Thermostabilization of a Cytochrome P450 Peroxygenase

Oriana Salazar, Patrick C. Cirino, and Frances H. Arnold*[a]

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Cytochrome P450 BM-3 is a soluble fatty acid hydroxylase composed of a heme domain and a reductase domain on a single polypeptide chain.[1] We recently described a laboratory-evolved variant of the P450 BM-3 heme domain which functions as an H_2O_2-driven hydroxylase ("peroxygenase") and does not require NADPH, O_2, or the reductase.[2] This variant, which we named 21B3, allows us to carry out cytochrome P450-catalyzed biotransformations under highly simplified reaction conditions: only the heme domain and hydrogen peroxide are needed for substrate (fatty acid) hydroxylation. Because its heme domain alone is competent for catalysis, P450 BM-3 peroxygenase 21B3 offers a unique opportunity to create a thermostable, functional cytochrome P450. Here we report further directed evolution of the 21B3 peroxygenase, resulting in an enzyme which is significantly more thermostable than wildtype cytochrome P450 BM-3 and retains much of the peroxygenase activity of 21B3.

Enzymes are often poorly stable under conditions encountered during production, storage, or use. Improving enzyme resistance to thermal denaturation has been a major focus of protein engineering efforts.[3–5] Improved thermostability often correlates with longer shelf-life, longer life-time during use (even at low temperatures), and a higher temperature optimum for activity.[6, 7] There have been no reports of stabilizing the relatively unstable cytochrome P450 enzymes by protein engineering, however, primarily because the P450s comprise multiple subunits and contain thermolabile cofactors.

P450 BM-3 heme domain containing the single amino acid substitution F87A (mutant HF87A) is significantly more active than wildtype heme domain (HWT) in reactions driven by H_2O_2.[8, 9] Variant 21B3 is much more active than HF87A in 10 mM H_2O_2, but is also less thermostable than HWT and HF87A. We therefore sought to improve the thermostability of 21B3 while maintaining its improved peroxygenase activity. Six cycles of random mutagenesis or DNA shuffling and screening for retention of peroxygenase activity after heat treatment (see the Methods section) yielded the thermostable peroxygenase variant 5H6.

[a] *Prof. F. H. Arnold, Dr. O. Salazar, P. C. Cirino*
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, CA 91125 (USA)
Fax: (+1) 626-568-8743
E-mail: frances@cheme.caltech.edu

To characterize the thermostability of the peroxygenase variants identified during screening, we measured the fraction of folded heme domain remaining after heat-treatment, which we determined from the fraction of the ferrous heme-CO complex that retained the 450 nm absorbance peak characteristic of properly-folded P450. Figure 1 shows the percentage of properly-folded heme domain protein remaining after 10-minute incubations at different, elevated temperatures. To allow comparison to the wildtype full-length enzyme (BWT), whose stability is limited by the stability of the reductase domain and therefore cannot be determined from the CO-binding measurement, we determined the residual (NADPH-driven) activity of BWT following 10-minute incubations at the same temperatures. Data in Figure 1 were fit to a two-state model, and the resulting calculated temperatures corresponding to half denaturation for the 10-minute heat incubations (T_{50}) are listed in Table 1. These values are in good agreement with the midpoints of the melting curves of Figure 1. According to this measure of stability, variant 5H6 ($T_{50} = 61\,^{\circ}C$) is much more thermostable than the natural catalytic system, BWT ($T_{50} = 43\,^{\circ}C$). It is also significantly more thermostable than HF87A and 21B3.

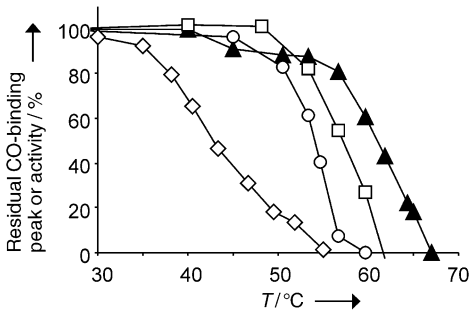

Figure 1. Percentage of 450 nm CO-binding peak of cytochrome P450 BM-3 heme domain HWT (□), HF87A (○), and 5H6 (▲) remaining after 10-minute incubations at the indicated temperatures. For the holoenzyme BWT (◇), the percentage of initial NADPH-driven activity remaining after 10-minute incubations is shown.

