Addition of N\(^a\)-acetyl-lysine to the Genetic Code of *Escherichia coli*
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Supplementary Figure 1: Purified His6 MnSOD and His6 MnSOD (K44AcK). 800 ng of His6 MnSOD and His6 MnSOD (K44AcK) were resolved on 4-20 % gradient SDS-PAGE. The acetylated protein reproducibly runs slightly slower than the non-acetylated protein, consistent with its decreased positive charge.
Supplementary Figure 2: ESI-MS of His6 MnSOD and His6 MnSOD (K44AcK).
Electrospray ionization mass spectrometry of purified rat mitochondrial His6 MnSOD (blue) and purified rat mitochondrial His6 MnSOD (K44AcK), (orange) was performed as described in the methods. The found and expected masses are as follows: rat mitochondrial His6 MnSOD (Found= 23663.5 +/- 2.5 Da; expected = 23661.7 Da); rat mitochondrial His6 MnSOD (K44AcK), (Found= 23706 +/- 2.5 Da; expected = 23703.74 Da). The mass difference between the two proteins 42.5 +/- 2.5 Da compares well with the expected mass difference of 42 Da.
Supplementary Figure 3: MS/MS Collision induced dissociation of His6 MnSOD (K44AcK)

MS/MS fragmentation of His6 MnSOD (K44AcK) was carried out as described in the methods. The sequence of a tryptic peptide corresponding to residues 30 to 51 of MnSOD (top). K* is acetyl-lysine. The labelled fragmentation spectra are shown (bottom).
Supplementary Figure 4: Activity of His6 MnSOD and His6 MnSOD (K44AcK).

The activity of identical concentrations of His6 MnSOD and His6 MnSOD (K44AcK) were determined using the SOD Assay Kit-WST from FLUKA, as described in the methods. 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. In each experiment reactions were done in quintuplicate. At least three independent experiments were performed. The experiments were performed with three His6 MnSOD and His6 MnSOD (K44AcK) samples from three independent expressions and purifications to account for preparation-to-preparation variability in SOD activity. The error bars represent the standard error. Our experiments do not rule out the possibility that the his-6 tag interacts differently with mutant and wild-type proteins.
Supplementary Methods

The complete sequences of genes and proteins used and created.

A Wildtype proteins

> H6-MnSODrat (MnSOD rat sequence is a translation from Genbank accession number BC070913.1)
MGGSNHHHHHGHYAGKSHFSLPDLPYDGALEPHINAQIIMQLHSHKHYVNNLNVTEEHYH
EALAKGDVTTQVALQPKALFGNGHINHISISIFWNTLSPKGGEPKELLEAIKRDFGSFEEK
FKELTAVSGVQGSGWGLGQNEQGRGLQTAACSNQDPLQGTGTLILGPLIDVWEHAYY
LQYKNVPRDYLKAIWNVENWAVSQRYIVCKK*

>Sperm whale Myoglobin-H6 (derived from Genbank accession number AB217144)
MVLSEGEQVLHƯWAKVEDAVGQGDLIRLFKSHIPETLEKFDRFKHLKTEAENKASE
DLKKHGVTVLALTILKKHHEAELKPLAQSHATKHKIPKYLEFISEAIIHLHSRH
PGDFGADAQGMNKALELFRKDIAAKYKELGYQGSGGHH**

> MbPyLS MS (Translated from Genbank accession number AY273828, protein ID: AAQ19545.1)
MDKKNPLDSLATSGLWMSRTGTLHÚKHHKHEVRKSRSKIYIEMACGDHLVNNRSCTRATARF
RHMYKRTCKCRCSRVDENNFLTRSTESKNSVKVRVSAPKVKAMPKSVRAPKLEN
SVSAKASTNTSVPSVPKTPSNSSVPSAPAPSLTRSLQDLKEASHLPSDEKISLNNAKP
FRELEPELVTRRRKNDPQRQLTNREDYLGKLERDITKFVDFGFLKISPIALAPYVER
MGINNDTELSQIFRVDSKMLRPLMLALTLYNFLRKLRLIPGPKIFEGECYKBRSSDG
KEHHLEFTMNLFRQMSGCTRENEALIKFLAYLDIEIFEVDSMCYVGDDFTLDMHGDL
ELSSAVGVPVSDLREWGDIPWIGAGFLERKLVHGFKNIRASRSEYSNGTNL

