

## Improving Nature's Enzyme Active Site with Genetically Encoded Unnatural Amino Acids

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**Abstract:** The ability to site-specifically incorporate a diverse set of unnatural amino acids (> 30) into proteins and quickly add new structures of interest has recently changed our approach to protein use and study. One important question yet unaddressed with unnatural amino acids (UAAs) is whether they can improve the activity of an enzyme beyond that available from the natural 20 amino acids. Herein, we report the >30-fold improvement of prodrug activator nitroreductase activity with an UAA over that of the native active site and a >2.3-fold improvement over the best possible natural amino acid. Because immense structural and electrostatic diversity at a single location can be sampled very quickly, UAAs can be implemented to improve enzyme active sites and tune a site to multiple substrates.

### Introduction

Since gaining the ability to modify proteins from the genetic level, defined amino acid changes have been used to modify and study protein function. Altering the chemical and physical properties of proteins and enzymes has been instrumental in their use as catalysts, therapeutics, and in the activation of therapeutics. Although amino acid mutagenesis is one of the most powerful tools for modifying protein function, it is restrictive because we have not yet truly moved past the 20 common amino acids with their limited structural and chemical diversity. The ability to site-specifically incorporate a diverse set of unnatural amino acids (>30) into proteins and quickly add new structures of interest has recently changed our approach to protein use and study.<sup>1–4</sup> One important question yet unaddressed with unnatural amino acids (UAAs) is whether they can improve the efficiency of an enzyme beyond that available from the natural 20 amino acids.

Although UAAs have been incorporated via a variety of methods into enzymes to probe their function, none has been reported to improve an enzyme's activity, begging the question whether additional amino acids would improve current biological function.<sup>5,6</sup> Since the practicality of obtaining large quantities of protein containing unnatural amino acids has improved, it is now feasible to test whether unnatural amino acids can improve enzyme efficiency, as well as to perform structure activity relationship studies by systematically altering the protein instead of the substrate. To prove that the UAAs currently accessible

for site-specific *in vivo* incorporation into proteins can be used to tune and improve enzyme activity beyond that available with natural amino acids, we focused on a critical active-site amino acid in the prodrug activator nitroreductase (NTR).

Nitroreductase from *Escherichia coli* is being used as a prodrug activator in cancer therapy.<sup>7,8</sup> Currently employed prodrug CB1954 and new lead prodrug LH7 have been activated by NTR using antibody-directed and gene-directed enzyme methods. NTR is a 48 kDa homodimer that reduces nitroaromatic compounds by transferring hydrides from NAD(P)H through a bound FMN cofactor in a Ping-Pong Bi Bi mechanism. CB1954 is reduced to the corresponding cytotoxic hydroxylamino derivative **1**, whereas LH7 activation proceeds through the hydroxylamino intermediate which releases anionic cytotoxic mustard **3** (Figure 1a). Activated drug toxicity results from the formation of poorly repaired, interstrand DNA cross-links. The current therapeutic limitation of CB1954/NTR drug delivery system is the high dosage of drug needed because of the high  $K_M$  of CB1954 for native NTR.

To improve the efficacy of the NTR/CB1954 system by increasing the activity of NTR with CB1954, NTR structural information helped guide the alteration of all relevant active site residues.<sup>7,9</sup> Active site residue Phe124 emerged as critical for substrate binding and was modified to all possible natural amino acids. These modifications were found to have a range of effects on CB1954 activation. The F124K and F124N mutations emerged as the best for improving the activity of NTR for CB1954. Structural studies have suggested that Phe124 pi stacks with the aryl ring of the prodrug or NADH when the active site is occupied. Improved activity with K124 or N124

