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A *p*-nitrothiophenolate screening system for the directed evolution of a two-component epoxygenase (StyAB)

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Abstract

Epoxygenases are attractive enzymes for synthesizing important chemical synthons. Directed evolution of epoxygenase properties to production demands have been limited until recently by a lack of screening systems. The previously reported *p*-nitrothiophenolate (pNTP) screening system was validated through improving styrene epoxidation activity of P450 BM-3 from *Bacillus megaterium*. Unlike the catalytically self-sufficient P450 BM-3, most epoxygenases are multi-component systems and often significantly less active. We improved the pNTP screening system for a two-component epoxygenase, styrene monooxygenase StyAB from *Pseudomonas* species, by enhancing the sensitivity of the pNTP assay from 400 to 140 μM and reducing styrene evaporation from 72 to 52%. These improvements were achieved using methylated β-cyclodextrins (mβ-CD) as inclusion host for styrene. Incorporation of mβ-CD increases styrene availability over the assay period and thus enables screening for improved mutants. The pNTP screening procedure for StyAB was subsequently verified in 96-well microtiter plate screens by gas chromatography analysis of styrene conversions.

Keywords: Directed evolution; High-throughput screening; Cyclodextrins; Monooxygenase; Epoxidation

1. Introduction

Epoxides are an important and versatile class of chemicals used as intermediates for fine chemicals and pharmaceutics production [1]. Chiral epoxide synthesis has seen impressive progress, for instance through the use of metallosalen catalysts [2]. Biocatalysis presents attractive alternatives for the production of epoxides that often complement substrate and product spectra of chemical catalysts [3–6]. Advantages of biocatalysts comprise an often adjustable and improvable regio- and enantioselectivity, as well as their ability to function under mild conditions at ambient temperature using molecular oxygen as oxidant. Cytochrome P450s [7,8], styrene monooxygenases [9,10], toluene monooxygenase [11] and alkene monooxygenase [12] are examples of well-studied epoxygenases.

For use in chemical syntheses it is important to improve epoxygenase properties such as activity, selectivity, organic solvent resistance, and tolerance towards molar concentration of substrates and products. Directed evolution steers the laboratory adaptation of biocatalysts in iterative cycles of generating genetic diversity and identifying improved protein variants [13–15]. A prerequisite for any directed evolution experiment is a reliable screening system that reflects application conditions and enables identification of improved biocatalyst variants in mutant libraries. For epoxidations, two microtiter plate screening systems have been reported recently [16–18]. The chromogenic *p*-nitrothiophenolate (pNTP) screening system was used for doubling the cytochrome P450 BM-3 activity toward styrene epoxidation and unexpectedly inverting its enantiopreference [18]. Cytochrome P450 BM-3 is, unlike most secondary metabolites converting epoxygenases, a catalytic self-sufficient monooxygense where the heme and reductase domains are on a single polypeptide [19]. Most epoxygenases are two- or even three-component systems [8]. Increased complexity of these multi-component systems tends to reduce turnover frequency. For example P450 BM-3 has an initial

Abbreviations: β-CD, β-cyclodextrins; GC, gas chromatography; IPTG, isopropyl-β-D-thiogalactoside; mβ-CD, methylated β-cyclodextrins; pNTP, *p*-nitrothiophenolate.

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epoxide turnover number of 600 min^{-1} for arachidonic acid [20], and 3126 min^{-1} for linolenic acid [21]. Multi-component monooxygenases such as human P4502C8 epoxidizes arachidonic acid at an initial turnover number of 0.7 min^{-1} [22] and rabbit P4502CAA epoxidizes linolenic acid at an initial turnover number of 1.7 min^{-1} [23].

StyAB from *Pseudomonas* species VLB120 is a two-component styrene monooxygenase which belongs to the class E flavoprotein monooxygenase [10,24]. StyAB has successfully been expressed heterologously in *Escherichia coli* and used to produce (*S*)-styrene oxide with >99% *ee* and a volumetric productivity of 2.2 g/L h^{-1} (*S*)-styrene oxide [25]. To further increase the attractiveness of this enzyme for industrial application, we seek to improve its catalytic rate of turnover. Unfortunately StyAB activity could not be detected using the published pNTP screening procedure [18] due to evaporation of styrene.