B. Modified proteins

> H6-MnSODrat K44acK
MGGSNHHHHHGHYAGKSHFSLPDLPYDGALEPHINAQIIMQLHSHKHYVNNLNVTEEHYH
EALAKGDVTTQVALQPKALFGNGHINHISISIFWNTLSPKGGEPKELLEAIKRDFGSFEEK
FKELTAVSGVQGSGWGLGQNEQGRGLQTAACSNQDPLQGTGTLILGPLIDVWEHAYY
LQYKNVPRDYLKAIWNVENWAVSQRYIVCKK*

> Myoglobin-H6 S4AcK
MVL(AcK)EGEQVLHƯWAKVEDAVGQGDLIRLFKSHIPETLEKFDRFKHLKTEAENKASE
DLKKHGVTVLALTILKKHHEAELKPLAQSHATKHKIPKYLEFISEAIIHLHSRH
PGDFGADAQGMNKALELFRKDIAAKYKELGYQGSGGHH**

> AcKRS-1
MDKKNPLDSLATSGLWMSRTGTLHÚKHHKHEVRKSRSKIYIEMACGDHLVNNRSCTRATARF
RHMYKRTCKCRCSRVDENNFLTRSTESKNSVKVRVSAPKVKAMPKSVRAPKLEN
SVSAKASTNTSVPSVPKTPSNSSVPSAPAPSLTRSLQDLKEASHLPSDEKISLNNAKP
FRELEPELVTRRRKNDPQRQLTNREDYLGKLERDITKFVDFGFLKISPIALAPYVER
MGINNDTELSQIFRVDSKMLRPLMLALTLYNFLRKLRLIPGPKIFEGECYKBRSSDG
KEHHLEFTMNLFRQMSGCTRENEALIKFLAYLDIEIFEVDSMCYVGDDFTLDMHGDL
ELSSAVGVPVSDLREWGDIPWIGAGFLERKLVHGFKNIRASRSEYSNGTNL