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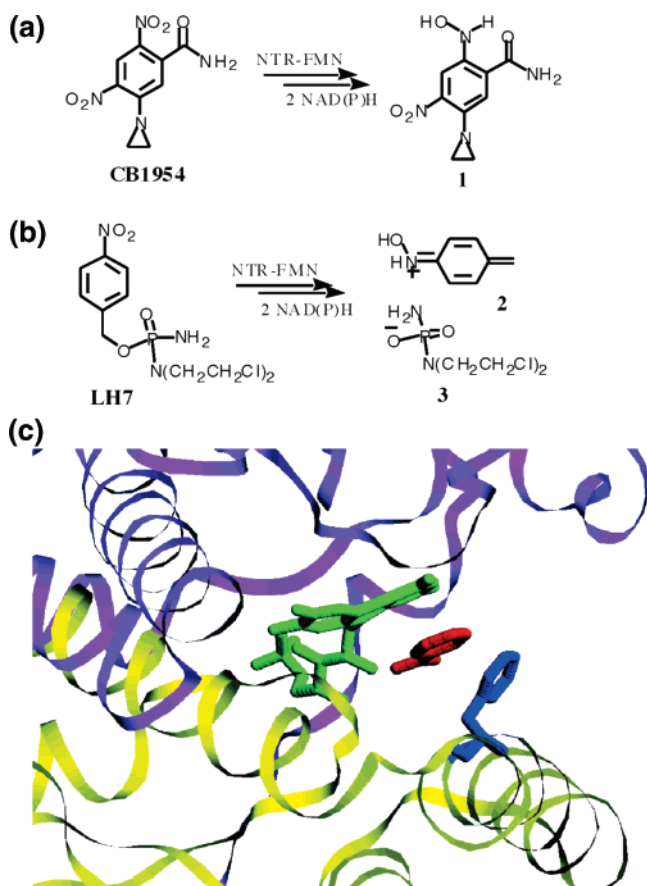
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**Figure 1.** Enzymatic activation of prodrugs with NTR: (a) CB1954 activation. (b) LH7 activation. (c) NTR active site with homodimer represented as yellow and purple strands (PDB code 1ICR). Bound at the active site is FMN in green and inhibitor, nicotinic acid, in red. The modified active site phenylalanine 124 is in blue.

could be explained by NH- $\pi$  face stabilizing interaction with the electron deficient prodrugs but other stabilizing interactions are possible. Phe124 or the amino acid replacing it not only aids in directing and facilitating the reaction of the substrate with bound cofactor, FMN, but it can also interact with the opposite subunit when the active site is closed, as well as interact with substituents on the aryl ring which project out of the active site. The exhaustive analysis of all natural amino acids at site Phe124 to improve the currently used prodrug CB1954 system, and the diversity of possible stabilizing interactions that are possible for this site, directed us to test this site in NTR to determine whether a protein could be improved with unnatural amino acids.

The incorporation of different UAAs at site 124 in NTR was accomplished by using an orthogonal, evolved *Methanococcus jannaschii* suppressor tRNA/aminoacyl-tRNA synthetase pair (MjTyrRS/tRNA<sup>Tyr</sup><sub>CUA</sub>) for each UAA. Using evolved synthetase we were able to site-specifically incorporate additional UAAs similar in structure to the evolved synthetases UAA, providing us with a total of eight UAAs to compare with natural amino acids.<sup>1,2,10–12</sup> Comparing the catalytic proficiency of the best natural amino acids with eight UAAs at this critical F124

position, we demonstrate that UAAs can tune activity for different substrates and improve the efficiency of enzymes beyond that accessible with the natural amino acids.

## Results and Discussion

**Generation of Natural NTR Variants.** The gene encoding NTR, nfnB, was cloned from *E. coli* K12 into a standard protein expression plasmid, pTrcHisA, which added an NH<sub>2</sub>-terminal His6-tag to facilitate purification. Site-directed mutagenesis of nfnB gene in pTrcHisA provided the F124K and F124N mutants. Site-directed mutagenesis also converted the codon for Phe124 to a TAG stop codon resulting in pTrc-NTR-124TAG, which when expressed alone in DH10B cells produces no purifiable protein. When pTrc-NTR-124TAG is combined in cells with the orthogonal *Methanococcus jannaschii* suppressor tRNA/aminoacyl-tRNA synthetase pair (MjTyrRS/tRNA<sup>Tyr</sup><sub>CUA</sub>), a full-length protein is produced with tyrosine incorporated at site 124. The genes for MjTyrRS/tRNA<sup>Tyr</sup><sub>CUA</sub> resides in a medium copy plasmid (pDule-Tyr).<sup>13</sup> These expression systems provided access to large quantities of pure protein depicted as the natural set of NTR variants in Figure 2a.

