Characterization of the Epoxide Hydrolase NcsF2 from the Neocarzinostatin Biosynthetic Gene Cluster

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Supporting Information

General experimental procedures

Substrates including racemic styrene oxide, (*R*)-styrene oxide, and (*S*)-styrene oxide, and products including racemic 1-phenyl-1, 2-ethanediol, (*R*)-1-phenyl-1, 2-ethanediol, (*S*)-1-phenyl-1, 2-ethanediol, and [^{18}O]-H_2O were purchased from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT) was purchased from Research Products International Corp (Mt. Prospect, IL). Complete protease inhibitor tablet, EDTA-free, was from Roche Applied Science (Indianapolis, IN). Medium components and buffers were from Fisher Scientific (Pittsburgh, PA). Synthetic DNA oligonucleotides were purchased from the University of Wisconsin-Madison Biotechnology Center. PCR was performed with a PerkinElmer GeneAmp 2400. Electrospray ionization-mass spectroscopy (ESI-MS) was performed with an Agilent 1100 MSD SL ion trap mass spectrometer (Agilent Technologies, Inc. Santa Clara, CA). NMR spectra were recorded using a Varian UI-500 spectrometer (Varian, Inc., Palo Alto, CA). High performance liquid chromatography (HPLC) analyses were carried out on a Varian HPLC system equipped with Prostar 210 pumps, a photodiode array (PDA) detector, and an Alltech Appolo C18 reverse phase column (5 μm, 4.6 × 250 mm, Grace Davison Discovery Sciences, Deerfield, IL), using a 20 min linear gradient from 0 to 60% acetonitrile in water. The enantiomeric separation was performed on a Waters HPLC system equipped with 600 pumps, a 996 photodiode array (PDA) detector, and a Chiralcel OD-H chiral column (5 μm, 4.6 × 250 mm, Grace Davison Discovery Sciences) using a 60 min isocratic elution with 2.5% isopropanol in *n*-hexane.

Cloning, overproduction, and purification of NcsF1 and NcsF2

The gene encoding NcsF1 and NcsF2 were amplified by PCR from cosmid pBS5004[1] as template. PCRs were performed with Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) using the following primers: forward 5′- GAC GAC GAC AAG ATG CGG CCG TTT CAG GTC C -3′ and reverse 5′- GAG GAG AAG CCC GG TCA GAG CTT CTT GAG TGT CC -3′ for NcsF1; forward 5'- GAC GAC GAC AAG ATG GAG CCG TTC CGT ATA G -3' and reverse 5'- GAG GAG AAG CCC GGT CTA GGA CGT CCG CAG CG -3' (starting and stop codons underlined) for NcsF2. The gel-purified PCR products were directly cloned into the pCDF-2 Ek/LIC vector using ligation-independent cloning as described by Novagen (Madison, WI) to give pBS5041 (for NcsF1) and pBS5042 (for NcsF2) for *E. coli* expression. The over-expression in *E. coli* BL21 (DE3) and purification of the recombinant protein by affinity chromatography using NTA-Ni agarose column (Qiagen, Valencia, CA) were performed following the previously described procedure for SgcF purification.[2] After the purified protein was desalted using a PD-10 column (GE Healthcare, Piscataway, NJ) twice to completely remove glycerol and then concentrated with a 10 K MWCO Vivaspin ultrafiltration device (Sartorius, Edgewood, NY), the NcsF2 protein was stored at -80 ^{o}C in 100 μL aliquots. The purity of the purified protein was assessed by 12% SDS-PAGE and the concentration was determined from the absorbance at 280 nm using a molar absorptivity (ε= 68.41 mM^{-1} cm^{-1} for NcsF1, 82.34 for NcsF2) calculated using the program Protparam (http://www.expasy.ch/tools/protparam.html).

EH activity assays with styrene oxide as a substrate mimic

HPLC-based assays were carried out in 200 μL reaction mixtures containing 2 mM racemic styrene oxide and 50 mM phosphate buffer (pH 7.5). The reaction was initiated by the addition of 50 μM enzyme (NcsF1 or NcsF2) and incubated at 25 ºC for 1 h. The reaction was quenched by extraction with 200 μL ethyl acetate for three times and organic extracts were combined. After solvent was removed using a speed-vac, the resulting residue was dissolved in 50 μL acetonitrile and 25 μL was subjected to HPLC analysis. Control reactions were carried out under the identical conditions except with enzyme that had been boiled for 5 min.

