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Protocol for Mutagenesis of Alkene Monooxygenase and Screening for Modified Enantiocomposition of the Epoxypropane Product

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Alkene monooxygenase (AMO) from *Rhodococcus rhodochrous* B-276 is a 3-component enzyme system encoded by the 4-gene operon *amoABCD*, which catalyzes the stereoselective epoxidation of aliphatic alkenes yielding primarily the R enantiomer. With propene as the substrate, wild-type AMO yields R-epoxypropane with an enantiomeric excess (e.e.) of 83%. The presumed site of alkene oxidation is a dinuclear iron center situated within the large subunit of the epoxygenase component, AmoC. Substantial problems with the expression of recombinant AMO were previously overcome. In this study, the authors have further developed this expression system to allow *amoC* to be subjected to mutagenesis by means of error-prone PCR, with the aim of developing a system that could be used to manipulate the enantioselectivity of the enzyme. The mutants were screened for altered stereoselectivity in the propene/epoxypropane reaction by a whole-cell assay, solvent extraction, and chiral gas chromatography analysis protocol that is suitable for scale up to several thousand mutants and that is estimated to detect differences in e.e. of as little as 5%. (*Journal of Biomolecular Screening* 2006:553-556)

Key words: alkene monooxygenase, epoxygenase, directed evolution, propene, epoxypropene

INTRODUCTION

THE USE OF BIOLOGICAL SYSTEMS for the environmentally friendly production of enantiopure chemicals is a major opportunity for the application of biocatalytic technology.[1] The production of enantiopure epoxides is one such process for which biocatalysts offer an alternative to traditional chemical-based synthesis. Enantiopure epoxides are valuable because of the diverse range of reactions that the epoxide functional group can undergo and hence their versatility for introducing chiral centers into molecules.[2] Short-chain chiral epoxides lacking other functionalities, such as epoxypropane, are particularly valuable, but chemical routes to their synthesis are challenging. For that reason, biological systems that can produce them have and continue to arouse interest.

One system that is able to produce chiral epoxypropane is the alkene monooxygenase (AMO) of *Rhodococcus rhodochrous* B-276. This organism is able to use several aliphatic alkenes as its sole source of carbon and energy.[3] AMO is the first enzyme in the alkene metabolism pathway, and it stereoselectively inserts 1 oxygen atom from dioxygen across the alkene double bond, yielding the chiral epoxide, with the other oxygen being reduced to water by electrons provided from NADH.[4] Whole cells of *R. rhodochrous* B-276, or purified AMO, can be used for the stereoselective oxidation of terminal and subterminal alkenes to their corresponding epoxides, usually with the R enantiomer having high enantiomeric excess (e.e.). For example, propene is converted to R-epoxypropane with an e.e. of up to 83%.[3]

Sequence analysis and spectroscopic studies show that AMO belongs to a family of enzymes termed *bacterial multicomponent monooxygenases* (BMMs).[5,6] This family of carboxylate-bridged nonheme diiron proteins are capable of oxidizing a broad range of hydrocarbons including C_1-C_8 alkanes, alkenes, and aromatic compounds. There are 4 subclasses of BMMs: the soluble methane monooxygenases (sMMOs) produced by some methane-oxidizing bacteria, the phenol hydrolases, the 4-component alkene/arene monooxygenases or toluene monooxygenases (TMOs), and AMO.[6] Like the sMMOs, the rhodococcal AMO is a 3-component enzyme system.[4] The 3 components of the AMO enzyme system are the epoxygenase, reductase, and coupling protein, which are encoded by the 4-gene operon *amoABCD*.[5] Unlike BMMs of the other subclasses in which the terminal oxygenase components exist as $\alpha_2\beta_2\gamma_2$ hexamers, the