**Generation of NTR-124-UAA Proteins.** Each UAA in generation 1 (Figure 2a) was incorporated with a unique orthogonal, evolved *Methanococcus jannaschii* suppressor tRNA/aminoacyl-tRNA synthetase pair (MjTyrRS/tRNA<sup>Tyr</sup><sub>CUA</sub>).<sup>1,2,10,11</sup> The genes for each synthetase/tRNA<sup>Tyr</sup><sub>CUA</sub> pair reside in a medium-copy plasmid (pDule-UAA), allowing a standard high-copy protein expression plasmid for use in production of UAA containing NTR (Figure 2b). All UAA-proteins were produced only when cells containing both plasmids pDule-UAA and pTrc-NTR-124TAG were induced to produce protein in modified minimal media supplemented with the UAA. The expression of all UAA containing NTR with and without UAA present in the media was monitored by silver stain SDS-PAGE analysis (Supporting Information Figure 1).<sup>13</sup>

Using the chemical diversity of site-specifically incorporated UAAs, we can combine functionalities and alter electrical factors of the Phe124 aromatic ring. In exploring the activity of the first generation unnatural-NTR, we found that the pAF substitution modestly improved NTR activity above the best natural amino acids at that position (Figure 3). To further explore and improve the electronic factors of this para position amine, we sought the substitutions indicated as generation 2 in Figure 2a, but evolved synthetases for these amino acids did not exist at the start of the study. The ptfmF synthetase was evolved using standard selection methods and was found to also incorporate the p-MF with high selectivity and fidelity.<sup>12</sup> We also discovered that using the evolved synthetase for pAF we were able to site-specifically incorporate the pAMF and the pNF with high fidelity but at lower yields (Figure 2b).

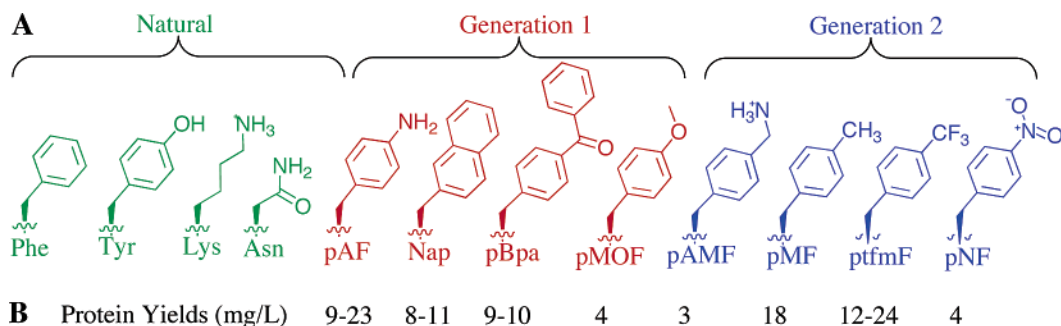
To verify that discrete incorporation of each UAA only takes place at site 124, each pure protein was digested by trypsin and the fragments were analyzed by LC-ESI MS. The only peptide fragment containing the desired change was the expected F(X)-ADMHR fragment with a minimum of >50% sequence coverage for all protein samples (MS/MS, Mascot search). These results along with the SDS-PAGE gel analysis confirm the high fidelity and efficiency of UAA incorporation into proteins with

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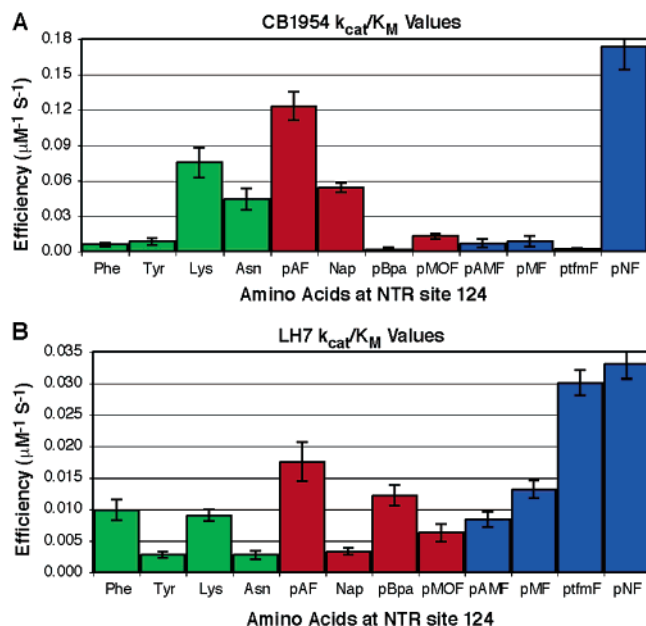
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**Figure 2.** NTR modifications: (a) Natural and unnatural amino acids incorporated at site 124 in NTR. Generation 1: *p*-aminophenylalanine (pAF), naphthylalanine (Nap), *p*-benzoylphenylalanine (pBpa), *p*-methoxyphenylalanine (pMOF). Generation 2: *p*-aminomethylphenylalanine (pAMF), *p*-methylphenylalanine (pMP), *p*-trifluoromethylphenylalanine (ptfmF), *p*-nitrophenylalanine (pNF). (b) Purified unnatural-NTR yields from modified minimal media expressions.