Mutations in AcKRS-1: D76G, L266V, L270I, Y271F, L274A, C313F

C. DNA sequences

H6-MnSODrat (from Genbank accession number BC070913.1)
atggggggtctcatcatcatcatcatcatgtatggtagcaagcacacagt
cctccctgaacctgtctcatacactgtgataggccgctgagccgcgtccatattaacgc
cgcaagatcatcgacacgacacggaagacgacacccgaccctgtgacctgacg
aatcataatcactagttgctctctcaacctctgactgaatgctagttgcctagc
gccatataaatccagcagctttttctgcaacaacttgacacttcaaaaggtgtgt
gagacacactaaagctgattccagccctactgtagcacaagcagccgagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
tatcggccgccttcctaaactaggcaccacttcgcaagagaaaccagagctgacagtttctttgacagttacagtgcacag
atgggtctgctgtctgaaggtgaatggcagctggttctgcatgtttgggctaa
agttgaagctgacgtcgctggtcatggtcaggacatcttgattcgactgt
tcaaaacttcctacccgaaatcctgaaaataattcgatggtttcaaaacactctg
taaatcgtagctgaatgaattcctgcaagatctgacatcttgacattacgcacaaggtctggtgcggcgcaatcctaccgattaccggggcagc
ctgtttcgtcatcataaataccgcaa
aacctgcaaacgttgccgtgtgagcgatgaagatatcaacaactttctga
ccctcggaccaccgcgaagcacaacacggcaacactggaagattcaccatggttaacttttgccaaatgggcagcggctgcacccgtgaaaacctggaagcgctgaccaagattcctggattatctggaaatcggacttcgaaattgtgggcgatagctgcatggtgtatggc
gataccctggatattatgcatggcgatctgggaactgagcagcgcggtggtgggtccggttagcctggatcgtgaatggggcattgataaaccgtggattg
gcgccggttttggcctggaacgtctgctgaaagtgatgcatggcttcaaa
aacattaaacgtgcgagccgtaagacagctataacggcattagcacagaaacctgtaa
MbPylT (strain MS, from Genbank accession number AY064401)
gggaacctgatcatgtagatcgaatggactctaaatccgttcagccgggt
tagattccgcggggtttccgcca
AcKRS-1 (mutations relative to MbPylS MS in lower case)
ATGGATAAAAAACCGCTGGATGTGCTGATTAGCGCGACCGGCCTGTGGATGAGCCGTACCGGCACCCTGCATAAAATCAAACATCATGAAGTGAGCCGCAAGCAAAATCTATATtgAAATGGCGTGCGGCGATCATCTGGTGGTGAACAACAGCCGTAGCTGCCGTACCGCGCGTGCGTTTCGTCATCATAAATACCGCAAACCTGCAAACGTTGCCGTGTGAGCGgTGAAGATATCAACAACTTTCTGAGCCCGTAGCACCGAAAGCAAAAACAGCGTGAAAGTGCGTGTGGTGAGCGCGCCGAAAGTGAAAAAAGCGATGCCGAAAAGCGTGAGCCGTGCGCCGAAACCACCCTGGAAAATAGCGTGAGCGCGAAAGCGAGCACCAACACCAGCCGTAGCGTTCCGAGCCCGGCGAAAAGCACCAGGACACAGCGTTCCGGCGTCTGCGGGCACCGAGCCTGACCCGCAGCCAGCTGGATCGTGTGGAAGCGCTGCTGTCTCCGGAAGATAAAATTAGCCTGAACATGGCGAAACCGTTTCGTGAACCTGGAACCGGAACTGGTGACCCGTCGTAAAAACGATTTTCAGCGCCTGTATGGAAATTAAAAGCCCGATTCTGATTCGGCGGAATATGTGGAACGTATGGGCATTAACAACGACACCGAACTGAGCAAAACAAATTTTCCGCGTGGATAAAAACCTGTGCTGCGTCGATGgTtGCACCGACCaTTttTAACTATgctCGTAAACTGGATCGTATTCTGCCGGGTCGATCAAAATTTTTGAAGTGGGCCCGTGCTATCGCAAAGAAAGCGATGGCAAAAGAACACCTGGAAGAATTCACCA
tGGTTAACTTTTttCCAAATGGGCAGCGGCTGCACCCGTGAAAACCTGGAAGCGCTGATCAAAGAATTCCTGGATTATCTGGAAATCGACTTCGAAATTGTGGGCGATAGCTGCATGGTGTATGGCAGTACCCTGGATCTGCTGGAACGTGACTAGCGGCAACAGCGCTGGTGGCCGTCGGACCGTGGAGCCCTGGATCGTGAATGGGGCATTGATAAACCGTGGATTGCGCGGGTTTTGGCCTGGAACGTCTGCTGAAAGTGATGCATGGCTTCAAAACATTAAACGTGCGAGCCGTAGCGAAAGCTACTATAACGGCATTAGCACGAACCTGTAA

Methods

Construction of plasmids

Plasmid pMyo4TAG-PylT encodes a sperm whale myoglobin gene, with codon 4 replaced by an amber codon, under the control of an arabinose promoter. It also contains the PylT gene from Methanosarcina barkeri MS with an lpp promoter and rrnC terminator. pMyo4TAG-PylT was generated by the ligation of two PCR products. One PCR product was generated using pBADJYAMB4TAG1 as template in a PCR reaction that amplified the entire vector except the MjtRNA_CUA gene. This PCR used the primers pMyoNotF (5’-CAA GCG GCC GCG AAT TCA GCG TTA CAA GTA TTA CA-3’) and pMyoPstR (5’-GAC CAC TGC AGA TCC TTA GCG AAA GCT-3’). The second PCR product was generated by amplifying the PylT gene from pREP-PylT using primers PYLTPST13 (5’-GCG ACG CTG CAG TGG AAA CCC CGG GAA TC-3’) and PYLNTOT15 (5’-GGA AAC CGC GCG GCC GCG GGA ACC TGA TCA TGT AGA TCG-3’). The two PCR products were digested with NotI and PstI and ligated with T4 DNA ligase to form pMyo4TAG-PylT.

pMnSOD PylT was created by replacing the myoglobin gene in pMyo4TAG-PylT
with a DNA fragment coding for an N-terminal His$_6$-tag and rat MnSOD [gb:Bc070913.1]. The fragment was obtained by PCR using primers PylTMnSODf (5'-AGG AAT AAA CCA TGG GGG GTT CTC-3') and PylTMnSODr (5'-AGT GGT ACC TCA CTT GCA AAC TAT GTA TC-3') and MnSOD cloned into NheI and EcoRI sites of pTrcHisa as template. The codon for Lys 44 was replaced by an amber stop codon by Quickchange mutagenesis using primers MnSODK44TAGf (5'-GTC ACC GAG GAG TAG TAC CAC GAG GCG CTG GCC AA-3') and MnSODK44TAGr (5'-CCT CGT GGT ACT ACT CCA GCA AAC TAT GTA TC-3') to create pMnSOD (44TAG) PylT.