In this report, we increased the sensitivity of the pNTP screening system and reduced styrene evaporation by using methylated β-cyclodextrin (mβ-CD) for styrene complexation. Cyclodextrins are macrocyclic oligosaccharides consisting of 6(α), 7(β) or 8(γ) α-1,4-linked D-glucopyranose units. Cyclodextrins improve solubility of hydrophobic compounds and reduce their volatility [26]. Solubility of various hydrophobic compounds was increased including styrene [27] and drugs like hydrocortisone [26]. Volatility has for instance been reduced for sulfluramide [28] and methanol [26]. The pNTP screening procedure was thereby for the first time applied and validated for a two-component monooxygenase (StyAB).

2. Experimental

2.1. Material

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma–Aldrich Chemie (Taufkirchen, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), mβ-CD was from Fluka (product no. 66292, Taufkirchen, Germany) and pNTP was from Acros Organics (Geel, Belgium). All enzymes were purchased from New England Biolabs (Frankfurt, Germany), Fermentas (St. Leon-Rt, Germany), Sigma–Aldrich Chemie and Codexis (Redwood city, CA, United States). *E. coli* strains XL2-Blue and BL21-Gold(DE3) were purchased from Stratagene (La Jolla, CA, United States) and vector pET28a(+) from Novagene (EMD Biosciences, San Diego, United States). Purification kits used during cloning were from Qiagen (Hilden, Germany) and Machery-Nagel (Düren, Germany).

All PCRs were performed using a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany). Cell cultivations were performed in the shaking incubator Multitron II (Infors GmbH, Einsbach, Germany). Liquid handling machine Multimek 96 Beckman (Fullerton, CA), microplate shaker IH-S-1500 (Incutec GmbH, Mössingen, Germany) and microplate reader FlashScan S12 (Analytik Jena AG, Jena, Germany) were used for StyAB screening system development in 96-well microplates. Oxygen consumption was monitored using oxygen probe (Fibox 3, Precision Sensing GmbH, Regensburg, Germany) and GC analysis was performed using Shimadzu GC-2010 fitted with Optima®5 column (25 mm × 0.25 mm, Macherey-Nagel, Düren, Germany). All concentrations are in % (w/v) unless stated otherwise.

2.2. Cloning of styrene monooxygenase StyAB into pET28a(+)

The *StyAB* gene (GenBank accession no. AF031161) was amplified by PCR (95 °C for 2 min, 1 cycle/95 °C for 30 s, 58 °C for 30 s, 72 °C for 4 min, 30 cycles/72 °C for 10 min, 1 cycle) using dNTP (0.2 mM), pSPZ10 template (10.6 ng) [25], primers FPblunt (5′-CATGAAAAAGCGTATCGGTATTGTTGG-3′; 0.4 μM) and RPXhoI (5′-TCTGCTCGAGTTAATTCAGTGGCAACG-3′; 0.4 μM), and *Pfu* polymerase (2.5 U). Amplification product was purified by gel extraction, digested using XhoI (20 U), and purified using PCR purification kit. Cloning vector pET28a(+) was digested with NcoI (20 U), purified by a PCR purification kit and blunt ended with mung bean nuclease (10 U). The blunt ended pET28a(+) was PCR kit purified, digested with XhoI (20 U), and finally purified using gel extraction kit. Digested StyAB PCR product and pET28a(+) were ligated using T4 DNA ligase (2 U) to give pET28a-StyAB and transformed into *E. coli* XL2-Blue and BL21-Gold(DE3) using the TSS method [29].