**Figure 3.** Catalytic efficiency of modified NTR enzymes: (a)  $k_{cat}/K_M$  values for natural and unnatural NTRs with CB1954 and (b)  $k_{cat}/K_M$  values for natural and unnatural NTRs with LH7.

the pDule plasmids. Although it seems obvious that UAAs of similar shape should be utilized by unnatural synthetases which have been evolved to discriminate against only the natural amino acids, this method of quickly expanding the subset of current orthogonal unnatural synthetases has yet to be embraced.

**Kinetic Evaluation of NTR Proteins.** The activity of NTR proteins was monitored spectrophotometrically by observing the formation of the hydroxylamine products at 420 nm for CB1954 studies and following the loss of NADH at 340 nm for LH7 studies.<sup>7,8</sup> To ensure the special electronic characteristics of unnatural amino acids were not unfairly targeted, assay conditions for each prodrug were optimized using native NTR and kept constant for all proteins. Low prodrug solubility, and the range of NTR–UAA catalytic efficiencies, prevented the comparison of individually optimized NTR–prodrug assays. The CB1954 activities of the natural versions of NTR match well with previous studies, showing slight improvement with Tyr-NTR and significant improvement with Lys-NTR and Asn-NTR (Figure 3a). Combining the amine and aryl ring in pAF–NTR improved both prodrug binding and catalytic activity resulting in a higher efficiency than feasible with any natural amino acid. Focusing on the electrostatic and structural aspects of pAF, the

amine was separated from the aryl ring by a methylene unit in pAMF, making the basicity more like Lys and the ring polarization more like Phe. Unnatural protein pAMF–NTR produced not only the highest catalytic activity of any amino acid tested but also the highest  $K_M$ . Reducing aryl ring polarization and removing basicity by moving to pMF and ptfmF showed similar results to Phe-NTR and Tyr-NTR. However, increasing ring polarization by switching to a para nitro functionality in pNF–NTR improved both the prodrug binding and catalytic activity of pAF–NTR, resulting in the best unnatural-NTR (>30 times better than the native NTR, and >2.3-fold better than the best natural).

The complexity of this multistep reaction has made it difficult to elucidate the role of natural amino acids in prodrug binding and activation, but structural work on natural active site variants are starting to provide clues. Unfortunately the flexibility of lysine in the crystal structure of NTR-124 prevents elucidation of its role in improving catalytic efficiency. Of particular interest within the improved efficiency for the NTR–UAAs: (NTR–pAF, NTR–Nap and NTR–pNF) is the significant drop in  $K_M$  because poor prodrug solubility and high prodrug dosage prevent wider use of this system for cancer treatment. We expect that future catalytic analysis and crystal structure studies with these NTR variants will help elucidate how improved prodrug binding is achieved and can be further improved.

Limited *in vitro* kinetic work has been done with LH7 and NTR but our results show that the improvements seen with natural amino acids for activating CB1954 are not seen with LH7 (Figure 3b). Multiple UAA-NTRs showed improved activation of LH7 over the natural-NTRs tested with a >3-fold improvement from both ptfmF–NTR and pNF–NTR. We suspect with LH7 the reduced  $K_M$  provided by the polarized aromatics at this site will lead to the highest efficiency for this prodrug. Interestingly, ptfmF–NTR activates prodrug LH7 but not CB1954 and could be used simultaneously with CB1954 treatment to access different bystander effects on different targeted cell types.

