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ARTICLE

Enhanced turnover rate and enantioselectivity in the asymmetric epoxidation of styrene by new T213G mutants of CYP 119

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New CYP 119 T213G mutants were constructed and characterized. Introduction of T213G mutation into the wild-type CYP 119 significantly enhances the turnover rate for the peroxide-dependent styrene epoxidation by 4.4-fold to 346.2 min^{-1}, and the double T213G/T214V mutant improves the ratio of the S- and R-enantiomers of the epoxide products by 2- fold to 5.8. The molecular modelling and docking results support our initial design and experimental data.

Introduction

Cytochromes P450 enzymes (P450s or CYPs) are heme-containing enzymes that catalyze a vast range of reactions and tolerate a diversity of substrates.[1] In humans, the P450s serve in highly specific oxidations of drugs, pro-drugs and xenobiotics.[2] These oxidations include the regio- and stereoselective epoxidation of olefins and hydroxylation of hydrocarbon chains, which make P450s highly promising catalysts in fine chemical synthesis and biopharmaceutical production.[3] However, the practical potential of these enzymes in industrial process has been restricted because of the deficiency in thermostability, the high costs of cofactors such as NADH, the inappropriate docking between substrate and active site pocket and the consequent inability through protein engineering to the desired properties.

CYP 119 as one of the first thermophilic P450 enzymes provides improvements for the above limitations in application process.[4] CYP 119 cloned from the hyperthermophilic archaebacteria *sulfolobus acidocaldarius* has demonstrated enhanced stability for high temperature and pressure.[5-7] The molecular structure of CYP 119 was determined by X-ray crystallography after heterologous expression in *E. coli* and purification, [8] which contributes to the determination of substrate specificity and the consequent modification of the heme active site. The rigid active site structure of thermostable CYP 119 makes it an attractive system for the formation of the Compound I species by the peroxide shunt in the P450 reaction mechanism, [9-14] which enables substrate oxidation without the use of expensive electron equivalents from NADH. The initial studies of styrene epoxidation were carried out with hydrogen peroxide as an electron donor of oxidizing equivalents to assay the catalytic activity of the wild-type CYP 119, and very low turnover rate was given with 0.6 nmol min^{-1} $nmol^{-1}$ at 30 ^{o}C.[15] Recently, Niemeyer et al described that the optimized reaction conditions led to an increased turnover rate in the peroxide-dependent epoxidation catalyzed by the wild-type CYP119.[16] Montellano et al reported that the structural modification of CYP 119 in the active pocket enhanced its catalytic activity with no impact on its thermal stability.[15,17] For example, the site-directed mutation of Thr214 to valine was found to increase the rate by 3-fold in the H_2O_2-dependent styrene epoxidation at 30 °C, while the rate was kept at a very low value when Thr213 was mutated to valine, phenylalanine or tryptophan that had a larger residue size.[15] In addition, the asymmetric epoxidation of styrene suffers from very low enantioselectivity for most of the P450s although important progresses have been made by using chemical catalysts such as the Katsuki-Jacobsen or Shi catalyst and other biocatalysts such as styrene monooxygenase.[18,19] CYP 119 can catalyse the enantioselective epoxidation of styrene with the formation of the *S*- and *R*-enantiomers in an approximate ratio of 3.[15] These findings led us to propose that the site-directed mutation of Thr213 to glycine with the smallest side chain might enhance the rate of the peroxide-dependent styrene epoxidation under the optimized conditions, as well as improve the ratio of the *S*- and *R*-epoxides due to the changed steric effect.

Herein, we report the expression, purification, construction and characterization of new CYP 119 T213G mutants by an optimized method. We demonstrate that the introduction of new T213G mutation into the wild-type CYP 119 and its T214V mutant can significantly enhance the catalytic rate and enantioselectivity of the peroxide-dependent epoxidation of styrene respectively. Furthermore, these results are in agreement with our studies on the molecular modelling and docking of styrene into the active site pocket of the wild-type and T213G mutant.

