Global Incorporation of Norleucine in Place of Methionine in Cytochrome P450 BM-3 Heme Domain Increases Peroxygenase Activity

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Abstract: In this study we have replaced all 13 methionine residues in the cytochrome P450 BM-3 heme domain (463 amino acids) with the isosteric methionine analog norleucine. This experiment has provided a means of testing the functional limits of globally incorporating into an enzyme an unnatural amino acid in place of its natural analog, and also an efficient way to test whether inactivation during peroxide-driven P450 catalysis involves methionine oxidation. Although there was no increase in the stability of the P450 under standard reaction conditions (in 10 m*M* hydrogen peroxide), complete substitution with norleucine resulted in nearly two-fold-increased peroxygenase activity. Thermostability was significantly reduced. The fact that the enzyme can tolerate such extensive amino acid replacement suggests that we can engineer enzymes with unique chemical properties via incorporation of unnatural amino acids while retaining or improving catalytic properties. This system also provides a platform for directing enzyme evolution using an extended set of protein building blocks. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **00**: 000–000, 2003.

Keywords: protein engineering; norleucine; methionine oxidation; directed evolution; cytochrome P450; peroxygenase

INTRODUCTION

The cytochrome P450 monooxygenases are among the most widely studied enzymes. Their physiological roles are important in drug metabolism and drug design, and their catalytic capabilities are increasingly valued in chemical synthesis and bioremediation (see Guengerich, 2002; McGinnity and Riley, 2001; Ortiz de Montellano, 1995; Wong, 1998). Cytochrome P450 BM-3 is one of the fastest fatty acid hydroxylases known, preferring straight-chain substrates with 12 to 18 carbons (Miura and Fulco, 1975; Ruettinger et al., 1989). Hydroxylation occurs at subterminal positions, primarily ω-1, ω-2, and ω-3. P450 BM-3 contains a heme domain (~463 amino acids) attached to a reductase domain (~594 amino acids). The reductase transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to the heme during catalysis, which additionally requires dioxygen.

We are attempting to improve the efficiency of the BM-3 peroxide "shunt" pathway, a mechanism inherent in heme oxygenases by which catalysis can be driven by peroxide, eliminating the need for the reductase domain, NADPH, and O_2. The overall reaction equation using hydrogen peroxide (H_2O_2) is:

$$RH + H_2O_2 \rightarrow ROH + H_2O$$

This "peroxygenase" activity is not naturally utilized by P450 BM-3 and is very inefficient (Cirino and Arnold, 2002). The H_2O_2 concentrations (m*M*) required to drive this reaction cause rapid inactivation. Our goal was to use directed evolution to isolate mutants with higher k_{cat} values for the peroxygenase reaction, lower K_m values for H_2O_2 binding, and/or greater stability to peroxide. After several rounds of mutagenesis and screening for peroxygenase activity, we generated a thermostable BM-3 heme domain variant ("TH-4") with more than 100-fold improved peroxygenase activity compared with wild-type BM-3 and approximately 10-fold improved peroxygenase activity compared with BM-3 mutant F87A (Cirino and Arnold, unpublished results). Although it is more active, mutant TH-4 is still rapidly inactivated by H_2O_2; that is, in 10 m*M* H_2O_2, the enzyme is essentially inactive after 10 min.

H_2O_2-mediated inactivation of TH-4 is primarily the result of heme degradation, as indicated by a decrease in the heme absorbance peak in the presence of H_2O_2 (not shown). We nonetheless sought to determine whether protein modification plays a role in inactivating the enzyme. Methionine residues are prime targets for oxidation by peroxide (Caldwell and Tappel, 1964; Schechter et al., 1975; Stauffer and Etson, 1969), and methionine oxidation often results in a decrease or loss of biological function (Brot and Weissbach, 1991; Estell et al., 1985; Kim et al., 2001; Stauffer and

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Etson, 1969; Vogt, 1995). Both buried and surface methionines can be oxidized (Keck, 1996), and the rates of peroxide-mediated oxidation of the methionines within a single protein can vary greatly (Lu et al., 1999). BM-3 mutant TH-4 contains 13 methionines, and it would be tedious to introduce unnatural methionine analogs or other natural amino acids at all 13 positions in a site-directed fashion. We decided therefore to test whether global incorporation of a methionine analog might enhance stability to peroxide.