2.3. Construction of StyAB error-prone PCR libraries

Mutant libraries of StyAB were created using epPCR (94 °C for 2 min, 1 cycle/94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min 45 s, 30 cycles/72 °C for 10 min, 1 cycle) with dNTP (0.2 mM), pET28a-StyAB template (10 ng), primers FPXbaI (5′-TCCCCTCTAGAAATAATTTTGTTTAAC-3′; 0.4 μM) and RPBamHI (5′-TCGGCGGATCCAGACTGATG-3′; 0.4 μM), $MnCl_2$ (0.02 or 0.05 mM) and *Taq* polymerase (2.5 U). PCR product was purified using a gel extraction kit. PCR product and pET28a-StyAB plasmid were digested using XbaI and BamHI (20 U each), purified by PCR purification kit and gel extraction kit, respectively. The digested StyAB mutant libraries were ligated into the double digested pET28a-StyAB using T4 DNA ligase (0.5 U) and transformed into *E. coli* BL21-Gold(DE3).

2.4. Solid-phase indigo assay

pET28a-StyAB mutant libraries in *E. coli* BL21-Gold(DE3) were plated on LB_{kan} plates (room temperature for ∼12 h, followed by 24–48 h at 4 °C for indigo formation). Blue colonies were transferred into 96-well microplate containing 150 μL of LB medium (50 mg/L kanamycin, 1% glucose) and cultivated overnight (37 °C, 900 rpm, 70% humidity).

2.5. 96-Well microplate expression of StyAB

Replica of mutant libraries were cultivated overnight (37 °C, 900 rpm, 70% humidity) in LB medium (150 μL, 50 mg/L

kanamycin, 1% glucose). The overnight cultures were used as inoculum (5 μL) in enriched TB medium (550 μL, 50 mg/L of kanamycin, 1% glucose, US*-trace element solution [30]; 2.2-mL deep well plates (square well/round bottom)). After 2.5 h of cultivation (37 °C, 900 rpm, 70% humidity), TB medium supplemented with isopropyl-β-D-thiogalactoside (50 μL, 50 mg/L kanamycin, 1% glucose, US*-trace element solution [30], 6 mM IPTG) was added to induce StyAB expression (30 °C, 700 rpm, 9.5 h). *E. coli* cells (400 μL) were harvested by centrifugation (4 °C, 3220 × *g*, 15 min) in V-bottom microtiter plates. The supernatant was discarded and cell pellets were stored at −20 °C.

2.6. Expression of StyAB in culture flask

StyAB was expressed in culture flasks for investigating the effects of mβ-CD. Enriched TB media (50 mL in 300-mL flask, 50 mg/L of kanamycin, 1% glucose, 50 μL US*-trace element solution [30]) was inoculated with an overnight culture of *E. coli* BL21-Gold(DE3)/pET28a-StyAB (100 μL). Cell density was monitored during cultivation (37 °C, 250 rpm) and StyAB was induced at an OD_{578} value between 0.6 and 0.8 by adding IPTG (0.5 mM). Expression was continued (30 °C, 250 rpm, 9.5 h) and cells were harvested by centrifugation (4 °C, 3220 × *g*, 20 min) and stored at −20 °C overnight. Cells were then resuspended in buffer (10 mL, 50 mM Tris pH 7.5, 25% sucrose, 10% (v/v) glycerol), disrupted using high-pressure homogenizer and cell debris was removed using centrifugation (4 °C, 20,000 × *g*, 30 min) followed by filtration through a 0.45 μm filter membrane. Cell lysates were subsequently used for measuring oxygen consumption rates.

2.7. Measurement of initial oxygen consumption rate

Measurement of oxygen consumption was performed in a 4.5-mL glass vial (2 mL reaction volume). Cell lysate (0.5 mL) from culture flask expression was added to the reaction mixture (1.4 mL, 100 mM potassium phosphate pH 7.5, 0–1.70% mβ-CD, 2 mM styrene, 2% (v/v) ethanol) and incubated with stirring in the closed vial for 2 min to allow stabilizing of oxygen concentration. NADH (0.25 mM) was then added to initiate the StyAB conversion of styrene and oxygen concentration was recorded using the oxygen probe (Fibox 3) at a sampling rate of 1/s.

