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Stabilization of cyclohexanone monooxygenase by a computationally designed disulfide bond spanning only one residue

Hugo L. van Beek, Hein J. Wijma, Lucie Fromont, Dick B. Janssen, Marco W. Fraaije *

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

Enzyme stability is an important parameter in biocatalytic applications, and there is a strong need for efficient methods to generate robust enzymes. We investigated whether stabilizing disulfide bonds can be computationally designed based on a model structure. In our approach, unlike in previous disulfide engineering studies, short bonds spanning only a few residues were included. We used cyclohexanone monooxygenase (CHMO), a Baeyer–Villiger monooxygenase (BVMO) from *Acinetobacter* sp. NCIMB9871 as the target enzyme. This enzyme has been the prototype BVMO for many biocatalytic studies even though it is notoriously labile. After creating a small library of mutant enzymes with introduced cysteine pairs and subsequent screening for improved thermostability, three stabilizing disulfide bonds were identified. The introduced disulfide bonds are all within 12 Å of each other, suggesting this particular region is critical for unfolding. This study shows that stabilizing disulfide bonds do not have to span many residues, as the most stabilizing disulfide bond, L323C–A325C, spans only one residue while it stabilizes the enzyme, as shown by a 6 °C increase in its apparent melting temperature.

1. Introduction

Enzyme stability is an important parameter in biocatalysis, as an increase in stability will improve the economics of enzyme use in industrial applications [1]. Unfortunately most enzymes are relatively unstable, not evolved to operate at high temperatures or in the presence of organic solvents. Improvements in thermostability are often laborious and usually require the creation and screening of large enzyme mutant libraries [2]. In most proteins, stabilizing mutations are found in certain critical regions, suggesting structural regions that allow early unfolding steps of an enzyme, while mutations elsewhere in the protein have a much smaller effect on thermostability [3–5]. Therefore, there is a need for methods to locate such critical regions [6], to allow enzyme engineering efforts to focus and so to reduce the amount of labor required to stabilize an enzyme [7,8].

Recently, we published an improved computational method for disulfide bond design to boost enzyme stability. For limonene epoxide hydrolase, this resulted in identification of 9 stabilizing disulfide bonds out of the 18 that were experimentally tested [9]. The stabilizing disulfide bonds were clustered at a critical region for stability, where also stabilizing point mutations had most effect [9]. This study showed that by introducing computer-designed disulfide bonds, it is possible to locate the critical region for stabilization using relatively few variants. However, this was done for a protein for which an X-ray structure was available and it is not obvious whether such a strategy would work for a protein for which only a homology model is available. Homology models are structurally far less accurate than (high resolution) X-ray structures [10], which could hinder the computational design of stabilizing disulfide bonds and thus a higher fraction of non-stabilized disulfide bonds can be expected.

Introduced disulfide bonds that contribute to protein stability typically involve disulfide bonds that bridge a large number of residues. The use of disulfide bonds spanning only a few residues is a surprisingly unexplored method to stabilize proteins. Disulfide bonds in natural proteins in majority encompass <30 residues, with most bonds spanning ~11 residues [11]. However, in protein stability engineering, short disulfide bonds are avoided, because it is assumed that the obtained stabilization by a disulfide bond is dominated by the number of residues that the disulfide bond spans across [12]. This proposed correlation between the obtained stabilization and the number of spanned residues is however poorly supported by experimental data. Therefore it appeared

Abbreviations: BVMO, Baeyer–Villiger monooxygenase; CHMO, cyclohexanone monooxygenase; DTT, dithiothreitol; MD, molecular dynamics; PAMO, phenylacetone monooxygenase

* Corresponding author. Tel.: +31 50 363 4345; fax: +31 50 363 4165.
E-mail address: m.w.fraaije@rug.nl (M.W. Fraaije).

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worthwhile to investigate the effect of introducing very short disulfide bonds, which might have been overlooked earlier [13].

In this study, we test whether computational design of disulfide bonds can be used to find a region critical for unfolding and to stabilize a coenzyme-dependent and cofactor-containing enzyme, cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB9871 (CHMO), of which no crystal structure is available. While our previous successful disulfide design was based on an X-ray structure of the target enzyme, this time we applied our in-house developed computational design protocol on a homology model. An additional modification was the inclusion in the *in silico* design of short disulfide bonds spanning <15 residues.

The target enzyme, CHMO, can be considered as the prototype enzyme for Baeyer–Villiger monooxygenases (BVMOs), an enzyme class that is attracting an ever-increasing interest for being applied in biocatalytic processes [14–20]. CHMO has been studied for its biocatalytic potential in numerous studies which has revealed that it (1) accepts a wide range of aliphatic substrates, (2) is able to perform chemo- and regioselective oxidations, and (3) yields absolute enantioselectivity for many of the tested conversions [19,21]. In the last decade, it has been the subject of several directed evolution and site-directed mutagenesis studies, aiming at altering the substrate specificity or improving the stability [22–25]. Recently this enzyme was used by Codexis as a starting point in a directed evolution approach to improve the enantioselective production of (*S*)-omeprazole, a blockbuster pharmaceutical [26]. So far, CHMO has defied all crystallization attempts which may partly be due to its notoriously poor thermostability [27–29]. Yet, to guide enzyme engineering attempts of CHMO, crystal structures of the homologous phenylacetone monooxygenase (PAMO) are available [30,31], and more recently the structure of the more closely related CHMO from *Rhodococcus* sp. was elucidated [32,33].