Table 1. *Thermostability and activity parameters for evolved and parental P450s.*

Mutant[a]	T_{50} for 10-minute incubations[b] [°C]	$t_{1/2}$ at 57.5 °C [c] [min]	Peroxygenase Activity[d] [min^{-1}]
BWT	43	0.46	< 5
HWT	57	n.d.	< 5
HF87A	54	2.3	23
21B3	46	n.d.	430
5H6	61	115	220

[a] BWT = full-length, wildtype P450 BM-3; HWT = wildtype P450 BM-3 heme domain; HF87A = P450 BM-3 heme domain containing mutation F87A; 21B3, 5H6 = evolved heme domain peroxygenase variants. [b] Calculated from the data in Figure 1, fit to a two-state denaturation equation. [c] Calculated from the data in Figure 2, fit to a first-order exponential decay equation. [d] Reported as initial rates at room temperature on 12-pNCA in 10 mM H_2O_2 and 6% DMSO. n.d.: not determined.

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Peroxygenase activities were measured at room temperature, using a colorimetric assay with 12-*p*-nitrophenoxycarboxylic acid (12-pNCA) as substrate (see ref. [10] and Methods Section). 5H6 retains ~50% of the high activity of 21B3 and is almost ten times as active as HF87A (Table 1). Another useful measure of enzyme stability comes from the rate of inactivation at high temperature. Figure 2 shows the percentage of activity that remains for the

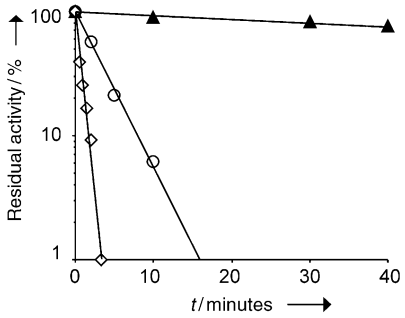

Figure 2. Heat-inactivation of cytochrome P450 BM-3 holoenzyme BWT (◇) and peroxygenase mutants HF87A (○) and 5H6 (▲), calculated as the percentage of activity remaining after incubation at 57.5 °C for the indicated periods of time. Peroxygenase activity was measured for HF87A and 5H6, while NADPH-driven activity was measured for BWT.

different variants upon heating at 57.5 °C. The activities decay exponentially with time (first-order), and the half-life ($t_{1/2}$) of each catalytic system is listed in Table 1. HF87A (which is less thermostable than HWT) is significantly more resistant to inactivation at 57.5 °C compared to BWT. (The half-life of HF87A is also higher than that of BWT at room temperature (data not shown)). The half-life of 5H6 at 57.5 °C is 50 times longer than that of HF87A and 250 times that of BWT. The fraction of peroxygenase activity remaining after heat treatment correlated with the fraction of remaining CO-binding peak for HF87A and 5H6. (Residual activity of HWT could not be correlated to the remaining CO-binding peak because HWT has essentially no peroxygenase activity.)

Enzyme thermostabilization often leads to a shift in the activity-temperature profile to higher temperatures, reflecting the higher stability of the folded protein.[6] Measurements of peroxygenase activity at different temperatures, however, showed no significant increase in the optimum temperature for activity for 5H6 compared to HF87A (both were 25 – 30 °C).

Thermostable peroxygenase 5H6 contains eight new amino acid substitutions compared to 21B3 (see ref. [2]), and 15 substitutions compared to HF87A. 5H6 also contains a deletion resulting in the removal of one His residue from the 6-His sequence included at the C terminus. The M145V substitution that had been found previously in mutant 21B3 reverted to Met upon back-crossing with HF87A (see Methods Section). Five of the substitutions are conservative with regard to hydrophobicity and size: L52I, A184V, L324I, V340M and I366V. Ser106 was converted to a positively charged Arg residue (S106R) and a negatively-charged Glu residue was converted to a positively-charged Lys (E442K). It is difficult to rationalize how these mutations increase the enzyme's stability. According to the P450 BM-3 heme domain crystal structure,[11] substitutions S106R, L324I, V340M, I366V, and E442K are located on the protein surface; the others are buried. Four stabilizing mutations are close to positions where mutations that improved peroxygenase activity accumulated in earlier experiments: L52I (in β-sheet 1 – 2) is adjacent to I58V (helix B) from 21B3, S106R (in a loop connecting helices C and D) lies next to mutation F107L from 21B3, E442K (in β-sheet 4 – 2) lies adjacent to K434E (in β-sheet 4 – 1) from 21B3, and the reversion to M145 (helix E) is adjacent to S274T (helix I) from 21B3. The new stabilizing mutations may serve to alleviate structural perturbations introduced by the original (activity) mutations.