2.8. Effect of mβ-CD on pNTP/styrene oxide reaction

The effects of mβ-CD on the pNTP/styrene oxide reaction were determined in 96-well polypropylene plates. The reaction mixture (200 μL) consisted of potassium phosphate (100 mM), styrene oxide (0–1.0 mM), ethanol (2%, v/v) and pNTP (0.48 mM). The reaction mixture was incubated (30 °C, 900 rpm, 1 h) and absorbance was measured after the colorimetric reaction at 405 and 443 nm using a microplate reader.

2.9. 96-Well format epoxide detection colorimetric assay

Cell lysate was prepared for pNTP activity assay in 96-well plates by resuspending each cell pellet in lysozyme (140 μL, 14000 U, 100 mM potassium phosphate pH 7.5, 10% glycerol), followed by incubation (37 °C, 1 h), and centrifugation (4 °C, 3220 × *g*, 15 min). 100 μL of lysed cell supernatant was transferred to each well of a 96-well polypropylene microplate. A reaction mix (42.5 μL) and NADH (7.5 μL, 5 mM) were supplemented reaching a final 150 μL reaction volume that consisted of mβ-CD (0.57%), styrene (4 mM), ethanol (2%, v/v), flavin adenine dinucleotide (13.3 μM), sodium formate (2.5 mM), catalase (55 U), formate dehydrogenase (0.1 U) and NADH (0.25 mM). The microtiter plates were incubated (750 rpm, 37 °C, 5 min) for styrene conversion. pNTP (40 μL, 2.5 mM in 0.25 M NaOH) and isopropanol (5 μL) were added sequentially to the reaction mixture, followed by a second incubation period (900 rpm, 30 °C, 30 min) for styrene epoxide quantification. Discoloration of pNTP was measured at 443 nm using a microplate reader. Calibration curves for pNTP discoloration were determined using the above protocol except that a gradient concentration of styrene oxide was added instead of styrene. Measurements for all styrene oxide concentrations were performed in triplicates. The pNTP dye was freshly prepared before use in 0.25 M NaOH instead of 0.1 M Tris buffer pH 8.0 [18].

Experiments for standard deviation calculations were performed in 96-well plate format using lysed cell supernatant from *E. coli* BL21-Gold(DE3) lacking StyAB, and in a separate experiment lysed cell supernatant with StyAB wild-type. Apparent standard deviation was based on discoloration obtained during a 30 min reaction between pNTP and styrene oxide in a 96-well microtiter plate of StyAB. The true standard deviation was calculated by subtracting the background discoloration of the microplate lacking StyAB from the apparent values.

2.10. Extraction and analysis of styrene oxide using gas chromatography

During screening procedure optimization and mutant characterization, all samples for GC analysis were prepared by solvent extraction using diethyl ether and 5 mM dodecane as internal standard. The extraction procedure was performed as previously described and samples were analyzed with gas chromatography (GC; Optima®5: 50 °C for 10 min; 50–170 °C at 20 °C min^{-1}; isothermic 170 °C for 6 min) [18]. Styrene and styrene oxide solutions were used to record a calibration curve for determining recovery, evaporation, and product formation.

3. Results and discussion

3.1. Indigo prescreen

Microorganisms produce indigo when expressing styrene monooxygenase in an active form [31]. Indigo was used as blue–white prescreen in the directed evolution experiments of StyAB to differentiate functional variants from non-functional ones. The solid-phase indigo prescreen is known to allow efficient selection from large variant libraries generated [32]. Based on the indigo prescreen, the mutagenic conditions of error-prone PCR were adjusted to 0.05 mM $MnCl_2$ which generates 30% non-functional StyAB variants. The solid-phase prescreen

allows selecting functional StyAB variants for subsequent liquid phase pNTP screening, thereby increasing screening efficiency. Indigo prescreen has a throughput of ~800 clones per petri dish and ~4000 clones were prescreened on agar plates before 2046 colonies were picked for subsequent liquid pNTP assay.