Results and Discussion

We first cloned out the gene of CYP119 from a hyperthermophilic archaebacteria (ATCC 35091). Cultures were grown at 75 ^{o}C in medium 1304 (ATCC). Originally, The genome of CYP119 was believed to derive from the thermophile *Sulfolobus solfataricus*, but more recently, it was reported to come from the closely related speices *Sulfolobus acidocaldarius* that contaminated an *S. solfataricus* culture.[16] In our procedure, the plasmid is constructed

by pET30a vector with 6 histidine tag before the C-terminal, which makes the protein easy to be eluted with low concentration of imidazole, and can avoid the bonding of excess imidazole and the protein. The utility of single Ni-NTA column equilibrated with the high concentration of NaCl and washed with linear gradient of imidazole can ensure the purity of the desired protein. The CYP119 enzyme in yield of ca. 23 mg per liter of culture is obtained after overexpression and purification by an optimized expression method.[20]

Then, the CYP119 T213G mutant were constructed by using QuickChange Lighting Site-Directed Mutagenesis Kit. The mutant was overexpressed and purified in the same manner as wild-type CYP119 in the pET30a vector. The yield (ca. 18 mg) of purified mutants was less than that of the wild-type. The purity of the T213G mutant was determined by SDS-polyacrylamide gel electrophoresis with single 43-kDa band eluted from the Ni-NTA column (Figure 1a). As shown by the UV-visible absorption spectra in Figure 1b, the substrate-free ferric form of the T213G mutant, similar to that of the wild type of CYP 119, has a Soret band at ca. 417 nm and two visible bands at ca. 534 and 568 nm. The reduced-CO difference spectrum of T213G with a Soret maximum at ca. 450 nm is very similar to that of the wild type. These spectral signatures of T213G mutant are indicative of a typical low spin state of P450 enzymes. We also constructed both the T214V mutant and the double T213G/T214V mutant, which exhibit an abnormal ferric P450 spectrum with a partial high spin character as reported earlier that the mutation at Thr-214 has a much greater effect on the spin state than mutation at Thr-213.[15] These mutants are shown to be completely in the undenatured P450 state by stable reduced CO difference spectra at 450 nm (Figure 1b).

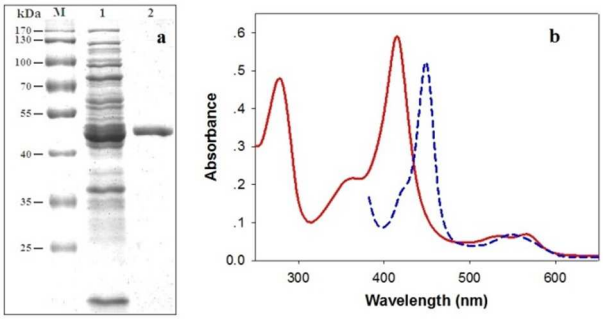

Figure 1. (a) SDS-polyacrylamide gel electrophoretic analysis of the purification of CYP119 T213G. Lane 1: supernatant after heating the crude lysate, Lane 2: after Ni-NTA column purification. (b) UV-vis spectra of CYP119 T213G. The ferric protein (solid line) and ferrous-CO complex protein (dashed line)

We carried out the styrene epoxidation reaction catalyzed by CYP 119 and its mutants at 70 °C and pH 8.5 in the presence of *tert*-butyl hydroperoxide (TBHP) as the oxidant on the basis of the optimized conditions reported earlier.[16] Within 10 min, the conversion of styrene by using CYP 119 and its mutants was almost complete with the formation of styrene epoxide, and no hydrolysis by-product was observed by HPLC and GC-MS analyses. The initial rate of the epoxide formation is determined with the aid of an external standard calibration curve prepared by using the pure epoxide. Figure 2 shows the double-reciprocal plot of these experimental data for the TBHP-dependent styrene epoxidation catalyzed by the CYP119 and its mutants. The kinetic constants calculated from the resulting regression equation are summarized in Table 1. Our result for the turnover rate of the wild-type CYP 119 was completely consistent with those reported in the literature at 70 °C (Entry 1).[16] The T213G mutant converts styrene to styrene epoxide with k_{cat} value of 346.2 min^{-1} and the T214V mutant turns over styrene at a rate of 191.6min^{-1} (Entry 2 and 3). Since both of the mutants apparently increase rates relative to the wild-type, the T213G/T214V double mutant was constructed to check if the turnover rate was additive, but only a reduced k_{cat} value of 42.7 min^{-1} was obtained in comparison with the wild-type (Entry 4). The K_m value of 30.4 mM for the T213G mutant shows an approximately 3-fold decrease in the affinity with styrene relative to those of the T214V mutant and the wild-type protein at 10.3 mM and 9.1 mM respectively. These results suggest that the increased turnover rate might not be attributed to the affinity to substrate, but to the reduced steric hindrance of the T213G mutation with the smallest side chain, as well as the increased hydrophobic environment of the T214V mutant with valine residues lacking affinity for water, which may enhance the accessibility of both styrene and TBHP to the catalytic active site cavity. This hypothesis was also supported by the findings from the H_2O_2-dependent styrene epoxidation at 30 °C, stating that the mutation of Thr213 to alanine or serine with smaller side chain had been found to have the opposite effect on turnover rate than mutation to valine, phenylalanine or tryptophan with larger side chain.[15]

