Article

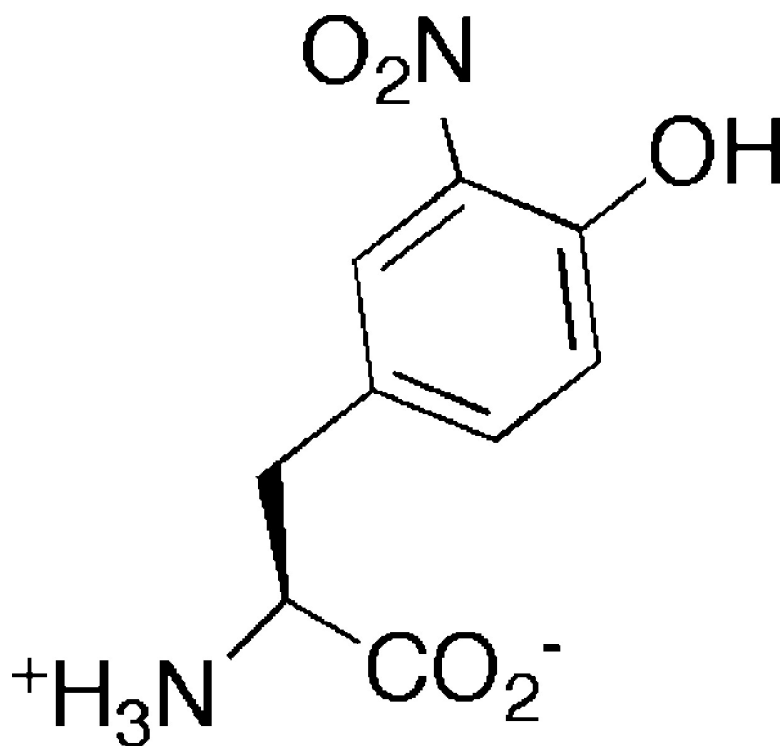# Genetically Encoding Protein Oxidative Damage

Heinz Neumann, Jennifer L. Hazen, John Weinstein, Ryan A. Mehl, and Jason W. Chin

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Genetically Encoding Protein Oxidative Damage

Heinz Neumann,[†] Jennifer L. Hazen,[‡] John Weinstein,[‡] Ryan A. Mehl,*,[‡] and
Jason W. Chin*,[†]

*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH,
United Kingdom, and Chemistry Department, Franklin & Marshall College,
Lancaster, Pennsylvania 17604*

Received November 7, 2007; E-mail: rmehl@fandm.edu; chin@mrc-lmb.cam.ac.uk

Abstract: Posttranslational modification of tyrosine residues in proteins, to produce 3-nitrotyrosine (3-NT), is associated with over 50 disease states including transplant rejection, lung infection, central nervous system and ocular inflammation shock, cancer, and neurological disorders (for example, Alzheimer's disease, Parkinson's disease, and stroke). The levels of 3-NT increase in aging tissue, and levels of 3-NT in proteins are a predictor of disease risk. Here we report the evolution and characterization of an aminoacyl-tRNA synthetase/tRNA pair for the cotranslational, site-specific incorporation of 3-NT into proteins at genetically encoded sites. To demonstrate the utility of our approach for studying the effect on protein function of nitration on sites defined in vivo, we prepared manganese superoxide dismutase (MnSOD) that is homogeneously nitrated at a site known to be modified in disease-related inflammatory responses, and we measured the effect of this defined modification on protein function.

Introduction

Posttranslational modification of tyrosine residues in proteins, to produce 3-nitrotyrosine (3-NT), is associated with over 50 disease states including transplant rejection, lung infection, central nervous system and ocular inflammation shock, cancer, and neurological disorders (for example, Alzheimer's disease, Parkinson's disease, and stroke).[1] The levels of 3-NT increase in aging tissue, and levels of 3-NT in proteins are a predictor of disease risk.[2]

Nitration of tyrosine residues in proteins occurs via reaction of the tyrosine side chain with radicals that are ultimately derived from nitric oxide.[3] Nitration of tyrosine reduces the pK_a of the phenolic hydroxyl by 2−3 units, such that at physiological pH 50% of 3-NT side chains are ionized, and alters the steric and electronic properties of the side chain. The modification is specific for a subset of proteins and a subset of tyrosine residues within a given protein and can alter protein conformation and lead to either a gain or loss of protein function.[3−5] Moreover several investigators have reported denitrase activities, raising the intriguing possibility that nitration is a reversible posttranslational modification that links nitric oxide signaling to protein function.[6,7]

While many investigators initially assumed that the treatment of cells or proteins with nitrating agents in vitro produced protein nitration patterns with in vivo relevance, the definition of nitrated residues in postmortem tissue[5,8] demonstrates that this is not a generally valid assumption. An understanding of the functional relevance of in vivo nitration therefore requires a general method to synthesize proteins containing the 3-NT modifications observed in vivo (Figure 1). Here we report the evolution and characterization of an aminoacyl-tRNA synthetase/tRNA pair for the cotranslational, site-specific incorporation of 3-NT into proteins at genetically encoded sites. To demonstrate the utility of our approach for studying the effect on protein function of nitration on sites defined in vivo, we prepared manganese superoxide dismutase (MnSOD) (that is homogeneously nitrated at a site known to be modified in disease-related inflammatory responses) and measured the effect of this defined modification on protein function.