pREP-PyIT was derived from pREP(2) YC-JYCUA$^{1, 2}$. The MjtRNA$_{CUA}$ gene in pREP(2) YC-JYCUA was deleted by Quickchange mutagenesis (Stratagene) creating unique BglII and SpeI sites downstream of the lpp promoter. This was performed using primers pREPDtf (5'-CTAGATCTATGACTAGTATCCTTTAGCAGAAAGCTAA-3') and pREPDtr (5'-ATACTAGTCATAGCTTAGCGTTACAAAGTATTACA-3'). The Methanosarcina barkeri MS PylT gene was made by PCR from primers pylTbegf (5'-GCT AGA TCT GGG AAC CTG ATC ATG TAG ATC GAA TGG ACT CTA AAT CCG TTC AGC C-3' and pylTendr (5'-GAT ACT AGT TGG CGG AAA CCC CGG GAA TCT AAC CCG GCT GAA CGG ATT TAG AGT C-3') and cloned between BglII and SpeI in the intermediate vector.

pBAR-PyIT (which contains a toxic barnase gene with amber codons at positions Q2 and D44 under the control of an arabinose promoter and Methanosarcina barkeri MS PylIT on an lpp promoter) was derived from pYOBB2 using the same strategy and primers used to create pREP-PyIT from pREP(2) YC-JYCUA.

**Library construction**

The Methanosarcina barkeri MS PylS gene [gb:AAQ19545.1] was codon optimized for E. coli and synthesized (Geneart). The complete sequence of this synthetic gene is available above. This ORF was cloned between the NdeI and PstI sites of pBK-JYRS$^1$ replacing the MjTyrRS gene and producing pBK-PylS. Three rounds of inverse PCR were performed on this template to randomize codons of L266, L270,
Y271, L274, C313 and W383, with the product of one round acting as a template for the next round. The following primers were used in each round of PCR reactions: (round 1) PylSC313f (5′-GCG CAG GAA AGG TCT CAA ACT TTN NKC AAA TGG GCA GCG GCT GCA CCC GTG AAA AC-3′) and PylSC313r (5′-GCG CAG AGT AGG TCT CAA GTT AAC CAT GGT GAA TTC TTC CAG GTG TTC TTT G-3′); (round 2) PylSL266f (5′-GCG CAG GTC TCA CCG ATG NNK GCC CCG ACC NNK NNK AAC TAT NNK CGT AAA CTG GAT CGT ATT CTG CCG GGT C-3′) and PylSL266r (5′-GCG CAG AGT AGG TCT CAT CGG ACG CAG GCA CAG GTT TTT ATC CAC GCG GAA AAT TTG-3′); (round 3) PylSW383f2 (5′-GCG CAG GAA AGG TCT CAA AAC CGN NKA TTG GCG CGG GTT TTG GCC TGG AAC GTC TGC TG-3′) and PylSW383r2 (5′-GCG CAG AGT AGG TCT CAG TTT ATC AAT GCC CCA TTC ACG ATC CAG GCT AAC CGG AC-3′). The enzymatic inverse PCR reactions were prepared in 100µL aliquots containing 1x PCR buffer with MgCl₂(Roche), 200 µM of each dNTP, 0.8 µM of each primer, 100 ng template and 7 U Expand High Fidelity Polymerase (Roche). PCR reactions were run in 50 µl aliquots using the following temperature program: 2 min at 95ºC, 9x(20 sec at 95ºC, 20 sec at 65ºC [-1ºC/cycle], 4 min at 68ºC), 31x(20 sec at 95ºC, 20 sec at 58ºC, 4 min at 68ºC), 9 min at 68ºC.

The purified PCR reactions were digested with DpnI and BsaI, ligated, precipitated and used to transform electrocompetent DH10B cells, as previously described. To increase the number of independent transformants after the last round of enzymatic inverse PCR the precipitated ligation product was amplified with Phi29 DNA polymerase in a 500 µl reaction, as previously described. The final transformation yielded a library of approximately 10⁸ mutants. The quality of the library was verified by sequencing twelve randomly chosen clones, which showed no bias in the nucleotides incorporated at the randomized sites.