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epoxygenase of AMO has only 2 types of subunit, of 38 and 57 kDa, encoded by *amoA* and *amoC*, respectively.[4,5] The AMO oxygenase has the same number of subunits as the propene monooxygenase (PMO) of *Mycobacterium* sp. strain M156,[7] which we propose should become the 2nd member of the AMO subclass of BMMs. X-ray structures for the terminal oxygenases of 2 sMMOs and 1 TMO have been solved, indicating a common global fold for the diiron-containing subunits.[8,9] As there is 56% similarity in primary structure between the large subunit of the AMO epoxygenase (AmoC) and the α-subunit of sMMO from *Mc. capsulatus* (Bath), it is reasonable to assume that AmoC shares the same fold. A homology model of the 3-dimensional structure of AmoC has been produced based on the structure of the terminal oxygenase component of sMMO.[10]

Conversion of alkenes to epoxides is common in BMMs. Interestingly, sMMO is able to oxidize alkenes to epoxides but with much lower e.e.s than observed with AMO (S. E. Slade, T. J. Smith, and H. Dalton, unpublished data). Several previous mutagenesis studies have sought to address the origin of stereoselectivity in BMMs. In a study of the mycobacterial PMO, several active-site mutants of PMO were designed to probe the control of stereoselectivity in this enzyme. A number of mutants with modified stereoselectivity were generated, but in only 1 case was there a slight enhancement (from 55% in the wild type to 62%) of e.e. during styrene epoxidation.[7] In another study, mutagenesis at position 103 in toluene 4-monooxygenase, a proposed gating residue controlling access to the active site, increased yield but not stereoselectivity during epoxygenation of butadiene.[9] The substrate specificity of toluene 4-monooxygenase has been manipulated by directed evolution[11] and that of sMMO by site-directed mutagenesis.[12] Site-directed and saturation mutagenesis using TMOs has generated enzymes with novel activities toward aromatic substrates.[13]

We were interested to draw on the success of directed evolution studies with other systems and especially the success achieved in modifying the substrate specificity of TMOs using such methods, to alter the stereoselectivity of AMO. We previously described a system for expression of recombinant rhodococcal AMO in *Streptomyces lividans* that overcame problems with low activity when the epoxygenase component was expressed in *Escherichia coli*.[14] Here we describe improvements in this expression system to allow generation and expression of large libraries of quasi-random mutants. This, combined with methodology for gas chromatography (GC)–based screening of mutants, which we also describe, allows directed evolution of AMO for modified enantioselectivity toward R-epoxypropane, which may be the smallest chiral metabolite in nature. The methodology outlined in this article also represents the 1st attempt at using GC analysis as a screen in a directed evolution analysis and paves the way for analysis reactions in which GC is the only viable screen.

MATERIALS AND METHODS

Preparation of mutant library by error-prone PCR

The complementary mutagenic primers AVRII01 (5′-GGC TCC GGC CTG CCT AGG CGC GGC CGC CGA CG-3′; *Avr*II restriction site underlined) and AVRII02 (5′-CG TCG GCG GCC GCG CCT AGG CAG GCC GGA GCC-3′; *Avr*II restriction site underlined) direct the insertion of a unique *Avr*II restriction site into the *amoC* gene via silent base pair changes. These primers were used to mutate pTJS104 (pET3a with a 4.0-kb insert containing *amoABCD* operon[14]) using the QuikChange method (Stratagene, La Jolla, CA). Subsequent restriction of this insert and ligation into the *S. lividans* expression vector pIJ6021 gave the plasmid pASH107. This plasmid has unique restriction sites for *Mlu*I and *Avr*II, flanking a 724-bp fragment that encodes amino acid positions 67 to 309 in AmoC. The modeled 3-dimensional structure of AmoC indicates that this fragment contains all the sites of interest within the active site of the enzyme.[10] These include the iron-binding residues, proposed hydrophobic binding site, and important equivalent residues identified in protein engineering of other BMMs.