Experimental Methods

General Methods. Chemical reagents and L-3-nitrotyrosine (3-NT) were purchased from Sigma−Aldrich and Fisher Scientific and used without further purification. Oligonucleotides, DH10B cells, and pTrcHisA were purchased from Invitrogen. Antibodies to 3-NT were purchased from Sigma (catalogue no. N0409), and penta-His-antibodies were from Qiagen (catalogue no. 34660).

Construction of Plasmids. pSUP 3NT plasmids were generated by excising the MjYRS gene from the pBK plasmids isolated from the library by use of restriction enzymes *Nde*I and *Pst*I. The DNA fragments were cloned into the respective sites on the pSUP plasmids. The MnSOD gene was amplified from a cDNA clone (RZPD,

[†] Medical Research Council Laboratory of Molecular Biology.
[‡] Franklin & Marshall College.
(1) Aulak, K. S.; Koeck, T.; Crabb, J. W.; Stuehr, D. J. *Methods Mol. Biol.* **2004**, *279*, 151−65.
(2) Kanski, J.; Hong, S. J.; Schoneich, C. *J. Biol. Chem.* **2005**, *280*, 24261−6.
(3) Radi, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4003−8.
(4) Ischiropoulos, H. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 776−83.
(5) Schopfer, F. J.; Baker, P. R.; Freeman, B. A. *Trends Biochem. Sci.* **2003**, *28*, 646−54.
(6) Irie, Y.; Saeki, M.; Kamisaki, Y.; Martin, E.; Murad, F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5634−9.
(7) Koeck, T.; Fu, X.; Hazen, S. L.; Crabb, J. W.; Stuehr, D. J.; Aulak, K. S. *J. Biol. Chem.* **2004**, *279*, 27257−62.

(8) Sharov, V. S.; Galeva, N. A.; Kanski, J.; Williams, T. D.; Schoneich, C. *Exp. Gerontol.* **2006**, *41*, 407−16.
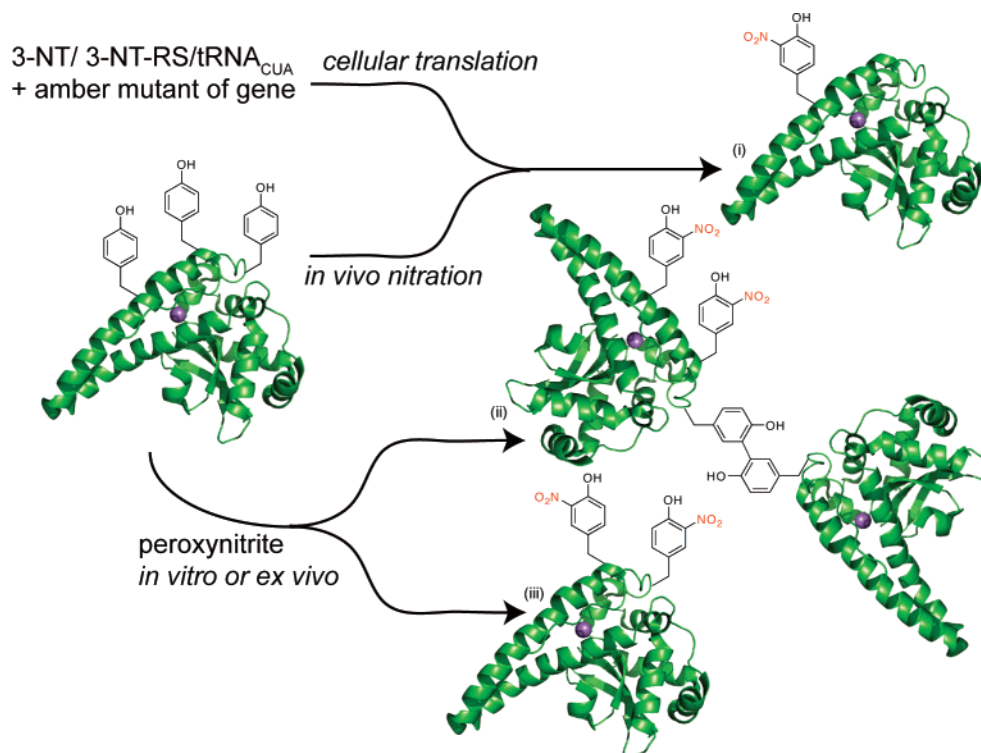
Figure 1. Genetic incorporation of 3-nitrotyrosine (3-NT) and in vivo vs ex vivo and in vitro nitration products. In vivo radicals derived from NO lead to specific nitration of tyrosine residues (i). In vitro or ex vivo nitration leads to a mixture of nitrated proteins (ii, iii). For some proteins the sites nitrated in vivo are nitrated in vitro with peroxynitrite, but other sites are also nitrated in vitro and a mixture of nonresolvable damaged proteins [of which only one is illustrated (ii)] is produced, which also includes dityrosine cross-linked proteins (ii). For other proteins the tyrosine residues nitrated in vivo are not nitrated under in vitro or ex vivo nitration conditions (iii). Cellular translation with evolved translational components allows genetic encoding of the exact nitration (i), for which evidence exists from in vivo samples. The structure figure was created by use of Pymol (www.pymol.com), with the structure of an MnSOD monomer (PDB ID 2ADQ). The tyrosine and 3-nitrotyrosine side chains shown are not to scale and their positions have been altered for illustrative purposes.