To determine the kinetic parameters of NcsF2-catalyzed hydrolysis of styrene oxide, a spectrophotometric assay method was adopted to achieve continuous and accurate determination of epoxide hydrolase activity.[3,4] Prior to kinetic analysis, the optimal pH for NcsF2 activity was tested in three different buffers: 50 mM sodium acetate (pH 5.0, 5.5, and 6.0); 50 mM sodium phosphate buffer (pH 5.5 to pH 8.5); and 50 mM glycine-NaOH (pH 8.5, 9.0, and 10.0). The reactions were carried out in 1 mL mixture containing 10 μL of 300 mM sodium periodate in DMF, 20 μL of 5 M styrene oxide in DMSO, and 50 mM buffer. The reactions were initiated by the addition of NcsF2 and carried out in triplicate. The absorbence at 290 nm was monitored in a 1-mL quartz cell and the velocity was calculated based on the rate of change of absorbance over several minutes. Steady-state kinetic parameters were obtained from reactions carried out in 50 mM phosphate buffer (pH 8.0) with varying styrene oxide concentrations [0.1-6.4 mM for (*R*)-styrene oxide with 2 μM NcsF2 and 1-16 mM for (*S*)-styrene oxide with 8 μM NcsF2]. The Michaelis-Menten equation was fitted to plots of velocity of 1-phenyl-1, 2-ethanediol formation versus substrate concentration to extract values for K_M and k_{cat}.

Large scale reaction of NcsF2

To prepare sufficient quantities of product for NMR characterization, a reaction was carried out in a 1-mL reaction mixture containing 10 mM styrene oxide and 200 μM NcsF2 in 50 mM phosphate buffer (pH 8.0). During the course of the reaction, 10 mM styrene oxide and 50 μM NcsF2 were added every hour. The reaction was allowed to proceed at 25 ºC for 8 h, after which the products were extracted from the reaction mixture with 5 x 0.5 mL of ethyl acetate. The solvent was removed by evaporation under reduced pressure. ^{18}O-labeled products were obtained following a similar procedure. The reactions were carried out in 0.5-mL reaction mixtures containing [^{18}O]-H_2O. For (*R*)-styrene oxide, 5 mM substrate and 6 μM NcsF2 were added every 30 min (~80% [^{18}O]-H_2O final concentration). For (*S*)-styrene oxide, 20 mM substrate and 15 μM NcsF2 were added every hour (~60% [^{18}O]-H_2O final concentration).

Enantioconvergent reaction using NcsF2/SgcF co-catalysis

An enantioconvergent enzymatic preparation of (*R*)-1-phenyl-1,2-ethanediol was performed in a 10 mL reaction containing 14 mM racemic styrene oxide (16.8 mg), 35 μM SgcF, and 10 μM NcsF2 in 100 mM phosphate buffer (pH 8.0). After the reaction was incubated at room temperature for 2 h, another batch of fresh enzyme (22.5 μM SgcF and 6.4 μM NcsF2) was added to the reaction mixture and incubated at room temperature for an additional 3 h. The reaction mixture was extracted 5 times with 10 mL ethyl acetate after the styrene oxide was consumed as judged by TLC. The organic extracts were combined and dried with anhydrous Na_2SO_4. The removal of solvent gave 16.8 mg of (*R*)-1-phenyl-1,2-ethanediol (87% yield and 99% ee) as determined by chiral HPLC analysis.

Figure S1. SDS-PAGE analysis on a 12% acrylamide gel of purified NcsF1 (lane 1) and NcsF2 (lane 3) both with expected masses of ~44.5 kDa, and molecular weight markers (lane 2).