Two thermostable cytochrome P450s, CYP119 and CYP175A1, from thermophilic organisms have recently been described[12, 13] and their (heme domain) crystal structures determined.[14–16] CYP119 exhibits a melting temperature of ~91 °C. Aromatic stacking, salt-link networks and shortened loops are believed to help stabilize these enzymes. Unfortunately, the functions of these P450s are not known, and reported activities are extremely low (for example, 0.35 min^{-1} in the NADH-driven hydroxylation of lauric acid).[17]

Directed evolution allows us to explore non-natural functions and properties of P450s. In this first example of engineering a P450 to resist thermal denaturation, we have used directed evolution to improve the thermostability of BM-3 peroxygenase variant 21B3. These results reinforce the biotechnological relevance of cytochrome P450 BM-3 and the catalytic potential of our biomimetic hydroxylase.

Methods

General Remarks: All chemical reagents were procured from Aldrich, Sigma, or Fluka. Restriction enzymes were purchased from New England Biolabs and Roche. Deep-well plates (96 wells, 1 mL volume per well) for growing mutant libraries were purchased from Becton Dickinson. Flat-bottom 96-well microplates (300 μL per well) for screening mutant library activities were purchased from Rainin.

Enzyme Expression and Purification: P450 BM-3 enzymes were expressed in catalase-deficient *E. coli*[18] using the β-D-thiogalactopyranoside (IPTG)-inducible pCWori+ vector.[19] The heme domain consisted of the first 463 amino acids of P450 BM-3 followed by a 6-His sequence at the C terminus, which had no significant influence on the activity. Cultures for protein production were grown and proteins were purified as described.[9] Purified enzyme samples were stored at −80 °C until use, at which time they were thawed at room temperature and then kept on ice. Concentrations of properly-folded P450 enzyme were determined from the 450 nm CO-binding difference spectra of the reduced heme, as described.[20]

Preparation of Mutant Libraries: Error-prone PCR libraries were prepared using standard protocols.[21] Starting with 21B3 as the parent, three rounds of error-prone PCR (using *Taq* DNA polymerase (Roche)) followed by screening were performed, and the most thermostable mutant which did not lose peroxygenase activity was chosen as the parent for the next generation. Two additional generations were prepared with the GeneMorph PCR Mutagenesis Kit (Stratagene). In the final generation leading to mutant 5H6, a recombinant library was prepared by DNA shuffling[22] using *Pfu* Ultra

DNA Polymerase (Stratagene). Parents for the recombinant library included HF87A, mutants from the previous generation which were more stable but less active, and mutants with increased activity.

Mutant Library Screening: Screening was performed essentially as described,[2] except cell lysates were additionally subjected to a heat inactivation step and screened for residual activity (see also ref. [23]). Briefly, cultures expressing mutants were grown in 96-well deep-well plates. After cell growth, the plates were centrifuged, cell pellets were frozen at $-20\,^{\circ}C$, and the cells were lysed in Tris-HCl buffer (100 mM, pH 8.2) containing lysozyme (0.5 – 1 mg mL^{-1}) and deoxyribonuclease I (1.5 – 4 units mL^{-1}). Clarified cell lysates were transferred to 96-well microplates for activity measurements at room temperature (described below). Lysates were also transferred to 96-well PCR plates (GeneMate) and heated to an appropriate temperature (48 – 57.5 °C) in a PTC200 thermocycler (MJ Research) for 10 – 15 minutes, rapidly cooled to 4 °C, and then brought to room temperature. The residual activities of these heat-treated lysates were then measured in the same manner as the initial activities. Clones showing a higher fraction of activity remaining after heat treatment and high initial activity were characterized further.

Activity Assay: Activity on 12-pNCA[10] was determined by monitoring the formation of *p*-nitrophenolate (pNP) (398 nm) at room temperature using a 96-well plate spectrophotometer (SPECTRAmax Plus, Molecular Devices), as described.[2] Reaction wells contained Tris-HCl buffer (140 µL of 100 mM, pH 8.2), a stock solution of substrate (10 µL of 4 mM 12-pNCA) in DMSO, and purified enzyme or clarified lysate. Reactions were initiated by the addition of an H_2O_2 stock solution (10 µL). Data for accurate determination of 12-pNCA turnover rates with purified enzyme were collected using a BioSpec-1601 spectrophotometer (Shimadzu), where absorbance changes could be registered every 0.1 seconds. Typical final concentrations were 250 µM 12-pNCA, 6% DMSO, 1 – 10 mM H_2O_2, and 0.1 – 1.0 µM P450. The extinction coefficient for pNP was determined from standard pNP solutions prepared under identical reaction conditions. NADPH-driven activity of BWT was determined spectrophotometrically from the initial rate of NADPH consumption (measured as the decrease in 340 nm absorbance) in the presence of myristic acid, as described.[24]

Data for T_{50} Determination: Purified enzyme samples (~20 µM) in Tris-HCl buffer (100 mM, pH 8.2) were incubated for 10 minutes at different temperatures. Samples were then cooled on ice, and the concentration of properly-folded heme domain (diluted 8x) was estimated from the 450 nm CO-binding difference spectra and compared to the CO-binding peak prior to heat treatment. Residual NADPH-consumption activity was measured for BWT. Data in Figure 1 represent average values from at least two experiments.