Previously we attempted to access CHMO-like activity in a thermostable enzyme by using PAMO from the thermophile *Thermobifida fusca* as a scaffold to introduce activities of related BVMOs [34]. PAMO has an apparent melting temperature (T_m) more than 20 °C higher than CHMO, but only has a limited substrate range [19,35]. We were able to expand the substrate specificity in a PAMO–CHMO chimera, but activity with the preferred substrate of CHMO, cyclohexanone, was not detected.

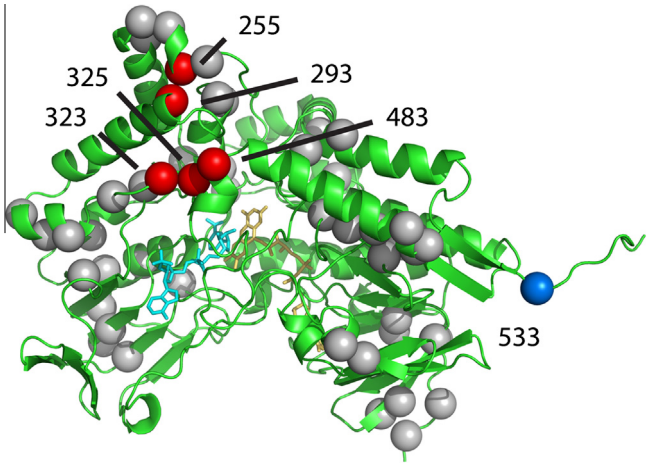

Fig. 1. Model structure of CHMO showing the introduced cysteines as spheres. Non-beneficial mutations are shown in gray, beneficial mutations shown in red. Residues 323–325 are mutated to cysteines in the best mutant, residues 255–293 and 325–483 were mutated in the other two described mutants. Of the truncation mutant, the last C-terminal residue is shown in blue. FAD is shown in yellow; $NADP^+$ is shown in cyan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Here we report on the introduction of disulfide bonds in CHMO, designed using a homology model based on the X-ray structure of a sequence-related CHMO (58% sequence identity). A total of 27 disulfide bond mutants were designed *in silico* and experimentally tested for their thermostability. Three stabilized mutants were identified, which were all located in the same region. This suggests that this particular region is important for the stabilization of CHMO. The most stabilized disulfide bond variant was L323C–A325C, with an apparent T_m increase of 6 °C and a 12-fold increased half-life, while its disulfide bonds spans only one residue. This illustrates the importance of taking extremely short disulfide bonds into consideration when designing more stable proteins.

2. Results

Disulfide bonds were designed in a model structure of *Acinetobacter* CHMO (see experimental section). To prevent the loss of catalytic activity, no disulfide bonds were allowed between residues that are within 8 Å from the FAD and $NADP^+$ cofactors bound in the homology model. Also, the loop between residues 487–504, which in the *Rhodococcus* CHMO adopts a different conformation in the open and closed form of the enzyme [32], was excluded from mutagenesis. While previously disulfide bonds that spanned <15 residues were not allowed [9], here no such restrictions were implemented. The selected disulfide bonds are distributed over the whole protein, to probe for the regions that are important for thermostability (Fig. 1). Of the 27 selected disulfide bonds, 8 spanned <15 amino acids (Table S1). Additionally, because in the homology model the C-terminal residues 534–545 could not be modeled (absent in the crystal structure), this C-terminus was removed in a separate mutant, as the removal of such disordered C-termini can also increase stability [11].

All mutants were grown in 50 mL TB medium and twenty disulfide bond mutants were expressed and purified in sufficient yield to determine the apparent melting point in the ThermoFAD experiment. In this experiment the enzyme is heated in a RT-PCR machine, and by following the increase in FAD fluorescence when it is released from the protein, the apparent melting point can be determined. Of the twenty purified mutants, three displayed a higher apparent melting temperature than the wild-type CHMO, while for other disulfide bond mutants the apparent melting temperature was similar to the wild-type CHMO or even significantly reduced (Table S1 and Fig. S1). The truncation mutant, missing 10 residues at the C-terminus, was expressed but turned out to be slightly less stable than the wild-type enzyme (Table 1). The best mutant, L323C–A325C, showed a 6 °C increase in apparent melting temperature, while two other mutants, A255C–A293C and A325C–L483C, showed an increase of 2.5 °C (Table 1). For these three mutants the melting curves showed only one maximum, indicating there are no subpopulations in which the disulfide bond is not formed, or formed improperly (Fig. S2).

In addition to the ThermoFAD measurements, enzyme activity was monitored at a single temperature to determine the half-life,

Table 1
Apparent melting temperatures of all studied CHMO variants.

CHMO variant	T_m (°C)
WT	38.0
A255C–A293C	40.5
A325C–L483C	40.5
L323C–A325C	44.0 (36.5)[a]
R534stop	37.0

[a] The melting point for the reduced form, obtained by incubating the enzyme o/n with 1.0 mM DTT.