding the six histidine codons of the pET22b(+) plasmid. PCR reactions were performed as described for the site-directed mutagenesis, with the primers CHMO_Histag_F (5′-CAC CAC CAC CAC CAC TGA GAT C-3′) and CHMO_Histag_R (5′-GGC ATT GGC AGG TTG CTT GAT ATC TGA AC-3′). After DpnI digestion and PCR cleanup, the products were circularized by use of a single-reaction phosphorylation and ligation step. Reaction mixtures (20 μL) consisted of product (5 μL), NEB ligation buffer (2 μL), T4 kinase (1.5 μL), and T4 ligase (1.5 μL, New England Biolabs). Phosphorylation and ligation were performed overnight at 4 °C, followed by transformation into *E. coli* DH5α. Mutations were again confirmed through sequencing of selected colonies.

Purification of wild-type and mutant proteins: The pET22-CHMO and mutant derivates were transformed into *E. coli* BL21-Gold (DE3) competent cells (Stratagene) and plated on LB plates containing carbenicillin (100 $\mu g\,mL^{-1}$). Expression was performed by using ZYP-5052 autoinduction medium containing carbenicillin (100 $\mu g\,mL^{-1}$). Cells were cultured for 24 h at 25 °C, after which they were harvested through centrifugation (8000 *g*, 10 min) and washed with Tris-HCl (pH 8, 50 mM). Washed cells were resuspended in Tris-HCl (pH 8, 50 mM) and broken by single passage through a cell disrupter (Constant Systems) at 207 MPa. The soluble fraction was separated from the crude extract by ultracentrifugation (100 000 *g*, 90 min). Recombinant C-terminally His_6-tagged CHMO and mutants were purified by metal-affinity chromatography and size-exclusion chromatography. The soluble fractions were loaded onto HisTrap FF columns (5 mL, GE Healthcare) equilibrated with Tris-HCl (pH 7.4, 50 mM) containing imidazole (40 mM) and NaCl (0.5 M). Unbound proteins were removed (5 $mL\,min^{-1}$) by use of the same buffer. Proteins of interest were then eluted in the same buffer with use of a linear gradient (100 mL) of imidazole up to 0.5 M. Fractions containing activity were pooled for subsequent purification. All protein samples were incubated with excess FAD before size-exclusion chromatography. Samples were concentrated to approximately 3 mL by ultrafiltration (10 kDa MWCO, Millipore) and loaded onto Sephacryl S-200HR columns (65×2.5 cm, Sigma) equilibrated with Tris-HCl (pH 8, 50 mM). Proteins were eluted with the same buffer at a flow speed of 1 $mL\,min^{-1}$. Expression levels were not significantly affected with typical yields of approximately 50 mg of purified protein from 1 L of expression culture.

Stability: The oxidative stabilities of the purified proteins were determined by incubation of the enzymes (0.2 $mg\,mL^{-1}$) for 3 h at 20 °C in buffers containing various concentrations of hydrogen peroxide. The thermostabilities were determined by incubation of the purified enzymes (0.2 $mg\,mL^{-1}$) at various temperatures for 10 min. Samples were immediately placed on ice, after which the residual activities were determined at room temperature.

Enzyme assay: Activities were measured by monitoring the oxidation of NADPH at 340 nm (Cary 300Bio UV/Vis spectrophotometer) with use of an extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. Assays were performed in Tris-HCl buffer (pH 8.5, 50 mM, 1 mL reaction volumes) containing NADPH (0.2 mM), cyclohexanone (0.5 mM), and the purified protein (10 $\mu g\,mL^{-1}$; final concentrations).

Analytical techniques: Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce), according to the manufacturer's instructions, with bovine serum albumin as the standard.

SDS-PAGE was performed by the protocol described by Laemmli,[25] with use of a resolving gel (10%) and a stacking gel (4%). Precision Plus protein standards (Bio-Rad) were used as molecular mass markers, and proteins were visualized by staining of the polyacrylamide gels with Coomassie brilliant blue R-250.

Acknowledgements

Financial aid from the Fonds der Chemischen Industrie is gratefully acknowledged. We also thank Jutta Rosentreter for the chiral GC analyses.

Keywords: Baeyer–Villiger monooxygenases · directed evolution · oxidative stability · protein engineering · thermostability

[1] Reviews of Baeyer–Villiger reactions: a) G. R. Krow, *Org. React.* **1993**, *43*, 251 – 798; b) M. Renz, B. Meunier, *Eur. J. Org. Chem.* **1999**, 737 – 750; c) G.-J. ten Brink, I. W. C. E. Arends, R. A. Sheldon, *Chem. Rev.* **2004**, *104*, 4105 – 4123.