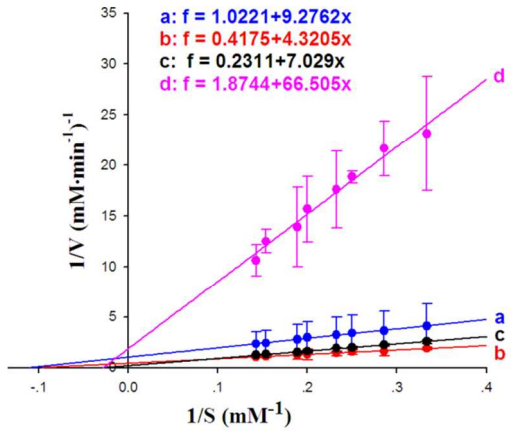

Figure 2. The double-reciprocal plot of kinetic data: (a) the wide type, (b) T214V mutant, (c) T213G mutant and (d) T213G/T214V mutant at 70 °C.

Table 1. The kinetic constants calculated from the double-reciprocal plot of CYP 119 and its mutants.

Entry	Enzyme	Tem. (°C)	pH	k_{cat} (min^{-1})	K_m (mM)	*S:R* epoxide ratio
1	WT	70	8.5	78.3±3.9	9.1±0.7	2.1
2	T213G	70	8.5	346.2±44.5	30.4±4.5	3.72
3	T214V	70	8.5	191.6±11.5	10.3±0.9	3.25
4	T213G/ T214V	70	8.5	42.7±8.4	35.5±7.9	4.6
5	WT	35	7.5	15.7±0.4	8.4±0.3	3.3
6	T213G	35	7.5	51.2±2.4	14.1±0.9	4.9
7	T214V	35	7.5	31.6±1.4	8.5±0.6	4.1
8	T213G/ T214V	35	7.5	-	-	5.8

In order to confirm whether the mutation in the active cavity can have effects on the catalytic activity of CYP 119

independent of its thermostability, we further investigated the TBHP-dependent styrene epoxidation at 35 °C with an adjusted pH value of 7.5 (Figure S4). As shown by Entry 5 in Table 1, the wild-type CYP 119 gave the k_{cat} value of 15.7 min^{-1}, which exhibits approximately 26-fold increase in turnover rate over the previously reported H_2O_2-dependent epoxidation with 0.6 nmol min^{-1} $nmol^{-1}$ at 30 °C.[15] The epoxide formation by using the T213G and T214V mutants gave rates of 51.2 and 31.6 min^{-1} respectively, which are apparently faster than the use of the wild-type enzyme (Entry 6 and 7 in Table 1). No turnover rate was observed for the double mutant under these conditions. The K_m values of the wild-type, T213G and T214V mutants are 8.4 mM, 14.1 mM and 8.5 mM respectively, which indicate higher affinity for styrene at 35 °C than at 70 °C. The above results demonstrate that the introduction of the mutation into the active site of the wild-type CYP 119 significantly improves its catalytic activity without affecting its thermal stability. Our results with the new T213G mutant at 70 °C exhibit about a 4.4-fold increase relative to the wild-type under the same condition.