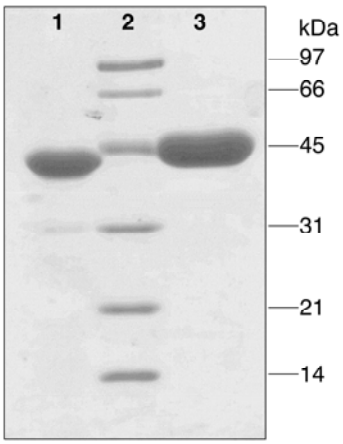
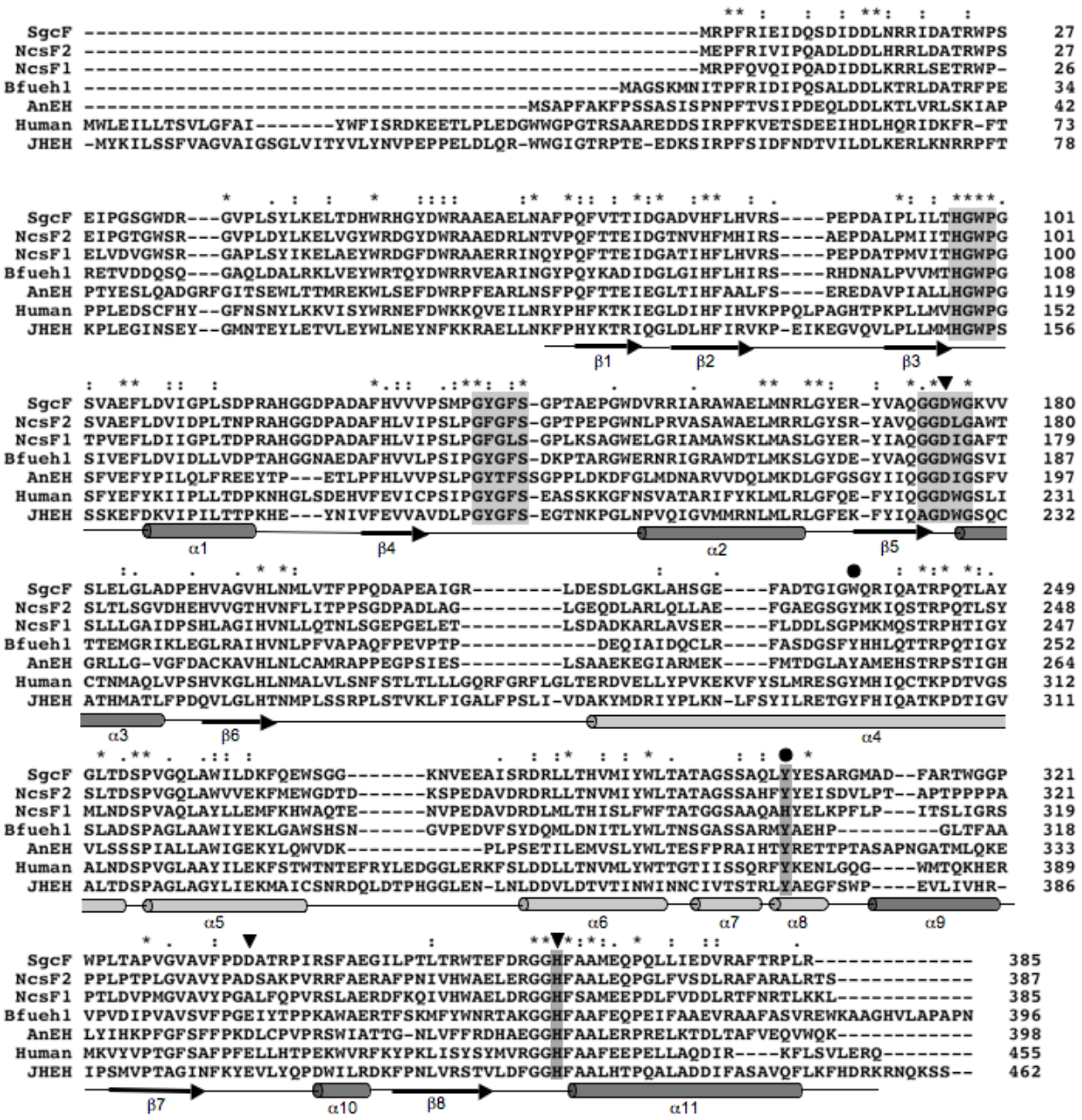


Fig. S2. Sequence alignment of NcsF1, NcsF2, and SgcF with selected epoxide hydrolase (EHs). The amino acid sequences were retrieved from NCBI with the following accession numbers: SgcF from *Streptomyces globisporus* (AAL06662); NcsF2 from *S. neocarzinostaticus* (AAM78002); NcsF1 from *S. neocarzinostaticus* (AAM77995); Bfueh1 from *Burkholderia fungorum* LB400 (ZP_00284192); AnEH from *Aspergillus niger* (CAB59813); human microsomal EH (P07099); juvenile hormone EH (Q25489). Shaded regions denote sequences conserved among EHs. Active site catalytic residues are marked as follows (NcsF2 numbering): ▼, catalytic triad residues D^{175}, D^{336}, H^{363}; ●, oxirane-binding residues Y^{235} and Y^{304}.



References

(1) Liu, W.; Nonaka, K.; Nie, L. P.; Zhang, J.; Christenson, S. D.; Bae, J.; Van Lanen, S. G.; Zazopoulos, E.; Farnet, C. M.; Yang, C. F.; Shen, B. *Chem. Biol.* **2005**, *12*, 293-302.
(2) Lin, S.; Horsman, G. P.; Chen, Y.; Li, W.; Shen, B. *J. Am. Chem. Soc.* **2009**, *131*, 16410-16417.
(3) Doderer, K.; Lutz-Wahl, S.; Hauer, B.; Schmid, R. D. *Anal. Biochem.* **2003**, *321*, 131-134.
(4) Mateo, C.; Archelas, A.; Furstoss, R. *Anal. Biochem.* **2003**, *314*, 135-141.