Data for $t_{1/2}$ Determination: Concentrated purified enzyme (70 µM) was added to pre-heated (57.5 °C) Tris-HCl buffer (100 mM, pH 8.2) and incubated at 57.5 °C. Samples were removed at time intervals (indicated in Figure 2), quenched by dilution into cold buffer, brought to room temperature, and assayed for residual activity. Data in Figure 2 represent average values from at least two experiments, with standard deviations less than 10%.

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[1] A. W. Munro, D. G. Leys, K. J. McLean, K. R. Marshall, T. W. Ost, S. Daff, C. S. Miles, S. K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, P. L. Dutton, *Trends Biochem. Sci.* **2002**, *27*, 250.
[2] P. C. Cirino, F. H. Arnold, *Angew. Chem.*, **2003**, *115*, 3421; *Angew. Chem. Int. Edit.*, **2003**, *42*, 3299.
[3] M. Lehmann, M. Wyss, *Curr. Opin. Biotechnol.* **2001**, *12*, 371.
[4] M. Lehmann, L. Pasamontes, S. F. Lassen, M. Wyss, *Biochim. Biophys. Acta* **2000**, *1543*, 408.
[5] P. L. Wintrode, F. H. Arnold, in *Evolutionary Protein Design, Vol. 55* (Ed.: F. H. Arnold), Academic Press, San Diego, **2001**, pp. 161.
[6] R. M. Daniel, M. J. Danson, R. Eisenthal, *Trends Biochem. Sci.* **2001**, *26*, 223.
[7] K. Martinek, V. V. Mozhaev, in *Thermostability of Enzymes* (Ed.: M. N. Gupta), Springer, Berlin, **1993**, p. 76.
[8] Q. S. Li, J. Ogawa, S. Shimizu, *Biochem. Biophys. Res. Commun.* **2001**, *280*, 1258.
[9] P. C. Cirino, F. H. Arnold, *Adv. Synth. Catal.* **2002**, *344*, 932.
[10] U. Schwaneberg, C. Schmidt-Dannert, J. Schmitt, R. D. Schmid, *Anal. Biochem.* **1999**, *269*, 359.
[11] K. G. Ravichandran, S. S. Boddupalli, C. A. Hasermann, J. A. Peterson, J. Deisenhofer, *Science* **1993**, *261*, 731.
[12] Y. T. Chang, G. Loew, *Biochemistry* **2000**, *39*, 2484.
[13] M. A. McLean, S. A. Maves, K. E. Weiss, S. Krepich, S. G. Sligar, *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166.
[14] J. K. Yano, L. S. Koo, D. J. Schuller, H. Li, P. R. Ortiz de Montellano, T. L. Poulos, *J. Biol. Chem.* **2000**, *275*, 31 086.
[15] S. Y. Park, K. Yamane, S. Adachi, Y. Shiro, K. E. Weiss, S. A. Maves, S. G. Sligar, *J. Inorg. Biochem.* **2002**, *91*, 491.
[16] S. Y. Park, K. Yamane, S. Adachi, Y. Shiro, K. E. Weiss, S. G. Sligar, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2000**, *56 (Pt 9)*, 1173.
[17] A. V. Puchkaev, L. S. Koo, P. R. Ortiz de Montellano, *Arch. Biochem. Biophys.* **2003**, *409*, 52.
[18] S. Nakagawa, S. Ishino, S. Teshiba, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 415.
[19] H. J. Barnes, M. P. Arlotto, M. R. Waterman, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5597.
[20] T. Omura, R. J. Sato, *J. Biol. Chem.* **1964**, *239*, 2370.
[21] P. C. Cirino, K. M. Mayer, D. Umeno, in *Directed Evolution Library Creation: Methods and Protocols* (Eds.: F. H. Arnold, G. Georgiou), Humana Press, Totowa, **2003**, pp. 3.
[22] W. P. Stemmer, *Nature* **1994**, *370*, 389.
[23] P. C. Cirino, R. Georgescu, in *Directed Enzyme Evolution: Screening and Selection Methods* (Eds.: F. H. Arnold, G. Georgiou), Humana Press, Totowa, **2003**, p. 117.
[24] H. Yeom, S. G. Sligar, *Arch. Biochem. Biophys.* **1997**, *337*, 209.

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