Quasi-random mutagenesis via error-prone PCR (epPCR) was carried out using the primers AMOCEP03 (5′-CCT CGA CGG CGC CGT GCG GAC GCG TG-3′) and AMOCEP05 (5′-GCG GAT CCG TCC GGC CTC GGA CTT GCG GCT TGA GCG ACT CAG CCA GC-3′) using Mutazyme DNA polymerase (Strategene). Restriction of the epPCR product with *Mlu*I and *Avr*II allowed direct ligation of the PCR product into the larger *Mlu*I and *Avr*II fragment of pASH107. Following ligation, the reaction mixture was used to transform protoplasts using the polyethylene glycol–assisted method, and transformants were selected using kanamycin.[15] After transformation into *S. lividans*, colonies were picked into 10-ml aliquots of yeast extract–malt extract (YEME) medium containing kanamycin (5 μg/ml) and grown at 30 °C, 180 rpm, for 14 days.[15] The *tipA* promoter was found to be sufficiently leaky that thiostrepton induction was not needed for AMO expression.

Epoxidation stereoselectivity assay

Samples of culture (0.5 ml) were removed into 2-ml GC vials with screw caps containing rubber/polytetrafluoroethylene septa (Chromacol, Welwyn Garden City, UK). Each assay was initiated by the injection of 1 ml of propene directly into the vial, and the vial was shaken (180 rpm) at 30 °C and 180 rpm overnight. The epoxypropane product was extracted via solvent extraction using 100 μl of diethyl ether injected into the vial. Following gentle inversion of the vial, the ether layer was removed for analysis. The assay was preformed in duplicate for each mutant.

Analysis was carried out using a GC 2010 gas chromatograph (Duisburg, Germany) fitted with an Alpha cyclodextrin

trifluoroacetyl capillary column of 20 m × 0.25 mm (Advanced Separation Technologies, Whippany, NJ) for chiral separation and flame ionization detection. The column temperature was 40 °C, and the split ratio was 1:50. The flow rate of the carrier gas (nitrogen) was 29.3 ml/min, and the injector and detector temperatures were 150 °C and 250 °C, respectively. Under these conditions, R- and S-epoxypropane eluted at 4.07 and 4.32 min, respectively. Peaks were assigned using 99% R- and S-epoxypropane standards (Sigma). Chromatogram analysis was preformed using GC solution software from Shimadzu (Kyoto, Japan), and peak areas were used to calculate e.e., where e.e. = $100 \times F(R) - F(S)$.

DNA sequencing

Cloned DNA was sequenced by reamplifying the mutagenised *amoC* gene fragment by means of PCR using primers AMOCEP03 and AMOCEP05. Dye termination sequencing was carried out by DBS Genomics at the University of Durham, United Kingdom.

RESULTS AND DISCUSSION

Construction of mutant library

Mutagenesis and cloning of the mutants in *S. lividans*, as described in the "Materials and Methods" section, yielded more than 100 colonies using 10 ng of library DNA, which was sufficient to allow production of several thousand mutants from a few tens of transformations. The conditions for epPCR were adjusted according to the Mutazyme kit manufacturer's instructions to produce a mutation frequency of 1 to 2 base pair changes per 1 kb of cloned product, which was verified by the sequencing of 10 clones.

Stereoselectivity assay

Chiral GC analysis of ether extracts from assay vials allowed accurate determination of e.e. values for the epoxidation reaction. Baseline resolution was observed for R- and S-epoxypropane with elution times of 4.07 and 4.32 min, respectively, as shown in **Figure 1**. Other peaks observed earlier in the chromatogram represent ether, propene, low-level contaminants in the ether, and coextractants from the assay. Ether extraction of assays preformed in the absence of propene indicated that the peaks at 4.07 and 4.32 min were correctly assigned to be enantiomers of epoxypropane. Achieving chiral resolution in such a relatively short time permits analysis of up to 10 samples per hour. This rate of analysis makes the screening of the several thousand mutants typically needed in the 1st round of a directed evolution approach feasible in a reasonable time scale. The detection limit for a single enantiomer is ~0.01 mM in the ether extract.