Although it is difficult to elucidate the electronic interactions by which the pAF or pNF is improving NTR activity, the inability to improve prodrug binding without disrupting reduction has plagued the development of new prodrugs. Different modes of stabilization might be accessed with different amino acids at site 124. We suspect that for the UAAs, a polarized aromatic ring at site 124 aids in pi stacking with the polarized aromatic substrate while also aiding in hydride transfer.

## Conclusion

We have demonstrated that UAAs, with their immense structural and electrostatic diversity, can provide proteins with improved catalytic properties not accessible with only natural amino acids. We report the >30-fold improvement of prodrug activator nitroreductase (NTR) efficiency with an UAA over that of the native active site, and a >2.3-fold improvement over the best possible natural amino acid for currently used prodrug CB1954. We have also shown that this quick and versatile approach to modifying proteins can be used to tune an active site to multiple substrates. Combining improved UAA-enzymes with analogous autonomous 21 amino acid bacteria could address the evolutionary fitness of natural 20 amino acid organisms. Because these unnatural amino acids are incorporated *in vivo*, any protein produced in *E. coli*, irrespective of size, can be explored with this method. Further studies are underway to understand the mechanism of activity enhancement so that future prodrugs and/or protected metabolites can be paired with improved unnatural active-sites.

## Experimental Section

**General Methods.** Chemical reagents were purchased from Sigma-Aldrich and used without further purification. Unnatural amino acids were purchased from Bachem and Peptech. Oligonucleotides, dh10B cells, and pTrcHisA were purchased from Invitrogen. CB1954 was donated by Dr. William Denny, Director of the Auckland Cancer Society Research Centre, The University of Auckland, New Zealand. LH7 was synthesized according to the procedure by Hu L. et al.<sup>8</sup>

**Overexpression of NTR Containing Natural Amino Acids.** The *nfnB* gene was amplified by PCR from *E. coli* K12 genomic DNA using the following primers: 5'-GTGCTGGGATCCGATATCATTTCT-GTCGCCTTAAAGCGTCATTCC-3' forward, 5' GCTCTTAGGAATTCGCCCGCAAGAGAGAATTAC-3' reverse. The amplified *nfnB* gene was digested with *Bam*HI and *Eco*RI and inserted into pTrcHisA producing pTrc-NTR. QuickChange site directed mutagenesis was performed on pTrc-NTR with primers 5'-GGTCGCAAGTTCAAAGCT-GATATGCACCG-3', 5'-GGTCGCAAGTTCAAACGCTGATATGCACCG-3', 5'-GGTCGCAAGTTCTAGGCTGATATGCACCG-3' to produce pTrc-NTR-K124, pTrc-NTR-N124, and pTrc-NTR-124TAG, respectively. Natural NTR proteins (NTR-native, NTR-124-Lys, and NTR-124-Asn) were produced in dh10B cells according to the procedure in the pTrcHisA manual. DH10B cells containing pDule-Tyr and pTrc-124TAG were grown, and protein was expressed according to the procedure outlined in Farrell et al. to produce NTR-124-Tyr.<sup>13</sup> Protein was purified to >95% according to the purification procedure found in the *BD Talon Metal Affinity Resins User Manual* (BD Biosciences 1/28/2003).

**Overexpression of Mutant NTR Containing Unnatural Amino Acid.** Overexpression of protein containing unnatural amino acid was performed according to the procedure outlined in Farrell et al.<sup>13</sup> Electrocompetent dh10B cells were transformed with plasmid DNA pTrc-NTR-124TAG (1  $\mu$ L of 25 ng/ $\mu$ L, enabling resistance to ampicillin) and pDule-UAA (1  $\mu$ L of 25 ng/ $\mu$ L, enabling resistance to tetracycline). Plasmid pDule-UAA encodes tRNA and synthetase for incorporation of a given unnatural amino acid into TAG codon. Transformed cells were placed into 1 mL of rescue media and incubated at 37 °C with shaking for 250 rpm. Cells containing both plasmids were selected on agar plates containing 100  $\mu$ g/mL ampicillin and 25 mg/mL tetracycline by incubating overnight at 37 °C. All overexpression cell lines for the production of protein containing unnatural amino acids were prepared in this manner. A single colony from the selection plates was used to inoculate 6 mL of 2 $\times$  YT broth, containing 100  $\mu$ g/mL ampicillin and 25 mg/mL tetracycline, and grown overnight with shaking at 250 rpm at 37 °C. Saturated cell culture (1 mL) was