IRBPp993F035D) by use of primers MnSODf (5′-ATG GCT AGC aag cac agc ctc cct gac ctg-3′) and MnSODr (5′-TTC GAA TTC tca ctt ctt gca aac tat gta tc-3′). The PCR product was cloned into the *Nhe*I and *Eco*RI sites of pTrcHisA. The amber codon at position 34 was introduced by Quickchange (Stratagene) by use of primers MnSODY34TAGf (5′-cca cgc gac cta Ggt gaa caa tct gaa cgt cac cg-3′) and MnSODY34TAGr (5′-cag att gtt cac Cta ggt cgc gtg gtg ctt gct gt-3′), giving plasmid pTrcMnSOD34 TAG).

Selection of 3-NT-Specific Aminoacyl-tRNA Synthetase.[9,10] The library of tRNA-aminoacyl synthetases was encoded on a kanamycin- (Kn-) resistant plasmid (pBK-3D-Lib) under control of the constitutive *Escherichia coli* GlnRS promoter and terminator. In the aminoacyl synthetases library (3D library) Leu65, His70, Gln155, and Ile159 were randomized to all 20 natural amino acids, Tyr32 was randomized to 15 natural amino acids (less Trp, Phe, Tyr, Cys), Asp158 was restricted to Gly, Ser, or Val, Leu162 was restricted to Lys, Ser, Leu, His, and Glu and Phe108 and Gln108 were restricted to the following pairs: Trp-Met, Ala-Asp, Ser-Lys, Arg-Glu, Arg-Pro, Ser-His, and Phe-Gln. The library plasmid, pBK-3D-Lib, was moved between cells containing a positive selection plasmid (pCG) and cells containing a negative selection plasmid (pNEG).

The positive selection plasmid pCG, (∼10 000 bp) encodes a mutant tRNA$^{Tyr}_{CUA}$, a TAG stop codon-disrupted chloramphenicol acetyltransferase, an amber-disrupted T7 RNA polymerase that drives the production of green fluorescent protein, and a tetracycline- (Tet-) resistant marker. The negative selection plasmid pNEG (∼7000 bp) encodes a mutant tRNA$^{Tyr}_{CUA}$, an amber-disrupted barnase gene under

control of an arabinose promoter and rrnC terminator, and the ampicillin- (Amp-) resistant marker. pCG electrocompetent cells and pNEG electrocompetent cells were made from DH10B cells carrying the respective plasmids and stored in 100 μL aliquots at −80 °C for future rounds of selection.

The synthetase library was transformed by electroporation into DH10B *E. coli* containing the Tet-resistant positive selection plasmid pCG. The resulting pCG/pBK-3D-Lib containing cells were amplified in 1 L of 2× YT with 25 μg/mL kanamycin and 25 μg/mL tetracycline with shaking at 37 °C. The cells were grown to saturation and then pelleted at 5525 rcf, resuspended in 30 mL of 2× YT and 7.5 mL 80% glycerol, and stored at −80 °C in 1 mL aliquots for use in the first round of selections.

For the first positive selection, 1 mL of pCG/pBK-3D-lib cells was thawed on ice before addition to 1 L of room-temperature 2× YT containing 25 μg/mL Kn and 25 μg/mL Tet. After incubation (12 h, 250 rpm, 37 °C), four 1 mL aliquots were pelleted (1000 rcf, 5 min), resuspended in 100 μL of 1.25% glycerol, combined, and diluted to 500 μL with 1.25% glycerol. A 50 μL aliquot of these cells was plated on 10 15-cm GMML−agar plates containing 50 μg/mL Kn, 12 μg/mL Tet, 60 μg/mL chloramphenicol (Cm), and 1 mM 3-NT. After the mixture was spread, the surface of the plates was allowed to dry completely before incubation at 37 °C for 3−4 days. In order to minimize surface reactions of 3-NT with air, plates containing 3-NT were poured within 15 min of plating and were prevented from drying prior to plating by keeping the plates closed. Once 3-NT was dissolved in solution, all work was done in the dark. To harvest the surviving library members from the plates, 10 mL of 2× YT (25 μg/mL Kn, 25 μg/ mL Tet) was added to each plate. Colonies were scraped from the plate with a glass spreader. The resulting solution was incubated (30

---

(9) Xie, J.; Schultz, P. G. *Methods* **2005**, *36*, 227−38.
(10) Xie, J.; Schultz, P. G. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 775−82.

min, 37 °C) to wash cells free of agar. The cells were then pelleted and plasmid DNA was extracted. For the first positive selection and first negative selection, a Qiagen Midiprep Kit was used to purify the plasmid DNA. For all other plasmid purification steps a Qiagen Miniprep Kit was used to purify the plasmid DNA. The smaller pBK-3D-Lib plasmid was separated from the larger pCG plasmid by agarose gel electrophoresis and extracted from the gel using the Qiagen gel extraction kit.