3.2. Expression of StyAB and cell lysate preparation in 96-deep well format

Expression of StyAB in 96-deep well plates was optimized using pET28a-StyAB plasmid in *E. coli* BL21-Gold(DE3). Expression conditions were optimized by varying IPTG concentrations (0.1, 0.2, 0.5 and 1.0 mM) and cultivation times (10, 12 and 24 h). Increasing IPTG from 0.1 to 0.5 mM showed significant increase in expression whereas only a small increase was observed by using 1.0 mM IPTG instead of 0.5 mM. Growth curves (OD_{578}) indicated that the *E. coli* host entered stationary phase of growth after 12 h.

Based on these results, the microtiter plate cultures (550 μL, enriched TB medium) were induced after 2.5 h using 0.5 mM IPTG and incubated for StyAB expression for 9.5 h (30 °C, 700 rpm, 70% humidity). The homogeneity of the cell density at cell harvest was determined at OD_{578} to have a standard deviation of 7%. Differing from previously published cell lysis protocol [18], glycerol (10% final concentration) was supplemented together with lysozyme during cell lysis to stabilize StyAB [33].

3.3. Effects of mβ-CD on StyAB activity and styrene oxide detection

The main modification to the previously reported pNTP screening system [18] is addition of mβ-CD. The cyclodextrins (α, β and γ) are commonly used for inclusion of hydrophobic compounds. In a comparative investigation of 8 different cyclodextrins, 9 out of 11 tested compounds (e.g. benzoic acid, salicylic acid and ibuprofen) have the lowest dissociation constants when complexed with β-cyclodextrin [34]. Methylation increases solubility of β-cyclodextrin. Since styrene and styrene oxide are known to form inclusion complexes with mβ-CD [27,35,36], mβ-CD was selected as host for styrene in the development of an enhanced pNTP screening procedure.

Effects of mβ-CD on the pNTP dye, styrene oxide detection and StyAB activity were investigated. mβ-CD causes a red-shift in the absorbance peak of pNTP, thus a wavelength of 443 nm was used instead of 405 nm for styrene oxide quantification via pNTP discoloration. A cyclodextrin-caused shift in fluorescence and absorbance spectra of dyes has been documented [26,37]. The observed red-shift in the absorbance peak of pNTP thus indicates a complex formation between mβ-CD and pNTP dye. Interestingly the reaction between pNTP and styrene oxide is improved in the presence of mβ-CD. Fig. 1 shows the increased reaction rate of pNTP with styrene oxide in the presence of various concentrations of mβ-CD (0.57–1.70%; 0.1 M potassium phosphate pH 7.5, 30 °C, 900 rpm) after a 1 h discoloration period. Under these conditions, the concentration of 0.57% mβ-CD is sufficient to improve detection sensitivity such that a production of 1 mM styrene oxide can clearly be identified with a change of 1.5 absorbance units compared to 0.7 absorbance units in the absence of mβ-CD. Differences in the starting absorbance are caused by the two different wavelengths used to determine conversions.

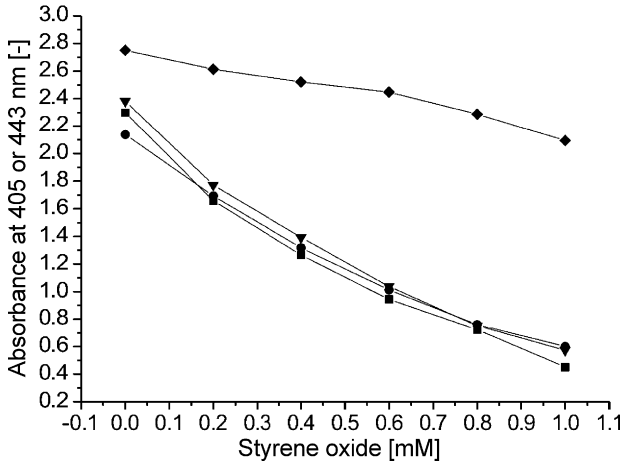


Fig. 1. Effects of mβ-CD on pNTP discoloration activity in presence of varied styrene oxide concentration after 1 h incubation (0.1 M potassium phosphate, pH 7.5, 30 °C, 900 rpm). (♦) No supplemented mβ-CD (405 nm); (●) in presence of 0.57% mβ-CD (443 nm); (■) in presence of 1.14% mβ-CD (443 nm); (▼) in presence of 1.70% of mβ-CD (443 nm).