**Selection of Nε-acetyl-lysine specific aminoacyl-tRNA synthetases**

*E. coli* DH10B harbouring the plasmid pREP-PylT were transformed with the library of mutant synthetase clones, yielding 10⁹ transformants. Cells were incubated (16 h, 37ºC, 250 r.p.m.) in 100 mL LB, supplemented with 12.5 µg ml⁻¹ tetracycline and 25 µg ml⁻¹ kanamycin (LB-KT). 2mL of this culture was diluted 1:50 into fresh LB-KT
containing 1 mM N\textsuperscript{ε}-acetyl-lysine (Bachem, used without further purification) and incubated (3-4 h, 37°C, 250 r.p.m.). 0.5 ml of the culture was plated onto LB-KT plates (24 cm x 24 cm) supplemented with 1 mM acetyl-lysine and 50 µg ml\textsuperscript{-1} chloramphenicol. After incubation (48h, 37°C) the plates were stripped of cells and plasmids isolated. The synthetase plasmids were resolved from the reporter plasmid by agarose gel electrophoresis and extracted using the Qiagen gel purification kit.

To select against synthetases that direct incorporation of natural amino acids in response to the amber codon plasmids isolated in this positive selection were used to transform DH10B containing plasmid pBar-PylT. After electroporation the cells were recovered (3 h, 37°C, 250 r.p.m.) in SOB medium. Approximately 10\textsuperscript{7} cells were plated onto LB-agar plates (24 cm x 24 cm) supplemented with 0.2% arabinose, 25 µg ml\textsuperscript{-1} kanamycin and 25 µg ml\textsuperscript{-1} chloramphenicol. The plates were incubated for 24 h at 37°C. Cells from the resulting colonies were harvested and the synthetase plasmids isolated as described above.

The third round of selection was performed in the same way as the first, except that instead of harvesting the pool of synthetase plasmids we picked individual colonies and grew these in parallel in 1mL of LB-KT. After overnight growth 200 µL of each culture was diluted 1:10 into fresh LB-KT and divided to give two identical 1 mL cultures derived from a single colony. One culture received 1 mM N\textsuperscript{ε}-acetyl-lysine and the other did not. After incubation (5 h, 37°C, 250 r.p.m.) the cells were pronged onto LB-KT plates with or without 1 mM N\textsuperscript{ε}-acetyl-lysine and containing increasing concentrations of chloramphenicol. Total plasmid DNA was isolated from 24 clones that showed strong N\textsuperscript{ε}-acetyl-lysine dependent chloramphenicol resistance. This DNA was digested with HindIII (which does not digest pBK-PylS, but does digest pREP-PylIT) and used to transform DH10B. To confirm that the observed phenotypes did not result from mutations in the cells genome or mutations in the reporter plasmid cells containing pREP-PylIT were transformed with the isolated pBK-PylS plasmids and tested for their ability to grow on increasing concentrations of chloramphenicol in the presence or absence of 2 mM N\textsuperscript{ε}-acetyl-lysine. Additionally, we analysed for the expression of GFP by scanning plates without chloramphenicol on a Storm Phosphoimager (Molecular Dynamics).
Protein expression and purification

Expression and purification of myoglobin: To express sperm whale myoglobin *E. coli* DH10B was transformed with pBKPy1S, AcKRS-1 or AcKRS-2 and pMyo4TAG-PylT. The cells were incubated (16 h, 37°C, 250 r.p.m.) in LB-KT. 1 liter of LB KT supplemented with 1 mM Nε-acetyl-lysine or Cyc (Sigma, used without further purification) was inoculated with 50 mL of this overnight culture. After 2 h at 37°C the culture was supplemented with 50 mM nicotinamide (Sigma) and grown for another 30 min. Protein expression was induced by addition of 0.2% arabinose. After a further 3 h cells were harvested and washed with PBS. Proteins were extracted by shaking at 25°C in 30 mL BugBuster (Novagen) supplemented with protease inhibitor cocktail (Roche), 1 mM PMSF, 50 mM nicotinamide and approximately 1 mg ml⁻¹ lysozyme. The extract was clarified by centrifugation (15 min, 2500 g, 4°C) and supplemented with 20 mM imidazole, and 50 mM Tris (pH 8.0) to give a total volume of 40 ml. 0.3 ml of Ni²⁺-NTA beads (Qiagen) 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 wash buffer (50 mM Tris, 20 mM imidazole, 200 mM NaCl). Proteins were eluted in 1 ml of wash buffer supplemented with 200 mM imidazole and immediately re-buffered to 10 mM ammonium carbonate (pH 7.5) using a sephadex G25 column. The purified proteins were analysed by 4-20% SDS-PAGE. Western blots were performed with antibodies against the hexahsitidine tag (Qiagen) and Nε-acetyl-lysine (Santa Cruz).