For all subsequent positive selections, 2 μL (or ~50 ng) of purified library DNA was transformed into 100 μL of pCG competent cells. The transformants were rescued for 1.5 h in 1 mL SOC at 37 °C with shaking, pelleted (1000 rcf for 5 min) and resuspended in 1 mL of 1.25% glycerol. A 50 μL sample of these cells was plated on three plates prepared as described in the first positive selection on modified GMML agar plates, but the Cm concentration was raised to 80 μg/mL.

The purified pBK-3D-Lib was then transformed into pNEG-containing DH10B *E. coli*. A 100 μL sample of pNEG electro-competent cells was transformed with 50 ng of purified pBK-3D-Lib DNA by electroporation. Cells were rescued in 1 mL of SOC for 1 h (37 °C, 250 rpm). In the first negative selection, the entire 1 mL of rescue solution was plated on three 15-cm LB agar plates containing 100 μg/mL Amp, 50 μg/mL Kn, and 0.2% L-arabinose. In sub-sequent negative selections, one plate was spread with 250 μL rescued cells and two plates were spread with 50 mL of rescued cells. Plates were grown for 12–16 h at 37 °C. Cells were collected from plates in the same manner as described above for positive selections.

After a total of three rounds of positive and negative selection, remaining pBK-3D-Lib members were transformed into positive selection cells and grown on modified GMML media plates in the presence of 1 mM 3-NT, 100 μg/mL Cm, 50 μg/mL Kn, 12 μg/mL Tet, and 0.002% arabinose. Individual colonies (36) were selected from the surviving library and screened in the same media in the presence and absence of 1 mM 3-NT and varying concentrations of Cm from 0 to 120 μg/mL.

GMML Media Composition. Glycerol based minimal media supplemented with leucine (GMML) was composed of M9 media plus 1.0% glycerol, 300 μM L-leucine, 136 nM $CaCl_2$, 1 mM $MgSO_4$, 4.0 μM D-biotin, 330 nM thiamin, and 1× heavy metal solution where indicated. The 250× heavy metal stock solution contains 500 mg of $MoNa_2O_4\cdot2H_2O$, 250 mg of $CoCl_2$, 175 mg of $CuSO_4\cdot5H_2O$, 1 g $MnSO_4\cdot H_2O$, 8.75 g $MgSO_4\cdot7H_2O$, 1.25 g $ZnSO_4\cdot7H_2O$, 1.25 g of $FeCl_2\cdot4H_2O$, 2.5 g of $CaCl_2\cdot2H_2O$, and 1 g of H_3BO_3 per 1 L. Minimal medium plates contained the same ingredients plus 2.25% dry agar. Plates were assembled by autoclaving a concentrated agar and glycerol solution (9 g of agar in 400 mL of 1.25% glycerol) and adding sterile stock solutions of the remaining ingredients.

Expression and Purification of MnSOD containing 3-NT. *E. coli* BL21 DE3 was transformed with pSUP 3NT/8 and pTrcMnSOD(34 TAG). The cells were grown overnight at 37 °C in LB in the presence of 100 μg/mL Amp and 25 μg/mL Cm. M9 medium (1 L) supplemented with the same antibiotics, 2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.5 mM 3-NT was inoculated with the pellet from a 40-mL overnight culture. The cells were grown overnight, 1 mM phenyl-methanesulfonyl fluoride (PMSF) was added, and protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG). After 4 h at 37 °C, cells were harvested and washed with phosphate-buffered saline (PBS). Proteins were extracted by shaking at 25 °C in 30 mL of BugBuster (Novagen) supplemented with protease inhibitor cocktail (Roche), 1 mM PMSF, and ca. 1 mg/mL lysozyme. The extract was clarified by centrifugation (15 min, 2500 g, 4 °C) and supplemented with 20 mM imidazole, 50 mM Tris, pH 8.0, and 200 mM NaCl to give 40 mL total volume. Ni^{2+}–NTA beads (0.5 mL) were added to the extract and incubated with agitation for 1 h at 4 °C.

Beads were poured into a column and washed with 40 mL of 50 mM Tris, 20 mM imidazole, and 200 mM NaCl. Proteins were eluted in 2 mL of the same buffer containing 200 mM imidazole and immediately rebuffered to PBS in a VivaSpin concentrator (10 kDa exclusion limit).