Incorporating unnatural amino acids into proteins is an established technique that has been useful for studying protein biosynthesis, probing protein structure and function, and adding unique functionality to proteins (see Cornish et al., 1995; Dougherty, 2000; Wang and Schultz, 2002). One simple method useful for incorporating some unnatural amino acids in place of their natural homologs is to exploit the promiscuity of the aminoacyl-tRNA synthetases. Methionine and norleucine (2-aminohexanoic acid) are isosteric amino acids, with norleucine the more hydrophobic (Schenck et al., 1996). Norleucine can substitute for methionine in the acylation of $tRNA^{Met}$ (Trupin et al., 1966), resulting in proteins containing norleucine in place of methionine. The replacement of methionine with norleucine often has little influence on protein structure or function (Anfinsen and Corley, 1969; Gilles et al., 1988; Naider et al., 1972; Randhawa et al., 1994; Rocchi et al., 1969), except in cases in which methionines play key functional roles (Yuan and Vogel, 1999). Gilles et al. incorporated norleucine into six methionine positions in adenylate kinase, resulting in 16% to 20% of the expressed kinase molecules resistant to CNBr cleavage and in improved stability in H_2O_2 (Gilles et al., 1988). In this investigation, we report the replacement of 13 methionines with norleucine in P450 BM-3 heme domain mutant TH-4 and the effects of this global replacement on enzyme activity and stability.

MATERIALS AND METHODS

All chemical reagents were obtained from Aldrich, Sigma, or Fluka. Hydrogen peroxide was purchased as a 35 wt% solution (Aldrich). Peroxide stock solutions were prepared fresh each day in 100 m*M* Tris-HCl (pH 8.2). Buffers and media were prepared according to standard protocols (Sambrook and Russell, 2001). Trypsin was purchased from Promega.

Tryptic digests contained 1 to 5 m*M* purified P450 and 8 ng/mL trypsin in 50 m*M* NH_4HCO_3. Following overnight digestion at room temperature, digests were quenched by addition of trifluoroacetic acid (TFA; final pH ~2) and cleaned using the $ZipTip_{C18}$ procedure (Millipore). Peptide fragments were eluted in 2 mL of 50% to 80% acetonitrile/1% TFA/water. The peptide samples were mixed (1:3 ratio) with an α-cyanohydroxycinnaminic acid solution (10 mg/mL in 50% acetonitrile/0.05% TFA/water) to form the matrix-assisted laser desorption ionization (MALDI) matrix. Mass spectra were recorded on an Applied Biosystems Voyager DE-PRO MALDI-time-of-flight (TOF) mass spectrometer operated in reflected ion mode.

The substrate 12-*p*-nitrophenoxycarboxylic acid (12-pNCA) was synthesized as described elsewhere (Schwaneberg et al., 1999), except hydrolysis of the ester was not performed enzymatically. Instead, the ester was dissolved into tetrahydrofuran (THF), mixed with an equal volume of an aqueous solution of 1 *M* KOH, and allowed to reflux with stirring for 6 h. After hydrolysis, the aqueous layer was separated, and 12-pNCA was crystallized by the addition of H_2SO_4 and then washed. The THF layer was saved for a second round of hydrolysis. The final product was verified by ^1H NMR, recorded on a Bruker DPX 300 spectrometer.

Enzyme Expression

P450 BM-3 heme domain (first 463 amino acids) mutant TH-4 was cloned into vector pCWori(+) (Barnes et al., 1991), allowing for isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression under the control of the double Ptac promoter. TH-4 includes a 6-His tag at the C-terminus, which has no noticeable effect on activity. Protein expression was performed using the medium shift method as described elsewhere (van Hest et al., 2000), using the *E. coli* methionine auxotroph, CAG18491 (*l⁻, rph-1, metEo-30769::Tn10*). Briefly, cells were transformed with the pCWori(+)(TH-4) plasmid and grown on Luria–Bertani (LB)-agar plates containing 100 mg/L ampicillin. A single, freshly transformed colony was used to inoculate 5 mL of M9AA medium supplemented with 1 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, 0.4 wt% glucose, 1 m*M* thiamine, and 100 mg/L ampicillin. This 5-mL culture was grown overnight and used to inoculate a 500 mL culture containing the same ingredients. A medium shift was performed when this culture reached an optical density at 600 nm (OD_{600}) of ~0.8. Cells were sedimented and resuspended in a 0.9% NaCl solution for washing, and this was repeated three times. Cells were then resuspended in 500 mL of the M9AA medium just described, without methionine. The culture was supplemented with 1 m*M* δ-aminolevulinic acid (a heme precursor) plus trace elements (Joo et al., 1999) and grown for 10 min at 37°C to deplete any remaining methionine. IPTG (0.5 m*M*) was then added to induce protein expression, and the culture was divided into four 125-mL cultures. One was grown without methionine or norleucine, as a negative control. The positive control, TH-4(Met), was grown with 100 mg/L L-methionine added. TH-4(Mix) was grown with 300 mg/L L-norleucine plus 10 mg/L L-methionine, and TH-4(Nor) was grown with 300 mg/L L-norleucine only. These cultures were grown at 30°C for 15 to 20 h.