Purification of MnSOD: DH10B cells were transformed with pBK AcKRS-1 and pMnSOD PylT or pMnSOD (44TAG) PylT and grown as above. Cells were resuspended in 30 ml of 50 mM Tris, 20 mM imidazole, 20 mM nicotinamide, 200 mM NaCl pH 8.0 supplemented with protease inhibitors and 1 mg ml⁻¹ lysozyme. Proteins were extracted by sonication (2 min, 30 W, 0°C) and the lysate was clarified by centrifugation (20 min, 4°C, 15000 rpm, SS34 rotor). Ni²⁺-purification of MnSOD was carried out in the same way as for myoglobin. The eluate of the Ni²⁺-purification was loaded onto a Tricorn 75 column (Amersham) equilibrated with 10 mM Tris/HCl pH 7.4, 50 mM NaCl. Proteins were eluted with a flow rate of 0.7 ml min⁻¹ and collected in 0.5 ml fractions. Protein content of the fractions was analyzed by SDS-
PAGE and the fraction with the highest MnSOD content rebuffered to 10 mM NH$_4$HCO$_3$ pH 7.6. Protein concentrations of these samples were measured by BCA assay (PIERCE) and relative concentrations confirmed by densitometry of Coomassie stained gels.

**Mass spectrometry**

Protein total mass was determined on an LCT time-of-flight mass spectrometer with electrospray ionization (ESI, Micromass). Proteins rebuffered to 10 mM ammonium carbonate (pH 7.5) were mixed 1:1 with 1% formic acid in 50% methanol. Samples were injected at 10 ml min$^{-1}$ and calibration performed in positive ion mode using horse heart myoglobin. 60-90 scans were averaged and molecular masses obtained by deconvoluting multiply charged protein mass spectra using MassLynx version 4.1 (Micromass). Theoretical masses of wild-type proteins were calculated using ProtParam ([http://us.expasy.org/tools/protparam.html](http://us.expasy.org/tools/protparam.html)), and theoretical masses for unnatural amino acid containing proteins adjusted manually.

To confirm the site of acetyl-lysine incorporation was as expected we performed tryptic digests and MS/MS fragmentation. 9 µM rat mitochondrial MnSOD in 10 µL 40 mM (NH$_4$)HCO$_3$ was alkylated and digested with trypsin overnight. To obtain the fragment series 10 µL of a 3-fold dilution of the tryptic peptide mixture was desalted and concentrated using a GELoader tip filled with Poros R3 sorbent (Perseptive Biosystems). The bound peptides were eluted with 1 µL of 60 % acetonitrile/ 2 % formic acid directly into a nanospray capillary and then introduced into an API QSTAR pulsar I hybrid quadropole-time-of-flight mass spectrometer (MDS Sciex). Product ion scans were carried out in positive ion-mode and MS survey scans for peptides measured. Selected ions (m/z= 656 4+) were fragmented by collision induced dissociation (CID) with nitrogen in the collision cell and the spectra of the fragment ions produced were recorded in a time-of-flight mass analyzer.

**MnSOD activity assays**

SOD activity was determined using the SOD Assay Kit-WST from FLUKA. WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) produces a watersoluble 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 O₂ and xanthine). SOD competes with this reaction by disproportionating superoxide anion into O₂ 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. 20 µl of each sample was mixed with 200 µl WST working solution and the reaction started by addition of 20 µl 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 using a Spectramax microtiter plate reader (Molecular Devices). OD₄₅₀ values of samples of known SOD activity (0.5-10 U 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 OD₄₅₀ values using the parameters obtained by the linear regression analysis of the standard samples. In each experiment reactions were done in quintuplicate. At least three independent experiments were performed.

References