SDS−PAGE and Immunoblotting. Protein samples were analyzed by SDS-PAGE on precast Tris/glycine 4−20% gels (Invitrogen) and either stained with GelCode Blue (Pierce) or transferred to nitrocellulose membrane. The membranes were blocked with 3% bovine serum albumin (BSA) in PBS for 30 min before addition of the antibody (anti-3-NT or anti-penta-His) at 1:500 dilution. After being shaken at ambient temperature for 3 h, the membranes were washed four times with PBS. HRP-coupled secondary antibodies [goat anti-rabbit for 3-NT or goat anti-mouse for penta-His (both Sigma)] were added 1:5000 in 3% BSA in PBS and incubated for 1 h with shaking. After being washed four times with PBS, proteins were detected by chemoluminescence using the enhanced chemiluminescence (ECL) detection kit (Amersham).

SOD Assay. SOD activity was determined by use of the SOD assay kit−WST from Fluka. WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction by superoxide anion. The rate of reduction is linearly related to the superoxide anion concentration (which is produced by xanthine oxidase from O_2 and xanthine). SOD competes with this reaction by disproportionating superoxide anion into O_2 and hydrogen peroxide. Therefore, high SOD activity results in decreased reduction of WST-1 and can be measured by comparing to samples of known concentration. In this assay one unit will, by definition, inhibit reduction of cytochrome *c* by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25 °C in a 3.0 mL reaction volume.

An aliquot (20 μL) of each sample was mixed with 200 μL of WST working solution, and the reaction was started by addition of 20 μL of enzyme working solution. The reactions were done in clear flat-bottom 96-well microtiter plates and incubated for 20 min at 37 °C. Absorbance at 450 nm was measured with a Spectramax microtiter plate reader (Molecular Devices). OD_{450} values of samples of known SOD activity (0.5−10 units mL^{-1}, Sigma S2515-3KU) were plotted against the logarithm of their activity and analyzed by linear regression. The SOD activity of unknown samples was then calculated from the observed OD_{450} values by use of the parameters obtained from linear regression analysis of the standard samples. In each experiment, reactions were done in quintuplicate. At least three independent experiments were performed.

ESI-TOF Mass Spectrometry. Proteins rebuffered in 10 mM ammonium carbonate, pH 7.5, were mixed 1:1 with 1% formic acid in 50% methanol. Total mass was determined on an LCT time-of-flight mass spectrometer with electrospray ionization (Micromass). Samples were injected at 10 mL min^{-1} and calibration performed in positive ion mode with horse heart myoglobin. Scans (60−90) were averaged and molecular masses were obtained by deconvoluting multiply charged protein mass spectra with MassLynx version 4.1 (Micromass).

MALDI MS/MS Sample Preparation. All protein samples processed for MS analysis were processed in parallel. Native and 3-NT-labeled proteins were overproduced in *E. coli*, affinity-purified on Co^{2+} resin to greater than 95% purity (silver stain SDS−PAGE), and then eluted into pH 7 buffer containing 50 mM sodium phosphate, 300 mM sodium chloride, and 150 mM imidazole. The samples were then buffer-exchanged into 20 mM ammonium acetate by gel filtration. The samples were analyzed at the Franklin & Marshall Mass Spectrometry Facility on their ESI-Q-TOF Ultima. Tryptic digestions were performed with dried samples of the same protein. Samples were dissolved in 100 mM ammonium hydrogen carbonate (1 μg/μL) for digestion with sequencing-grade trypsin (24 h) and then dried. The samples were checked by SDS−PAGE and LCMS for full digestion. Each sample was dried and analyzed.
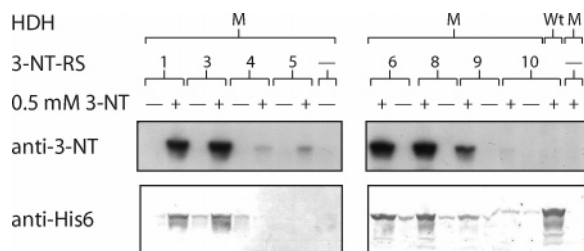
Figure 2. Production of HDH containing 3-NT, using evolved 3-nitrotyrosinyl-tRNA synthetase/tRNACUA pairs. Proteins were extracted from *E. coli* containing pTrc HDH(225 TAG) (labeled M) or pTrc HDH(Wt) (labeled Wt) and pSUP 3NT RS (labeled 3NT-RS) and grown in the presence and absence of 3-nitrotyrosine (3-NT), as indicated, by boiling a cell pellet from 1 mL of culture in sample buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and detected by anti-3-NT (upper panel) or anti-His (lower panel).