[2] Stereoselective BV reactions catalyzed by chiral transition metal complexes or organocatalysts: a) C. Bolm, G. Schlingloff, K. Weickhardt, *Angew. Chem.* **1994**, *106*, 1944 – 1946; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1848 – 1849; b) A. Gusso, C. Baccin, F. Pinna, G. Strukul, *Organometallics* **1994**, *13*, 3442 – 3451; c) T. Uchida, T. Katsuki, *Tetrahedron Lett.* **2001**, *42*, 6911 – 6914; d) K. Ito, A. Ishii, T. Kuroda, T. Katsuki, *Synlett* **2003**, 643 – 646; e) G. Strukul, *Angew. Chem.* **1998**, *110*, 1256 – 1267; *Angew. Chem. Int. Ed.* **1998**, *37*, 1198 – 1209; f) J.-C. Frison, C. Palazzi, C. Bolm, *Tetrahedron* **2006**, *62*, 6700 – 6706; g) M. Colladon, A. Scarso, G. Strukul, *Synlett* **2006**, 3515 – 3520; h) S.-I. Murahashi, S. Ono, Y. Imada, *Angew. Chem.* **2002**, *114*, 2472 – 2474; *Angew. Chem. Int. Ed.* **2002**, *41*, 2366 – 2368; i) Y. Imada, T. Iida, S.-I. Murahashi, T. Naota, *Angew. Chem.* **2005**, *117*, 1732 – 1734; *Angew. Chem. Int. Ed.* **2005**, *44*, 1704 – 1706; j) B. Wang, Y.-M. Shen, Y. Shi, *J. Org. Chem.* **2006**, *71*, 9519 – 9521; k) G. Peris, S. J. Miller, *Org. Lett.* **2008**, *10*, 3049 – 3052; l) A. V. Malkov, F. Friscourt, M. Bell, M. E. Swarbrick, P. Kočovský, *J. Org. Chem.* **2008**, *73*, 3996 – 4003; m) S. Xu, Z. Wang, X. Zhang, X. Zhang, K. Ding, *Angew. Chem.* **2008**, *120*, 2882 – 2885; *Angew. Chem. Int. Ed.* **2008**, *47*, 2840 – 2843.

[3] Reviews and selected key papers of BVMOs as catalysts in organic chemistry: a) M. M. Kayser, *Tetrahedron* **2009**, *65*, 947 – 974; b) M. D. Mihovilovic, *Curr. Org. Chem.* **2006**, *10*, 1265 – 1287; c) M. Kayser, G. Chen, J. Stewart, *Synlett* **1999**, 153 – 158; d) S. Flitsch, G. Grogan in *Enzyme Catalysis in Organic Synthesis, Vol. 2* (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**, pp. 1202 – 1245; e) V. Alphand, G. Carrea, R. Wohlgemuth, R. Furstoss, J. M. Woodley, *Trends Biotechnol.* **2003**, *21*, 318 – 323; f) P. C. Brzostowicz, D. M. Walters, S. M. Thomas, V. Nagarajan, P. E. Rouvière, *Appl. Environ. Microbiol.* **2003**, *69*, 334 – 342; g) N. M. Kamerbeek, D. B. Janssen, W. J. H. van Berkel, M. W. Fraaije, *Adv. Synth. Catal.* **2003**, *345*, 667 – 678; h) H. Iwaki, S. Wang, S. Grosse, H. Bergeron, A. Nagahashi, J. Lertvorachon, J. Yang, Y. Konishi, Y. Hasegawa, P. C. K. Lau, *Appl. Environ. Microbiol.* **2006**, *72*, 2707 – 2720; i) T. Kotani, H. Yurimoto, N. Kato, Y. Sakai, *J. Bacteriol.* **2007**, *189*, 886 – 893; j) A. Kirschner, J. Altenbuchner, U. T. Bornscheuer, *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1065 – 1072; k) D. E. Torres Pazmino, A. Riebel, J. de Lange, F. Rudroff, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* **2009**, *10*, 2595 – 2598; l) J. Rehdorf, M. D. Mihovilovic, U. T. Bornscheuer, *Angew. Chem.* **2010**, *122*, 4609 – 4611; *Angew. Chem. Int. Ed.* **2010**, *49*, 4506 – 4508.