Protein Purification

Purification of TH-4 took advantage of the 6-His tag using the QIAexpressionist kit (Qiagen) for purification under native conditions. Briefly, cultures were grown as just described and, after centrifugation and resuspension in lysis buffer (10 m*M* imidazole, 50 m*M* NaH_2PO_4 [pH 8.0], 300

m*M* NaCl), the cells were lysed by sonication. Cell lysates were centrifuged, filtered, and loaded onto a Qiagen Ni-NTA column. The column was washed with wash buffer (20 m*M* imidazole, 50 m*M* NaH_2PO_4 [pH 8.0], 300 m*M* NaCl), and the bound P450 was eluted with elution buffer (200 m*M* imidazole, 50 m*M* NaH_2PO_4 [pH 8.0], 300 m*M* NaCl). Aliquots of the purified proteins were buffer-exchanged with 100 m*M* Tris-HCl (pH 8.2) using a PD-10 desalting column (Amersham Pharmacia Biotech). Purified P450 concentrations were quantified from CO-binding difference spectra of the reduced heme as described elsewhere (Omura and Sato 1964), using an extinction coefficient of 91 mM^{-1} cm^{-1} for the 450-nm minus 490-nm peak. P450 total expression yields were estimated from CO-binding difference spectra of the clarified lysates.

Enzyme Activity Assay

P450 activity measurements using 12-pNCA were performed by monitoring the formation of *p*-nitrophenolate (pNP) (398 nm) at room temperature using a 96-well plate spectrophotometer (SPECTRAmax, Molecular Devices). Activity (hydroxylation of 12-pNCA) required folded enzyme displaying the characteristic 450-nm CO-binding absorbance peak. Enzyme samples containing either partially or fully denatured protein (as a result of guanidinium chloride treatment or heat treatment) were either less active than the native enzyme (for partially denatured samples) or not active at all (for fully denatured samples). Control reactions containing H_2O_2 and 12-pNCA with or without hemin chloride showed no background activity. A typical reaction well contained 140 μL of 100 m*M* Tris-HCl buffer (pH 8.2), 10 μL stock solution of substrate (4 m*M* 12-pNCA) in dimethysulfoxide (DMSO), and purified P450. Reactions were initiated by the addition of 10 μL of H_2O_2 stock solution. Final concentrations were 250 μ*M* 12-pNCA, 10 m*M* H_2O_2, and 0.1 to 0.2 μ*M* P450. DMSO (6% final concentration) was used to solubilize the 12-pNCA. In general, DMSO inhibits P450 BM-3 activity, although the peroxygenase activity of heme domain mutant TH-4 is not inhibited until DMSO concentrations exceed ~10% (not shown). The 398-nm absorbance reading for each well was blanked before addition of H_2O_2 so that end-point turnovers could be calculated. Rates of peroxygenase activity were calculated as the rate of pNP formation (or the increase in absorbance at 398 nm over time). The value for (extinction coefficient) × (path length) for pNP under the exact conditions used in the spectrophotometer assay was calculated from a standard curve generated with known concentrations of pNP. This factor was used to quantify turnover of substrate. Thermostability measurements were accomplished as just described, with the addition of a heat-inactivation step. Aliquots (50 μL) of each purified protein sample were heated to 57.5°C for 10 min in a PTC200 thermocycler (MJ Research), rapidly cooled to 4°C, and then brought to room temperature. The residual activities of these heat-inactivated samples were then determined.

RESULTS AND DISCUSSION

Table I lists the three culture conditions used during induced expression of TH-4, following the shift of cultures (OD_{600} of ~0.8) to methionine-free M9AA medium. Expression resulted in cultures containing TH-4 with only methionine incorporated [TH-4(Met)], TH-4 with both methionine and norleucine incorporated [TH-4(Mix)], and TH-4 with norleucine incorporated almost exclusively [TH-4(Nor)]. Table I also lists approximate P450 expression levels for each culture. As expected, expression levels and cell growth rates were significantly reduced under methionine-restricted conditions. The expression level in the control experiment indicates that a small percentage of TH-4 methionine positions will still contain methionine in TH-4(Nor). Coexpression of the methionyl-tRNA synthetase has been shown to increase expression of proteins incorporating norleucine (Kiick and Tirrell, 2000). In our case, however, coexpression of the synthetase had little effect on expression of TH-4(Nor) or cell growth (not shown). The reduced expression probably reflects norleucine's toxicity to cells (Bogosian et al., 1989) as well as the slow kinetics of norleucine incorporation (van Hest et al., 2000).