centrifuged at 1000  $\times$  g for 5 min, and 750  $\mu$ L of supernatant was removed. The remaining 250  $\mu$ L was resuspended and 62.5  $\mu$ L was used to inoculate 55 mL of minimal media, containing 0.01 mM riboflavin and 1 mM of unnatural amino acid where applicable.<sup>13</sup> Minimal media cultures contained 50  $\mu$ g/mL ampicillin and 10  $\mu$ g/mL tetracycline where appropriate. Cell cultures were allowed to grow to an OD<sub>600</sub>  $\approx$  0.8 at 37 °C with shaking (15–25 h). Cultures were induced to overexpress protein with 250  $\mu$ L of 200 mM IPTG and allowed to express 20–24 h. Overexpression cultures were centrifuged at 6000  $\times$  g for 20 min, and the procedure for protein purification found in the *BD Talon Metal Affinity Resins User Manual* (BD Biosciences 1/28/2003) was followed. Approximately 10  $\mu$ L of the first 500  $\mu$ L elution of each overexpression was analyzed using a 12% SDS-polyacrylamide gel (Supporting Information Figure 1). Protein was removed from elution buffer containing imidazole using Amersham PD10 columns and eluted into a 10 mM Tris-HCl buffer, pH 7.00. Protein concentrations were determined using BCA (bicinchoninic acid) *Protein Assay Kit* and instructions (Pierce 23225).

**Mass Spectrometric Analysis of UAA Proteins.** Proteins in 10 mM Tris buffer were dried overnight on a vacuum-line, resuspended in 0.1 M ammonium hydrogen carbonate, and then incubated at 55 °C for 30 min. Sequencing grade trypsin in 0.1 M ammonium hydrogen carbonate was added to each protein sample, and the samples were incubated at 37 °C overnight. Mass spectral analyses were performed on an Agilent 1100 series LC/MSD SL ion trap mass spectrometer with electrospray ionization and MS/MS capabilities (see Supporting Information).

**Enzyme Assays with Prodrugs.** Enzyme assays were performed spectrophotometrically in 10 mM Tris-HCl buffer, pH 7.00, at 25 °C in the presence of 100  $\mu$ M NADH and 4% DMSO and varying concentrations of a second substrate (Supporting Information Table 1). For CB1954, the reaction was initiated by the addition of 10  $\mu$ L of NTR solution to a final concentration of 4 nM. A 5 mM stock solution of CB1954 was made up in 4% DMSO, 10 mM Tris-HCl pH 7.00 buffer. Due to mild inhibition of NTR by DMSO, it was kept constant at 4% in all CB1954 assays. The progress of the reaction was monitored by observing the formation of the hydroxylamine products at 420 nm (with a molar absorbance of  $\epsilon = 1200 \text{ M}^{-1} \text{ cm}^{-1}$ ) at 10 s intervals for a period of 5 min and normalized for enzyme concentration.<sup>8</sup> CB1954 concentrations varied from 250 to 5000  $\mu$ M. For LH7, the reaction was initiated by the addition of 10  $\mu$ L of NTR solution to a final concentration of 40 nM. A 25 mM stock solution of LH7 was made up in 60% ethanol, 10 mM Tris-HCl pH 7.00 buffer. LH7 concentrations varied from 50 to 3000  $\mu$ M. The progress of the reaction was monitored at 340 nm by observing the oxidation of NADH. This was converted to a rate of reduction of LH7 using the molar absorbance of NADH ( $\epsilon = 6200 \text{ M}^{-1} \text{ cm}^{-1}$ ), assuming 2 mols of NADH are consumed per LH7, reduced to the hydroxylamine and normalized for enzyme concentration.<sup>8</sup> The observed rates (the observed slope of the reaction for 1 min) as a function of substrate concentration were fitted to a hyperbolic curve using Wilman Kinetic software to generate  $K_m$  and  $k_{cat}$  values for the data (Supporting Information Tables 3 and 4).

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**Supporting Information Available:** A description of SDS-PAGE gel silver stain protein analysis, mass spectrometry of proteins, kinetic assays, and catalytic constants are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>

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