We analyzed the enantioselectivity of the styrene epoxidation catalyzed by the wild-type and the mutants. The ratio of the *S*- and *R*-enantiomers of the epoxide at 35 °C and 70 °C are summarized in Table 1 respectively. The formation of the (*S*)-enantiomer by using the mutants is more favorable than those obtained by using the wild-type CYP 119. High temperature leads to decrease in the enantioselective oxidation probably because of the reduced affinity of the mutants for styrene at 70 °C over that at 35 °C. The double T213G/T214V mutant gave a 5.8 ratio of the *S*- and *R*-styrene oxide enantiomers at 35 °C, which showed an approximate 2-fold increase in enantioselectivity relative to the wild-type. The results in Table 1 suggest that the enantioselective improvement might be mainly due to the mutation at T213G with the introduction of the smallest side chain in the active site of CYP 119.

Similar results are found for various vinyl aromatic substrates including 4-fluoro, 4-chloro and 4-bromostyrene as shown in Table 2. The enantioselectivity of the epoxidation varies directly with the size of the substituents in the *para*-halogenated styrenes, but, no product was observed for the epoxidation of *para*-fluorostyrene catalyzed by the double mutant (Entry 1 and 2 in Table 2). The larger the para-substituent of styrene is, the lower the enantioselectivity of the epoxidation. For example, the epoxidation of *para*-fluorostyrene by using the T213G mutant yield a ratio of 5 for the *S*- and *R*- epoxide enantiomers at 35 °C, while that of *para*-bromostyrene gave a ratio of 2.9 under the same conditions (Entry 1 and 5 in Table 2). These results further confirm that the enantioselectivity is improved by decreasing steric hindrance of the peroxide-dependent epoxidation of styrene.

Table 2. Ratio of the *S*- and *R*-enantiomers from various styrenes epoxidation catalyzed by CYP 119 mutants.

Entry	Substrate	Tem. (°C)	pH	T213G	T214V	T213G/ T214V
1	4-fluorostyrene	35	7.5	5.0	4.5	-
2	4-fluorostyrene	70	8.5	4.0	4.0	-
3	4-chlorostyrene	35	7.5	2.9	2.8	2.6
4	4-chlorostyrene	70	8.5	2.7	2.5	2.2
5	4-bromostyrene	35	7.5	2.9	2.6	1.8
6	4-bromostyrene	70	8.5	2.4	2.3	1.3

We conducted the molecular modelling and docking of the prochiral styrene into the catalytic active cavity of the wild-type and the T213G mutant by using the *GOLD* program. As shown in Figure 3, the Gly213 residue in the T213G mutant and the Thr213 residue in the wild-type are 5.69Å and 3.25Å from an iron-oxo species of the heme respectively, which could give access to styrene and TBHP. When docking styrene into the catalytic active cavity of the T213G mutant (Figure 3a and b) and the wild-type (Figure 3c and d) respectively, we found that the distance of the vinyl group from either the Thr213 residue or the Gly213 residue in the *re* face interaction of styrene is always greater than that in the *si* face interaction. The interaction energy of the iron-oxo species known as Compound I with the *re* face of styrene is found to be always less than that with the *si* face, and the interaction energy differences for the wild-type and the T213G mutant are 6.7 kcal/mol and 4.4 kcal/mol respectively. This indicates that there should be enhanced preferences for the formation of *S*-epoxide enantiomer from the *re* face interaction by introduction of Gly213 mutation into the wild-type CYP 119. The molecular docking of styrene into the catalytically active cavity of the T213G mutant supports our initial hypothesis and experimental data that the reduced steric hindrance in the T213G mutant can significantly enhance the catalytic rate and enantioselectivity for the styrene epoxidation.