A further control experiment was performed to investigate whether mβ-CD affects the activity of StyAB. mβ-CD concentrations between 0.57 and 1.70% were investigated to determine an optimal concentration during styrene conversion by StyAB. Fig. 2 shows the initial oxygen consumption rates of 2 mM styrene epoxidation by StyAB in closed 4.5 mL vials. The ratio of air head-space in a closed reaction vial to volume of reaction mixture is 1.25, and this ratio is 1.0 in the microtiter plate screen. Under these comparable conditions no differences in oxygen consumption values could be observed in absence and presence of 0.57–1.70% mβ-CD.

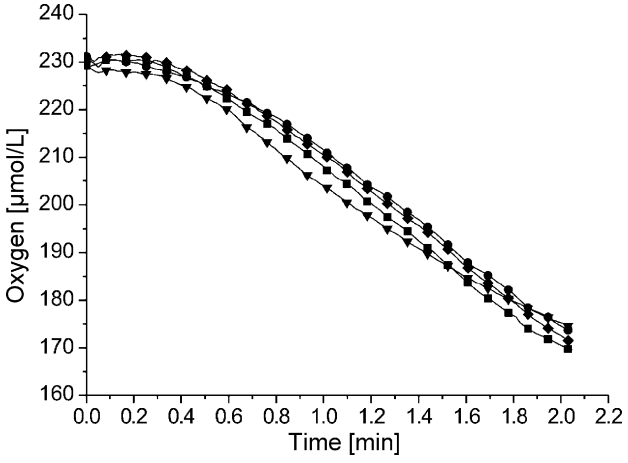
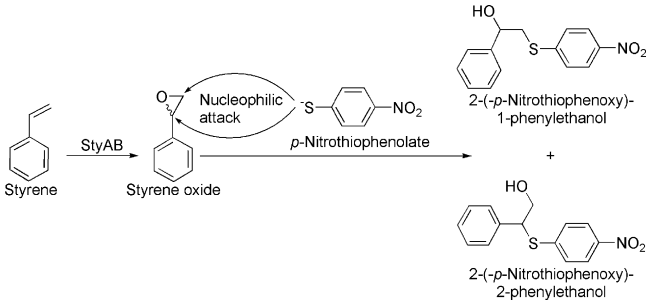


Fig. 2. Influence of mβ-CD on the epoxidation activity of StyAB monitored using oxygen consumption assay (2-mL reaction in 4.5-mL vial with oxygen probe Fibox 3); NADH is added at 0 min. (♦) No supplemented mβ-CD; (●) in presence of 0.57% mβ-CD; (■) in presence of 1.14% mβ-CD; (▼) in presence of 1.70% of mβ-CD.



Scheme 1. Reaction scheme of styrene conversion by StyAB and styrene oxide detection using pNTP.

3.4. Modified pNTP assay with improved sensitivity

The concentration of 0.57% mβ-CD was finally used in the multi-component enzyme/pNTP assay procedure. Substrate concentration of 2, 3 and 4 mM styrene were also investigated to identify an optimal styrene concentration for the multi-component enzyme/pNTP assay (Scheme 1) procedure. The K_m value of StyAB has been determined to be 0.38 ± 0.09 mM [10]. GC analysis shows that with 4 mM styrene substrate, 1.2 mM styrene (>3-fold higher than the K_m) remains after a 5 min reaction period. The calibration curves in Fig. 3 compares the discoloration values of pNTP assay at 443 nm (0.5 and 1 h incubation period) and at 405 nm (1 h incubation period) under gradient concentrations of styrene oxide [18]. These values show that sensitivity of 140–180 μM styrene oxide can be attained compared to the previously reported 400 μM [18]. In addition to an increased sensitivity, styrene evaporation during screening was reduced from 72 to 52% (based on GC analysis) by forming a well-known host/guest complex: mβ-CD/styrene [26]. Based on GC analysis (Optima® 5, 5 mM dodecane as internal standard; as described in Section 2.10) evaporation was further reduced to 39% (5 min incubation) when increasing mβ-CD concentration to 1.70% mβ-CD.