Figure 1 shows mass spectra of fragments from tryptic digests of the three purified TH-4 samples. Virtually complete substitution of methionine (MW 131.2) with norleucine (MW 113.2) in TH-4(Nor) was verified by the shift of 18 mass units in three fragments that each contain a single methionine. Low-abundance, nonshifted peaks confirmed that a small percentage of TH-4 methionine positions still contained methionine in TH-4(Nor). Partial incorporation was achieved in the mixed culture TH-4(Mix), as indicated by the presence of both sets of fragments. These three TH-4 samples displayed typical P450 spectra (450-nm CO-binding peak and 417-nm low-spin absorbance peak), indicating that their overall folds and heme environments were similar to those of wild-type BM-3. A small shoulder occasionally appeared at 420 nm in the CO-binding spectrum of TH-4(Nor). This is indicative of a P450 having an active site that is disrupted (Martinis et al., 1996), and suggests that stability was reduced as a result of norleucine substitution.

Table I. Methionine/norleucine concentrations used to supplement cultures following medium shift to methionine-free M9AA, and the resulting cytochrome P450 BM-3 mutant TH-4 expression levels.

Cultures	TH-4(Met)	TH-4(Mix)	TH-4(Nor)	Control
[L-Methionine] (mg/L)	100	10	—	—
[L-Norleucine] (mg/L)	—	300	300	—
P450 expression[a] (mg/L)	34	21	10	0.4

[a]P450 expression varies considerably between cultures. Here we report representative values, determined from the CO-binding difference spectrum of the clarified lysate, prior to purification.

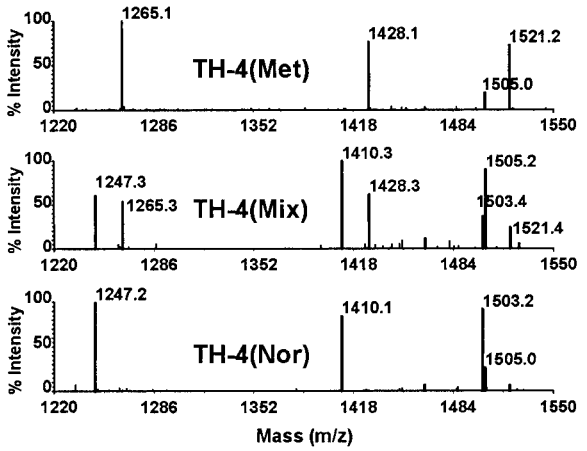

Figure 1. MALDI-TOF mass spectra of tryptic peptides derived from TH-4 expression in medium supplemented with methionine only [TH-4(Met)], methionine plus norleucine [TH-4(Mix)], and norleucine only [TH-4(Nor)]. Peaks at m/z = 1265, 1428, and 1521 arise from peptides that each contain a single methionine. A shift of 18 mass units indicates replacement of methionine with norleucine.

Specific peroxygenase activities of the three purified TH-4 samples were measured using 10 m*M* H_2O_2 and 12-*p*-nitrophenoxycarboxylic acid (12-pNCA) as substrate. Hydroxylation of 12-pNCA at the "terminal" carbon adjacent to the *p*-nitrophenoxy group results in release of pNP (Schwaneberg et al., 1999). TH-4 is 100% selective toward hydroxylating the C-12 carbon of ~12-pNCA (adjacent to the pNP group), resulting in complete conversion to pNP under substrate-limiting conditions. Product formation was measured by monitoring pNP absorption at 398 nm. Figure 2 shows the time course of pNP formation for the three TH-4 samples. The initial rate of product formation increased with the level of norleucine incorporation, and complete incorporation of norleucine resulted in almost two-fold improved activity over TH-4(Met). In repeated experiments

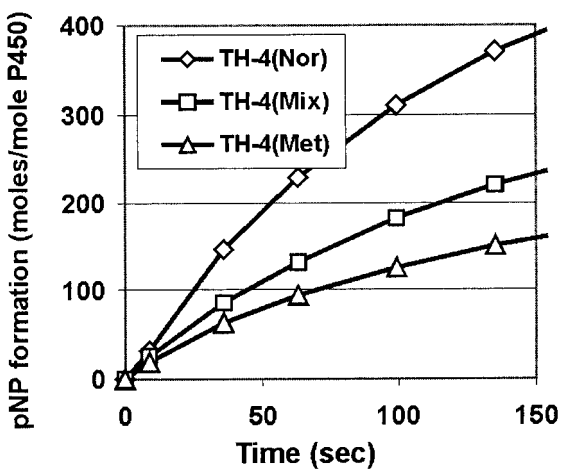

Figure 2. Time course of product formation during the peroxygenase reactions of TH-4(Met), TH-4(Mix), and TH-4(Nor) with 12-pNCA in 10 m*M* H_2O_2. Absorbance at 398 nm was monitored to measure formation of product (pNP). Data points represent averages from three experiments; standard deviations were <10%.