[4] Key mechanistic studies of BVMOs:[3] a) C. T. Walsh, Y.-C. J. Chen, *Angew. Chem.* **1988**, *100*, 342 – 352; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 333 – 343; b) J. M. Schwab, W. B. Li, L. P. Thomas, *J. Am. Chem. Soc.* **1983**, *105*, 4800 – 4808; c) D. Sheng, D. P. Ballou, V. Massey, *Biochemistry* **2001**, *40*, 11156 – 11167.

[5] a) G. E. Turfitt, *Biochem. J.* **1948**, *42*, 376 – 383; b) N. A. Donoghue, D. B. Norris, P. W. Trudgill, *Eur. J. Biochem.* **1976**, *63*, 175 – 192; c) Y.-C. J. Chen, O. P. Peoples, C. T. Walsh, *J. Bacteriol.* **1988**, *170*, 781 – 789.

[6] a) M. J. Taschner, D. J. Black, *J. Am. Chem. Soc.* **1988**, *110*, 6892 – 6893; b) M. J. Taschner, D. J. Black, Q.-Z. Chen, *Tetrahedron: Asymmetry* **1993**, *4*, 1387 – 1390; c) M. J. Taschner, L. Peddada, *J. Chem. Soc. Chem. Commun.* **1992**, 1384 – 1385.

[7] a) F. Secundo, G. Carrea, S. Dallavalle, G. Franzosi, *Tetrahedron: Asymmetry* **1993**, *4*, 1981 – 1982; b) S. Colonna, N. Gaggero, G. Carrea, P. Pasta, *Chem. Commun.* **1998**, 415 – 416; c) G. Ottolina, S. Bianchi, B. Belloni, G. Carrea, B. Danieli, *Tetrahedron Lett.* **1999**, *40*, 8483 – 8486; d) S. Colonna, V. Pironti, P. Pasta, F. Zambianchi, *Tetrahedron Lett.* **2003**, *44*, 869 – 871; e) S. Colonna, N. Gaggero, G. Carrea, G. Ottolina, P. Pasta, F. Zambianchi, *Tetrahedron Lett.* **2002**, *43*, 1797 – 1799.

[8] a) M. W. Fraaije, J. Wu, D. P. H. M. Heuts, E. W. van Hellemond, J. H. Lutje Spelberg, D. B. Janssen, *Appl. Microbiol. Biotechnol.* **2005**, *66*, 393 – 400; b) E. Malito, A. Alfieri, M. W. Fraaije, A. Mattevi, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13157 – 13162.

[9] a) F. Zambianchi, M. W. Fraaije, G. Carrea, G. de Gonzalo, C. Rodríguez, V. Gotor, G. Ottolina, *Adv. Synth. Catal.* **2007**, *349*, 1327 – 1331; b) C. Rodríguez, G. de Gonzalo, D. E. Torres Pazmiño, M. W. Fraaije, V. Gotor, *Tetrahedron: Asymmetry* **2008**, *19*, 197 – 203.

[10] M. Bocola, F. Schulz, F. Leca, A. Vogel, M. W. Fraaije, M. T. Reetz, *Adv. Synth. Catal.* **2005**, *347*, 979 – 986.

[11] a) M. T. Reetz, S. Wu, *Chem. Commun.* **2008**, 5499 – 5501; b) M. T. Reetz, S. Wu, *J. Am. Chem. Soc.* **2009**, *131*, 15424 – 15432; c) S. Wu, J. P. Acevedo, M. T. Reetz, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2775 – 2780.

[12] a) M. T. Reetz, F. Daligault, B. Brunner, H. Hinrichs, A. Deege, *Angew. Chem.* **2004**, *116*, 4170 – 4173; *Angew. Chem. Int. Ed.* **2004**, *43*, 4078 – 4081; b) 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; c) M. D. Mihovilovic, F. Rudroff, A. Winninger, T. Schneider, F. Schulz, M. T. Reetz, *Org. Lett.* **2006**, *8*, 1221 – 1224.

[13] a) A. Z. Walton, J. D. Stewart, *Biotechnol. Prog.* **2002**, *18*, 262 – 268; b) F. Zambianchi, P. Pasta, G. Carrea, S. Colonna, N. Gaggero, J. M. Woodley, *Biotechnol. Bioeng.* **2002**, *78*, 489 – 496; c) F. Secundo, F. Zambianchi, G. Crippa, G. Carrea, G. Tedeschi, *J. Mol. Catal. B* **2005**, *34*, 1 – 6.

[14] a) M. K. DiTursi, S. J. Kwon, P. J. Reeder, J. S. Dordick, *Protein Eng.* **2006**, *19*, 517 – 524; b) C. Ó'Fágáin, *Enzyme Microb. Technol.* **2003**, *33*, 137 – 149; c) T. Oshima, *Curr. Opin. Struct. Biol.* **1994**, *4*, 623 – 628.