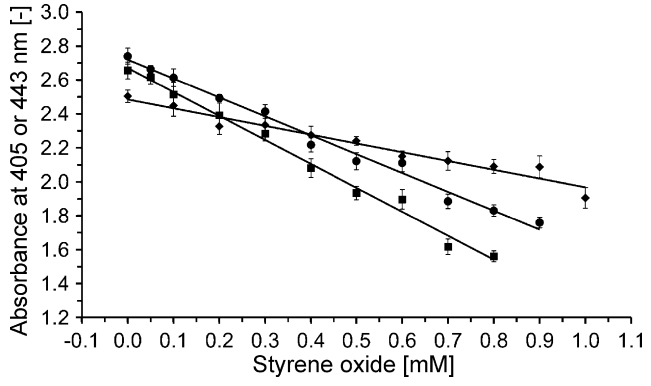


Fig. 3. Correlation between styrene oxide concentration and pNTP discoloration after reaction with styrene oxide under assay conditions. (♦) Absorbance measured at 405 nm as previously reported for P450 BM-3 (no mβ-CD and 1 h pNTP discoloration) [18]; (●) absorbance measured at 443 nm using current assay protocol for StyAB (0.57% mβ-CD and 0.5 h pNTP discoloration); (■) absorbance measured at 443 nm using current assay protocol for StyAB (0.57% mβ-CD and 1 h pNTP discoloration).

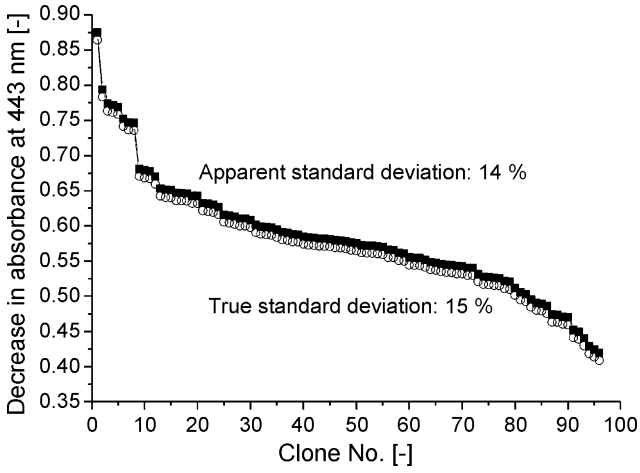


Fig. 4. Performance of pNTP screening system for the StyAB catalyzed styrene epoxidation in a 96-well plate. (■) Absorbance difference at 443 nm based on pNTP discoloration after StyAB wild-type epoxidation of styrene (5 min) and discoloration (30 min); (◯) absorbance difference of StyAB wild-type after subtracting the *E. coli* background.

Fig. 4 shows the performance of the multi-component enzyme/pNTP screen using a 5 min conversion period for styrene epoxidation followed by a 30 min pNTP discoloration period. Under these conditions a true standard deviation of 15% could be achieved for StyAB. Screening systems with standard deviations around 10–14% have been successfully used in directed evolution experiments of monooxygenases [18,38]. These performance criteria ensure that the mβ-CD enhanced pNTP screening system can be used for improving StyAB properties. The elaborated pNTP screening protocol has in a first directed evolution campaign been used to screen a mutant library of 2000 active StyAB clones in microtiter plate format and isolated variants are currently being studied in detail.