Results

To evolve an aminoacyl-tRNA synthetase for the site-specific incorporation of 3-NT, we began with a modified *Methanococcus jannaschii* (*Mj*) tyrosyl-tRNA synthetase/tRNA$_{CUA}$ pair,[9,10] which directs the incorporation of tyrosine in response to the amber (UAG) codon. This pair is orthogonal to the endogenous aminoacyl-tRNA synthetases and tRNAs in *E. coli*: *Mj* tyrosyl-tRNA synthetase does not appreciably aminoacylate endogenous tRNAs and *Mj* tRNA$_{CUA}$ is not aminoacylated by endogenous aminoacyl-tRNA synthetases. We used a library of the gene for this synthetase in which codons corresponding to nine amino acid residues (Tyr32, Leu65, His70, Phe108, Gln109, Gln155, Asp158, Ile159, and Leu 162) within 7 Å of the bound tyrosine in the active site were randomized.[9] The synthetase library was subjected to three rounds of alternating positive and negative selections in the presence and absence of 3-NT, respectively, essentially as previously described.[10,11] Thirty-six of the resulting aminoacyl-tRNA synthetase clones were isolated and transformed into cells bearing pREP2-JYCUA, which contains *Mj* tRNA$_{CUA}$ and confers amber suppressor-dependent expression of green fluorescent protein and chloramphenicol acetyltransferase on *E. coli*. Ten synthetase clones showed amino acid-dependent expression of both reporters, with the best clones surviving on chloramphenicol concentrations of 120 μg mL^{-1} in the presence of 3-NT but less than 10 μg mL^{-1} in the absence of 3-NT.

To further characterize the incorporation of 3-NT into proteins in response to the amber codon, we cloned the 10 most active synthetase clones into a pSUP vector[12] containing six copies of *Mj* tRNA$_{CUA}$. The resulting pSup-3NT plasmids (pSUP-3NT/1 to pSUP-3NT/10) were tested for 3-NT incorporation in response to an amber codon in histidinol dehydrogenase (HDH)[13] (Figure 2). The pSup-3NT plasmids that produced the most protein in the presence of 3-NT and no protein in the absence of 3-NT were sequenced, revealing four distinct clones (Table 1). The best clone, pSup-3NT/8, was used for all subsequent protein expression.

To produce a recombinant protein with nitration at a site defined from studies of postmortem tissue, we cotransformed

(11) Chin, J. W.; Martin, A. B.; King, D. S.; Wang, L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11020–4.
(12) Ryu, Y.; Schultz, P. G. *Nat. Methods* **2006**, *3*, 263–5.
(13) Farrell, I. S.; Toroney, R.; Hazen, J. L.; Mehl, R. A.; Chin, J. W. *Nat. Methods* **2005**, *2*, 377–84.

Table 1. 3NT-RS Sequences

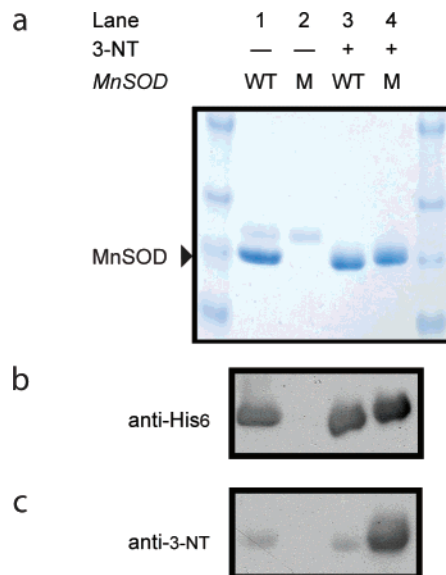|	residue no.								
	32	65	70	108	109	155	158	159	162
*Mj*YRS	Y	L	H	F	Q	Q	D	I	L
3NT-1	E	N	T	F	Q	Q	S	T	L
3NT-3	K	A	M	F	Q	Q	G	M	R
3NT-6	K	K	S	A	D	Q	G	Y	S
3NT-8	R	L	L	F	Q	M	G	L	H

Figure 3. An evolved 3-nitrotyrosinyl-tRNA synthetase/tRNACUA pair efficiently and specifically incorporates 3-nitrotyrosine in response to an amber stop codon in MnSOD. (a) MnSOD was expressed from plasmids pTrcMnSOD (WT) or pTrcMnSOD(34 TAG) (M) in the presence of pSUP3NT/8 and in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 0.5 mM 3-NT. Proteins were purified by Ni^{2+}–NTA affinity chromatography, analyzed by SDS–PAGE, and stained with Coomassie blue. In parallel experiments, proteins were purified and separated as described for panel a but were transferred to nitrocellulose and detected with an anti-His$_6$ antibody (b) or an anti-3-NT-antibody (c).

pSup-3NT/8 with pTrcMnSOD(34 TAG), which contains the gene encoding rat mitochondrial MnSOD with an amber codon at position 34 and an N-terminal hexahistidine tag. MnSOD is known to undergo near-quantitative nitration on tyrosine 34 in cardiovascular disease and aging and is also nitrated in acute and chronic inflammatory processes in both animal models and human disease.[14–16] In the presence of 3-NT, full-length MnSOD His$_6$-tagged protein was purified via Ni–NTA chromatography with a yield of 2 mg (L of culture)$^{-1}$, while no MnSOD was purified in the absence of amino acid (Figure 3). To further confirm the incorporation of 3-NT, we compared the mass of wild-type His$_6$-MnSOD with that of MnSOD produced from pTrcMnSOD(34 TAG) in the presence of 3-NT and pSUP-3NT/8 (Figure 4). The difference in mass observed