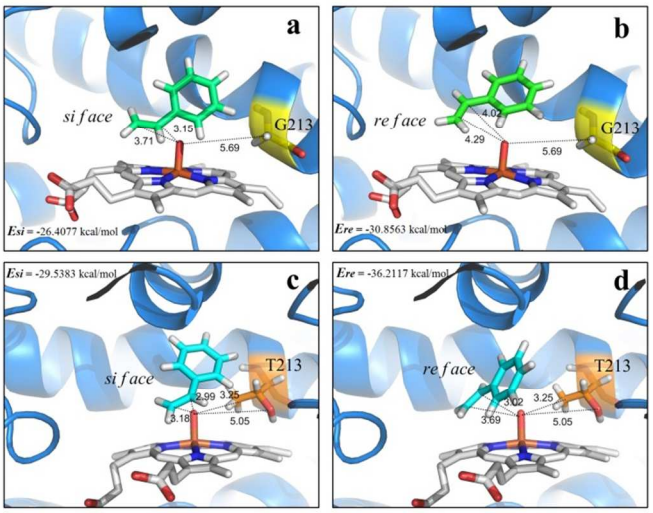

Figure 3. The molecular docking of prochiral styrene into the T213G mutant (a and b) and the wild-type CYP 119 (c and d).

Experimental Section

Cloning, Expression and Purification of CYP119

Sulfolobus acidocaldarius was purchased from ATCC (Strain 35091) and cultures were grown in medium 1304 (ATCC) at 75 °C. Chromosomal DNA was isolated by TIANamp Bacteria DNA Kit (TIANGEN Beijing, China). The gene encoding for CYP119 was

amplified by PCR with use of the forward primer CP1: 5'-CCGGAATTCATGTATGACT GGTTTAGTGA-3' and the reverse primer CP2: 5'-CCGCTCGAGTTATTCATTACTCTTCAACCTG ACC-3'. PCR amplification was carried out in a 50 μL reaction mixture containing 1 × reaction buffer, 1.5 mM $MgCl_2$, 1 μM of each primer, 200 μM of each dNTP, 50 ng template DNA, 1.25 units of Ex*Taq* Polymerase and sterile water to the final volume. PCR reactions were carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems Inc., California, USA). The thermocycling profile consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C and final extension step of 8 min at 72 °C. The PCR product was purified by a gel extraction kit E.N.Z.A.TM (Omega, Georgia, USA) and then ligated into pET30a vector using the *EcoR* I and *Xho* I sites. The sequence of plasmid pET30a-CYP119 was confirmed by DNA sequence analysis. The plasmid pET30a-CYP119 was transformed into BL21(DE3) plysS cells and selected for kanamycin and chloramphenicol resistance. For enzyme production, the overnight culture (2 mL) was used to inoculate supplemented TB medium (1 L), containing Kanamycin (50 mg/ L), chloramphenicol (34 mg/L), trace elements (250 μL/L), [20] and the expression was induced by the addition of IPTG (0.5 mM). The cells were cultivated for 48h at 30 °C. Cells were collected by centrifugation and the pellets were resuspended in 50 mM phosphate buffer with imidazole (5 mM), pH 7.4. The cells were sonicated and centrifuged. The supernatant was heated to 60 °C for 30 min. The soluble fraction was purified using a Ni-NTA superflow column (QIAGEN) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, 5 mM imidazole, containing two concentrations of NaCl (30 mM then 500 mM). The column was washed with a linear imidazole gradient (15–30 mM) and the protein was eluted with 80 mM imidazole. Fractions containing the highest A_{415}: A_{280} ratio was pooled. The pooled fractions were collected and concentrated using Amicon Ultra-15 Centrifugal Filter 10 kDa (Millipore) and buffer-exchanged with 50 mM phosphate buffer (pH = 7.5). Protein concentration was calculated with use of the molar extinction coefficient of ε_{415} = 104 mM^{-1}.[16] The purity of the protein was comfirmed by SDS-PAGE and by UV/Vis spectrophotometry.

Site-Directed Mutagenesis of CYP119

The mutants were constructed using QuickChange Lighting Site-directed Mutagenesis Kit (Agilent Techologies). The plasmid pET30a-CYP119 was amplified. The forward and reverse primers for the mutant T214V were 5'-GTAATGAGACT**GTT**ACTAAC TTAATATCAAACTCTGT-3', 5'-GATATTAAGTTAGT**AAC** AGTCTCATTACCCGCTATGAG-3'. Primers for the mutant T213G were 5'-TTCTCATAGCGGGTAATGAG**GGT**ACAACT AACTTAATATCAAA-3', 5'-TTTGATATTAAGTTAGTTGT**ACC** CTCATTACCCGCTATGAGAA-3'. Primers for the double mutant T213G/T214V were 5'-GGTAATGAG**GGTGTT**ACTAACTTA ATATCAAACTCTGT-3', 5'-TGATATTAAGTTAGT**AACACC** CTCATTACC CGCTATGAG-3' (Bold and lined face indicates the positions of the mutations). Over expression, and purification of CYP119 mutants were carried out the same as the WT CYP119, except for reducing the sonicated duty and time. The purity of the T213G mutant was determined by SDS-polyacryl-amide gel electrophoresis with single 43-kDa band eluted from the Ni-NTA column.