The mechanism for the faster pNTP/styrene reaction in the presence of mβ-CD (4.57 mM) remains speculative since styrene (<2 mM; binding constant of $31.606 \pm 3.350\ mM^{-1}$ for β-CD [39]), pNTP (0.5 mM; $0.075 \pm 0.010\ mM^{-1}$ for *p*-nitrophenolate and β-CD [40]) and styrene oxide (<1 mM; binding constant of $0.325 \pm 0.008\ mM^{-1}$ for dimethyl-β-CD [36]) all form complexes with mβ-CD. Polymerization of styrene [27,41] and copolymerization of dimethyl fumarate with styrene [35] are known to be accelerated when mβ-CDs form complexes with the hydrophobic substrates. Investigation of thermodynamic parameters by Li et al. suggests that cyclodextrin increases entropy and enthalpy of polymerization reactions and more importantly leads to a decrease in free energy of the complexed systems [42]. Whether styrene oxide and pNTP both react in mβ-CD complexed state or with at least one reactant partner being free in solution remains speculative. NMR spectroscopic studies indicate that styrene oxide is complexed with dimethyl β-CD through the phenyl-π-ring system within the hydrophobic cavity; the cyclic epoxide ring is located close to the rim of the β-CD [36]. Crystal structures of complexed dimethly β-CD and *p*-nitrophenol showed that *p*-nitrophenol is located at the intermolecular crevice and not found in the hydrophobic cavity of mβ-CD [43]. In summary, it is likely that the pNTP thiolate

group and an epoxide ring of styrene epoxide are sticking out of the hydrophobic mβ-CD cavity. A pNTP/styrene oxide reaction can therefore likely take place in complex states. The stable red-shift of mβ-CD complexed pNTP is a further indication for reaction of pNTP/styrene oxide in complexed formed.

3.5. Assay validation

Based on the calibration curve (Fig. 3), the amount of styrene oxide formed in the pNTP assay can be estimated. After 5 min incubation of styrene with StyAB, pNTP detection identified 0.51 ± 0.08 mM styrene oxide produced in the microtiter plate. To validate the accurate detection of styrene oxide by pNTP, the styrene oxide formed in the 96-well plate during screening was extracted and quantified using GC as previously described [18]. The GC analysis determined the formation of 0.6 mM styrene oxide which correlates with the estimated value of 0.51 ± 0.08 mM styrene oxide based on the calibration curve.

The pNTP assay represents one of the first applications of the well-known principle of host/guest complexes for hydrophobic compounds in directed evolution campaigns [44]. The host/guest screening principle might likely be of general interest for directed evolution since many cyclodextrin variants are known to form host/guest complexes with a large variety of hydrophobic compound classes. Examples include small aromatic molecules like styrene [27], large aromatic molecules like tri-terpenes and drugs like hydrocortisone [26], long hydrocarbon chains like oleic acid [45] and small alkyl compounds like methyl methacrylate [27]. Using host/guest complex in microtiter plate screens is significantly less laborious than employing two phase systems. Two phase systems require for instance organic solvent resistant microtiter plates, organic solvent resistance tips for liquid handling and controlled or vigorous mixing in microtiter plates for reducing diffusion barriers between phases. Further advantages of cyclodextrins comprise their high water solubility as well as their ability to improve activity and enantiomeric ratio, often due to reduced substrate or product inhibition [45,46].

4. Conclusion

In summary, the pNTP screening protocol was improved for the two-component StyAB system through: 1) reducing volatility of styrene by styrene/mβ-CD complex formation from 72 to 52%, and 2) increasing reaction rate between pNTP and styrene oxide due to styrene oxide/mβ-CD complex formation leading to enhanced sensitivity from 400 to 140 μM styrene oxide. A further implemented solid-phase indigo prescreen enables efficient selection of active StyAB variants for subsequent microtiter plate screens. The improved pNTP screening system was validated as an epoxygenase screening system for the two-component monooxygeanse (StyAB). The principle of using host/guest complexes (cyclodextrin/hydrophobic compound) for reducing substrate evaporation, improving catalytic activities, increasing solubility of hydrophobic substrates and enhancing sensitivity levels will likely be of general importance for designing screening systems in directed evolution experiments.

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