[15] a) J. Damborsky, J. Brezovsky, *Curr. Opin. Chem. Biol.* **2009**, *13*, 26 – 34; b) C. Jäckel, J. D. Bloom, P. Kast, F. H. Arnold, D. Hilvert, *J. Mol. Biol.* **2010**, *399*, 541 – 546; c) J. Tian, N. Wu, Y. Fan, *BMC Bioinformatics* **2010**, *11*:370.

[16] Reviews of protein engineering strategies for generating robust enzymes:[14] a) V. G. H. Eijsink, S. Gåseidnes, T. V. Borchert, B. van den Burg, *Biomol. Eng.* **2005**, *22*, 21 – 30; b) A. S. Bommarius, J. M. Broering, *Biocatal. Biotransform.* **2005**, *23*, 125 – 139; c) F. H. Arnold, *Acc. Chem. Res.* **1998**, *31*, 125 – 131; d) P. L. Wintrode, F. H. Arnold, *Adv. Protein Chem.* **2001**, *55*, 161 – 225.

[17] a) M. T. Reetz, J. D. Carballeira, A. Vogel, *Angew. Chem.* **2006**, *118*, 7909 – 7915; *Angew. Chem. Int. Ed.* **2006**, *45*, 7745 – 7751; b) M. T. Reetz, J. D. Carballeira, *Nat. Protoc.* **2007**, *2*, 891 – 903.

[18] M. T. Reetz, P. Soni, J. P. Acevedo, J. Sanchis, *Angew. Chem.* **2009**, *121*, 8418 – 8422; *Angew. Chem. Int. Ed.* **2009**, *48*, 8268 – 8272.

[19] a) J. R. Cherry, M. H. Lamsa, P. Scheider, J. Vind, A. Svendsen, A. Jones, A. H. Pedersen, *Nat. Biotechnol.* **1999**, *17*, 379 – 384; b) S.-S. Ju, L.-L. Lin, H. R. Chien, W.-H. Hsu, *FEMS Microbiol. Lett.* **2000**, *186*, 215 – 219; c) Y. Yang, L. Jiang, L. Zhu, Y. Wu, S. Yang, *J. Biotechnol.* **2000**, *81*, 113 – 118; d) L. Johnsen, G. Fimland, V. G. H. Eijsink, J. Nissen-Meyer, *Appl. Environ. Microbiol.* **2000**, *66*, 4798 – 4802; e) B. Morawski, S. Quan, F. H. Arnold, *Biotechnol. Bioeng.* **2001**, *76*, 99 – 107; f) K. H. Oh, S. H. Nam, H. S. Kim, *Protein Eng.* **2002**, *15*, 689 – 695; g) L.-L. Lin, H.-F. Lo, W.-Y. Chiang, H.-Y. Hu, W.-H. Hsu, C.-T. Chang, *Curr. Microbiol.* **2003**, *46*, 211 – 216; h) C. Miyazaki-Imamura, K. Oohira, R. Kitagawa, H. Nakano, T. Yamane, H. Takahashi, *Protein Eng.* **2003**, *16*, 423 – 428; i) A. Suemori, M. Iwakura, *J. Biol. Chem.* **2007**, *282*, 19969 – 19978.

[20] a) A. Slavica, I. Dib, B. Nidetzky, *Appl. Environ. Microbiol.* **2005**, *71*, 8061 – 8068; b) L. J. Perry, R. Wetzel, *Protein Eng.* **1987**, *1*, 101 – 105; c) Y. H. Kim, A. H. Berry, D. S. Spencer, W. E. Stites, *Protein Eng.* **2001**, *14*, 343 – 347.

[21] A. Mirza, B. J. Yachnin, S. Wang, S. Grosse, H. Bergeron, A. Imura, H. Iwaki, Y. Hasegawa, P. C. K. Lau, A. M. Berghuis, *J. Am. Chem. Soc.* **2009**, *131*, 8848 – 8854.

[22] D. J. Bougioukou, S. Kille, A. Taglieber, M. T. Reetz, *Adv. Synth. Catal.* **2009**, *351*, 3287 – 3305.

[23] J. D. Bloom, S. T. Labthavikul, C. R. Otey, F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 5869 – 5874.

[24] F. W. Studier, *Protein Expression Purif.* **2005**, *41*, 207 – 234.

[25] U. K. Laemmli, *Nature* **1970**, *227*, 680 – 685.

Received: August 11, 2010
Published online on November 15, 2010