Styrene Epoxidation

Styrene epoxidation experiments were carried out at 35 °C and 70 °C in the presence of *tert*-butyl hydroperoxide (TBHP) as the oxidant by using the wild-type, the T213G mutant, the T214V mutant and the double T213G/T214V mutant. Within 10 min, the conversion of styrene by using CYP 119 and its mutants was almost complete with the formation of styrene epoxide at 70 °C and pH 8.5 in the presence of TBHP. For reasons of comparison we used identical enzyme concentration previously reported.[16,17] The initial rate of the epoxide formation is determined with the aid of an external standard calibration curve prepared by using the pure epoxide. Kinetic constants were determined by double-reciprocal plot and calculated from the resulting regression equation. At 35 °C, the reaction were carried out in closed glass vials in total volumes of 80 μL containing CYP119 (12.5 μM), variable styrene concentrations (Styrene was added as a 0.25 M solution in acetonitrile). TBHP was used as the oxidant with the same concentration as styrene substrate. Phosphate buffer (pH7.5) was added to the final volume. Each reaction was allowed to proceed for 30 second at 35 °C and stopped by addition of acetonitrile (720 μL). At 70 °C, the reaction condition was carried out as the reaction at 35 °C except for some modifications: CYP119 mutants, variable styrene concentrations and TBHP were added in 50 mM glycine buffer, pH 8.5. All the experiments were carried out in triplicate. The products were analyzed by HPLC (Dionex U-3000) connected to a UV-detector set to 220 nm on a Waters SunFireTM C18 column (4.6 mm × 150 mm) with ddH_2O (30%) / acetonitrile (70%) as the mobile phase. Styrene and styrene epoxide were identified by comparison of the retention times with those of the pure substances under the same conditions. The retention times found with this condition were ca. 4.8 min for styrene and ca. 2.9 min for styrene epoxide.

Determination of Enantioselectivity

The enantioselectivity of styrene epoxidation catalysed by using the wild-type, the T213G mutant, the T214V mutant and the double T213G/T214V mutant was determined at 35 °C and 70 °C respectively. The reaction was performed as described above with 7 mM styrene, 4-fluorostyrene, 4-chlorostyrene and 4-bromostyrene respectively. After 10 min, each reaction was stopped by addition of CH_2Cl_2 (100 μL). Products were then extracted with CH_2Cl_2 for three times. CH_2Cl_2 layers were concentrated and then analysed by GC (SP3470) and GC-MS (ITQ900) on a CP-chirasl-Dex CB column (25m×0.25mm inner diameter). *R*-and *S*- styrene epoxides were used to identify the retention times. MS data of styrene epoxide was also verified by matching the fragmentation pattern with a database in the software. Authentic standards of the other epoxides were obtained by adding *m*-chloroperbenzoic acid to the *para*-halogenated styrene in $CHCl_3$ to react at 35 °C for 30 min. These products were detected by GC on a CP-chirasl-Dex CB column (25m×0.25mm inner diameter) and MS data were verified by matching the fragmentation pattern with a database in the software.