with enzyme from duplicate cultures and varying reaction conditions, TH-4(Nor) was consistently almost twice as active as TH-4(Met). Under substrate-limiting conditions, the same amount of pNP was formed with TH-4(Met) as with TH-4(Nor), eliminating the possibility that increased activity is due to altered regioselectivity. The increased peroxygenase activity of TH-4(Nor) was verified using styrene as substrate and quantifying styrene oxide by gas chromatography/FID (not shown). TH-4(Nor) is more active than TH-4(Met) in 2% DMSO as well as 6% DMSO (not shown).

To estimate the relative thermostabilities of the TH-4 samples, each was heated to 57.5°C for 10 min, cooled, and reassayed for activity at room temperature. Figure 3 shows the total turnover achieved by each enzyme sample before and after heat treatment. Thermostability decreased with increased norleucine incorporation such that TH-4(Nor) was almost completely inactivated by the heat treatment. Norleucine incorporation therefore decreases stability but increases peroxygenase activity. Because the majority of amino acid substitutions that can be introduced into an enzyme have neutral to deleterious effects on both activity and stability, the observed improvement in activity was unexpected. We are presently unable to offer an explanation for how norleucine increases peroxygenase activity.

Although initial activity is increased with norleucine incorporation, the time courses of product formation were very similar for the three mutants, indicating that enzyme inactivation was not affected by norleucine incorporation and TH-4 methionines were not oxidized under the peroxide concentrations used in the peroxygenase reaction ($\leq$10 m*M*) during these reaction times. Mass spectral analysis of fragments from trypsin, endoproteinase Lys C, and chymo-

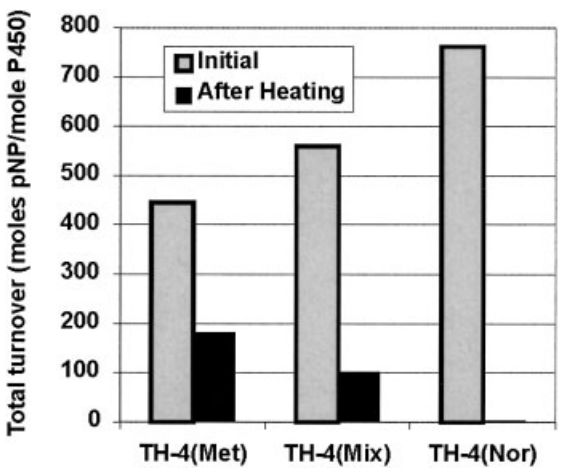

Figure 3. Total peroxide-driven turnover results achieved by TH-4(Met), TH-4(Mix), and TH-4(Nor) at before and after heat treatment. Reactions were performed at room temperature with 12-pNCA as substrate and initiated by the addition of 10 mM H_20_2. Also, 10 m*M* H_2O_2 was added to start each reaction. Heated samples were incubated at 57.5°C for 10 min and then cooled before measuring activity. Reactions were allowed to continue until pNP was no longer produced. Data represent averages from at least two experiments; standard deviations were <15%.

trypsin digests of peroxide-treated TH-4 verified that 8 of the 13 methionine residues were not oxidized. The remaining five residues were not represented by the detectable fragments, so oxidation at these positions could not be checked.

Peroxygenase activity of the P450 BM-3 heme domain mutant TH-4 was shown to be retained, and even improved, after global replacement of methionines with their oxidation-resistant, more hydrophobic analog norleucine. Our results are particularly encouraging in light of recent successes in the engineering of expression systems to utilize unnatural protein building blocks (Datta et al., 2002; Wang and Schultz, 2002). Novel properties can be engineered into an enzyme via unnatural amino acid incorporation without sacrificing the enzyme's natural catalytic functions, provided the substitutions are not overly disruptive. Directed evolution experiments will soon be extended to evolving enzymes expressed in the presence of unnatural amino acids, where one probable goal will be to improve the stability or function compromised as a result of analog incorporation. The system described herein provides a framework for such evolution experiments.

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