(14) Xu, S.; Ying, J.; Jiang, B.; Guo, W.; Adachi, T.; Sharov, V.; Lazar, H.; Menzoian, J.; Knyushko, T. V.; Bigelow, D.; Schoneich, C.; Cohen, R. A. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *290*, H2220–7.
(15) MacMillan-Crow, L. A.; Crow, J. P.; Kerby, J. D.; Beckman, J. S.; Thompson, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11853–8.
(16) MacMillan-Crow, L. A.; Crow, J. P.; Thompson, J. A. *Biochemistry* **1998**, *37*, 1613–22.
(17) Geoghegan, K. F.; Dixon, H. B.; Rosner, P. J.; Hoth, L. R.; Lanzetti, A. J.; Borzilleri, K. A.; Marr, E. S.; Pezzullo, L. H.; Martin, L. B.; LeMotte, P. K.; McColl, A. S.; Kamath, A. V.; Stroh, J. G. *Anal. Biochem.* **1999**, *267*, 169–84.
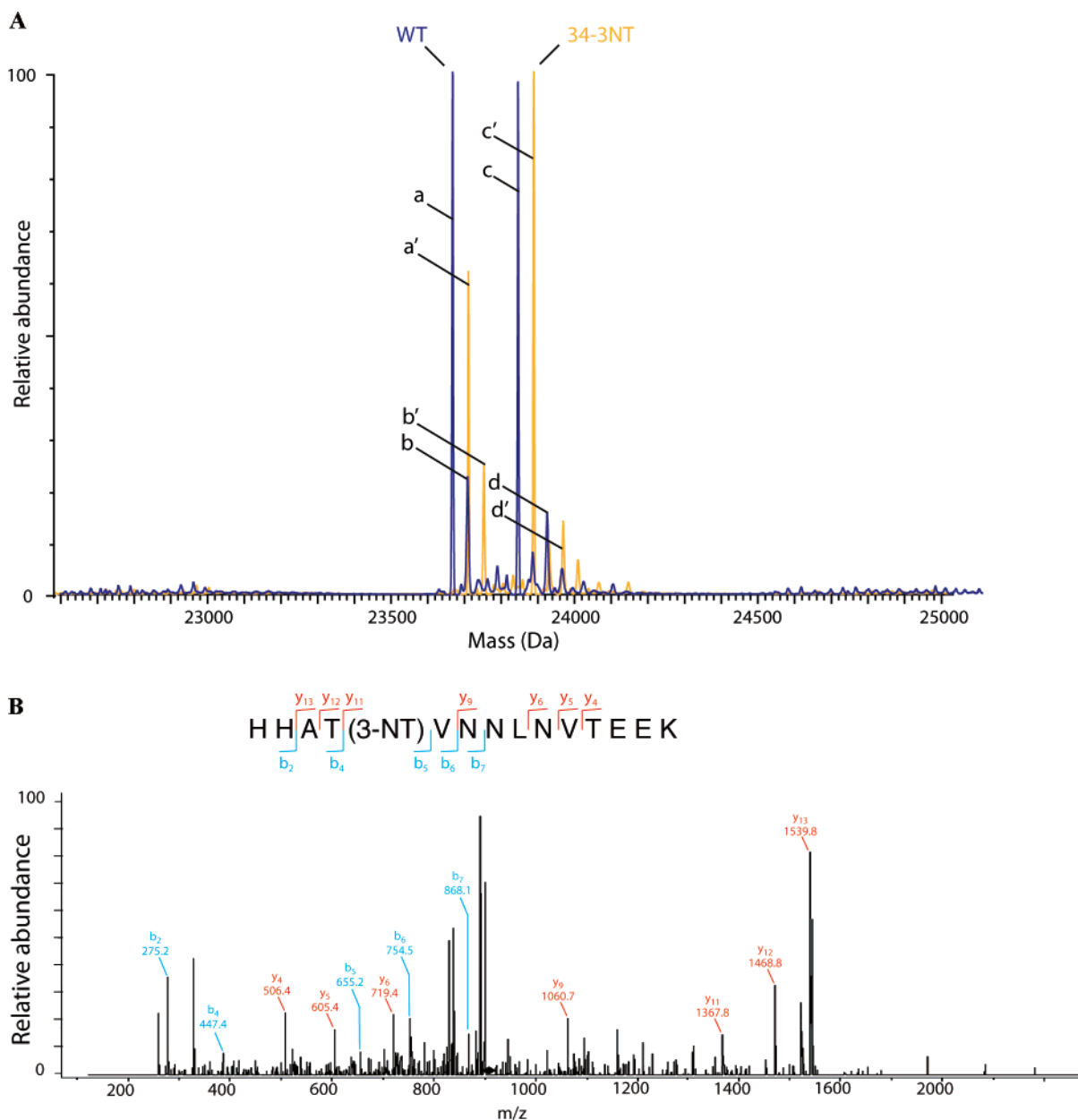
Figure 4. ESI-MS and MS/MS analysis of MnSOD demonstrates the efficient and site-specific incorporation of 3-nitrotyrosine in response to the amber stop codon. (A) ESI-MS TOF analysis of MnSOD(Wt) (blue) shows two major peaks at 23 667 (labeled a) and 23 846 Da (labeled c). The first corresponds to the wild-type protein lacking the N-terminal methionine residue (predicted: 23 661.7 Da); the second is caused by posttranslational α-N-D-gluconoylation of MnSOD[17] (Wt) ($\Delta m = 178$ Da). The spectrum of MnSOD(34 3-NT) (gold) shows two major peaks, a′ and c′, that are shifted by 44 Da from the a and c peaks in the MnSOD(Wt) spectrum due to the nitration of tyrosine-34 as expected. No peaks in the spectrum for MnSOD(34 3-NT) correspond to a protein that has a natural amino acid incorporated in response to the amber codon. Minor peaks in the MnSOD(Wt) and MnSOD(34 3-NT) spectra also differ by 44 Da (b and b′, d and d′). (B) MS/MS fragmentation of tryptic peptides derived from MnSOD(34 3-NT). The spectra confirm 3-NT incorporation at codon 34. The fragmentation sites are illustrated above the spectrum.