Molecular Modelling and Docking of the Wild-Type and the T213G Mutant

The crystal structure of wild-type CYP119 with access code 1F4T was obtained from the PDB database (http://www.pdb.org/). The crystal structure 1F4T was then used as the original template to build the structure of T213G mutant. A site-directed mutation was carried out to mutate residue Thr213 to Gly with the Build Mutants Protocol in Discovery Studio 3.1 (Accelrys Corporation). The minimum DOPE score was obtained and the optimum conformation with the larger cavity in the active pocket of the T213G compared with the wild-type CYP119. The conformation of Gly213 and its surrounding residues within 4.5Å cut-off radius was then optimized. Molecular docking was performed by using the *GOLD* (Genetic Optimization for Ligand Docking) program because of its considerable highly accuracy compared with the other docking program contained in

Discovery Studio. *GOLD* uses a genetic algorithm for docking flexible ligands into receptor binding sites. Firstly, for the wild-type CYP119, the crystal structure with PDB ID 1F4T was defined as the receptor. Crystal water molecules and heteroatoms were removed. Hydrogen atoms were added and CHARMM force field was applied, subsequently we defined the binding site approaching the heme which is the catalytic active region for prochiral styrene binding, then prepared the substrate of styrene using the ligands prepare protocol of the Discovery Studio, finally we carried out parameter setting (included inputting the receptor and ligands, choosing fitness function, GA parameter setting etc.) and running the *GOLD* program, The same method was used to dock the prochiral styrene into the catalytic active center of the T213G Mutant. Goldscore was selected as scoring function to evaluate that docking result by *GOLD*. Energy calculation and distance determination was performed by the interaction energy protocol and the distance measuring tool in the Discovery Studio. The optimizing figure was completed by the pymol software and relative software.

Conclusions

We constructed and characterized new CYP 119 T213G mutants by an optimized expression method. The introduction of the T213G mutation to CYP 119 can significantly enhance the catalytic turnover rate and enantioselectivity in the asymmetric epoxidation of styrene. We have demonstrated that the k_{cat} value of styrene epoxidation catalyzed by new mutant CYP 119 T213G at 70 ^{o}C exhibits approximately 4.4-fold increase relative to the wild-type under the same condition. The formation of the *S*-enantiomer with an approximate ratio of 5.8 by using the double T213G/T214 mutant is more favorable by about 2-fold than those obtained by using the wild-type CYP 119. These results were supported by information from the molecular docking of prochiral styrene into the wild-type and its T213G mutant that the significant improvements in catalytic rate and enantioselectivity come from the decreased steric hindrance in the T213G mutant. The significant improvements described here for the use of the T213G mutants over that of the wild type CYP 119 enzyme indicate that the protein engineering strategy has great potential in the development of novel CYP 119 biocatalytic systems for efficient asymmetric epoxidation.

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Notes and references

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Electronic Supplementary Information (ESI) available: HPLC, GC and MS spectra, molecular docking. See DOI: 10.1039/b000000x/

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Graphical Abstract

Enhanced turnover rate and enantioselectivity in the asymmetric epoxidation of styrene by new T213G

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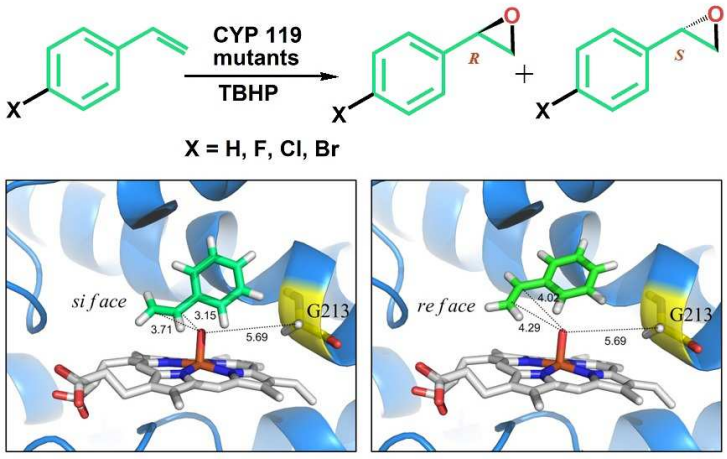
New CYP 119 T213G mutants were constructed and characterized. Introduction of T213G mutation into the wild-type CYP 119 significantly enhances the turnover rate for the styrene epoxidation by 4.4-fold to 346.2 min-1, and the double T213G/T214V mutant improves the ratio of the S- and R-enantiomers of the epoxide products by 2- fold to 5.8. The molecular modelling and docking results support our initial design and experimental data.



T213G: turnover rate up to 346.2 min^{-1}

T213G/T214V: *S* and *R* enantiomers ratio up to 5.8