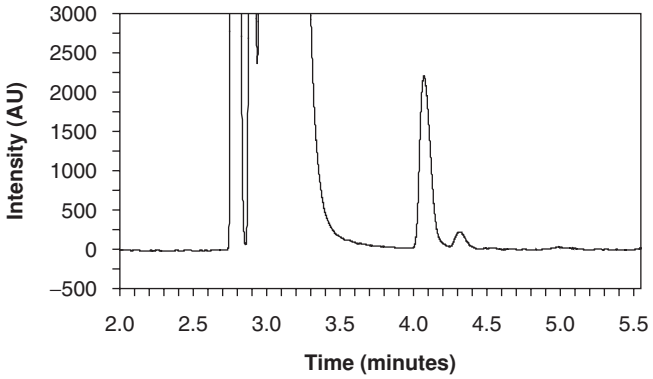

FIG. 1. Representative gas chromatography of products of propene oxidation during the screening procedure.

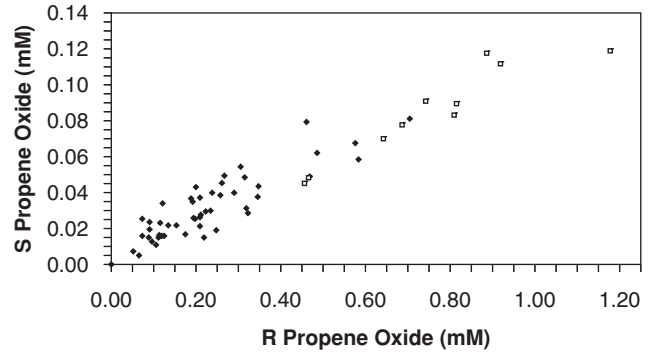

FIG. 2. Enantiocomposition of epoxypropane analyzed during screening. Solid symbols represent mutants; open symbols represent 10 control wild-type cultures.

The e.e. of R-epoxypropane formation observed for recombinant AMO in whole-cell cultures was found to be 79% ± 1% (assay preformed in triplicate), similar to the soluble extract of *S. lividans* expressing AMO, which gave an e.e. of 76% ± 4%.[14] A representative example of the screening results for 50 mutants is shown in **Figure 2**, from which it is evident that there is considerable variation in total epoxygenase activity observed across the cultures. To some extent, this variation in activity is due to the fact that *S. lividans* in liquid culture grows as compacted pellets of mycelium that are dispersed during incubation by using a baffle.[15] The extent of dispersion from culture to culture varies, affecting growth and ultimately the level of epoxygenase activity. Considerably greater variation was observed in AMO activity than the e.e. of the product. For 10 cultures expressing wild-type AMO, the e.e. was 79% ± 2%, whereas the concentration of product after ether extraction was 0.85 ± 0.23 mM. Thus, it is clear that the screening methodology outlined here is robust enough to enable the identification of mutants with altered stereoselectivity, despite the variations in total activity. Analysis of the data from **Figure 2** shows that the mutants have

on the whole lower e.e. values and epoxygenase activity compared to cultures expressing wild-type AMO. The mean e.e. and total epoxypropane synthesis for the mutants was 73% and 0.30 mM, compared to 79% and 0.85 mM for cultures expressing wild-type AMO. Statistical analysis (*t*-test) of these data demonstrated that this difference is significant ($p < 0.01$). Thus, at least some of the mutants produced have modified properties, most likely the result of detrimental random amino acid changes distant from the active site.

CONCLUSIONS

The results presented here demonstrate that the mutagenesis, expression, and screening system that we have developed permits sensitive screening of potentially thousands of AMO mutants for altered enantioselectivity in the oxidation of alkenes to epoxides. In the future, we propose to use this screen to identify mutants with altered e.e. via an iterative directed evolution process of screening, selection, and further rounds of mutagenesis. This is the focus of our ongoing research, which will show the extent to which e.e. in this reaction can be modified by directed evolution. It is also expected that analysis of mutants with altered e.e. will throw light on the mechanism of stereoselectivity in AMO and BMMs in general. Our results presented here may also pave the way for the use of directed evolution to manipulate the activity of other enzyme-catalyzed reactions in which GC is the only viable screen.

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