between these two proteins (44 ± 3 Da) corresponds to a single nitro group, confirming the incorporation of a single nitrotyrosine residue; this was further confirmed by anti-3NT Western blot. The site of 3-NT incorporation was confirmed by analysis of the tandem mass spectrometry (MS/MS) fragmentation series of the relevant tryptic peptide (Figure 4). To further demonstrate that there is no incorporation of 3-NT in response to sense codons in the gene, we performed Western blots with anti-nitrotyrosine antibodies on protein produced from pTrcMnSOD in the presence and absence of 3-NT (Figure 3). Any misincorporation would lead to greater immunoreactivity of the

protein produced in the presence of 3-NT. The identical immunoreactivity of wild-type MnSOD produced in the presence and absence of 3-NT (compare Figure 3, lanes 1 and 3) demonstrates that there is no measurable misincorporation of 3-NT in response to sense codons. Overall protein expression, MS, MS/MS, and immunoreactivity demonstrate the high-fidelity, site-specific incorporation of 3-NT at genetically programmed sites in MnSOD with no incorporation at nonprogrammed sites.

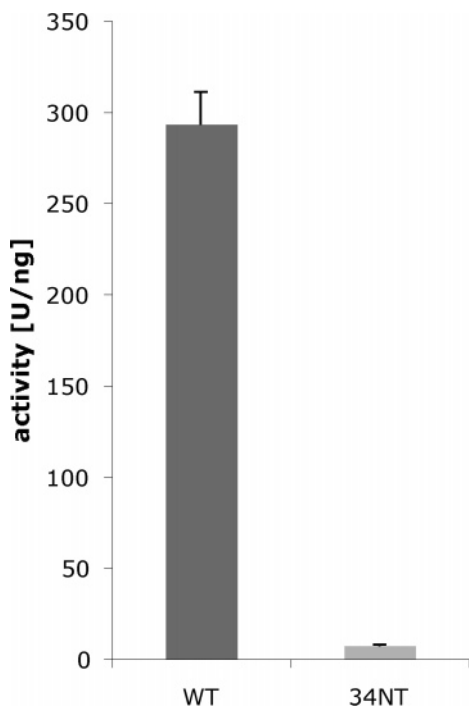To assess the effect of specific nitration of tyrosine 34 on SOD, we measured the ability of wild-type MnSOD and the

Figure 5. Nitration of tyrosine 34 of MnSOD regulates its catalytic activity. MnSOD(Wt) and MnSOD(34 3-NT) were expressed and purified under identical conditions and assayed for their catalytic activity. The specific activity was calculated by comparison to a known standard. The data shows the mean of 12 independent experiments. Error bars represent the standard deviation.

mutant containing 3-NT at position 34 to disproportionate the superoxide anion to oxygen gas and hydrogen peroxide using a coupled assay as described under Experimental Methods (Figure 5). Our results demonstrate that quantitative nitration of tyrosine 34 of MnSOD alone is sufficient to ablate at least 97% of its superoxide dismutase activity, in agreement with previous work.[15,16]

Conclusions

In summary, we have described a method for genetically encoding the protein oxidative damage adduct 3-NT at genetically defined sites in proteins. We have used this method to make MnSOD containing a Tyr to 3-NT mutation at position 34. Since our method is genetic and site-specific, it uncouples 3-NT incorporation from other forms of oxidative damage and therefore does not lead to dityrosine cross-links, nitration at nonprogrammed sites, or other artifactual oxidative damage that is commonly associated with in vitro nitration methods.[4] The synthesis of proteins containing chemically well-defined oxidative damage products, at sites defined from studies on post-mortem tissue, as reported here, should provide a new level of clarity in studies that aim to address the effects of protein oxidative damage on protein and cellular function.