Supporting information for “The molecular basis for the interaction of an electron transfer protein to a metal oxide surface”

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SUPPORTING EXPERIMENTAL SECTION:

Plasmid and Strain Construction:

To construct I5077, (Table S3), the plasmid coding for MtrF without a lipid anchor, we amplified the pBAD202 backbone and amino acids 25-669 of MtrF from vector LS271 using the "MtrF no lipidation Fwd" and "LS271 upstream-reverse" primers (Table S4). We also amplified the coding sequence of amino acids 1-24 of MtrB using the "MtrB Nterm Fwd and MtrB Nterm Rev" primers using plasmid I5049 as a template. The DNA fragment containing the MtrB N-terminal was joined to the MtrF-vector fragment using the Gibson assembly (New England Biolabs). The Gibson reaction solution was transformed into Mach1 cells, resulting in plasmid I5077 for expressing MtrF.

The I5085, I5083, and I5086 plasmids for expressing single point mutants of MtrF (supplemental Table 3) were constructed by Q5 Site-Directed Mutagenesis Kit (NEB) using the primers listed in Supplemental Table 4 and the I5077 plasmid as a template.

I5087, I5088 and I5089 plasmids that contain three mutations at heme 6-7 region of MtrF were constructed using mutagenesis as described above, the MtrF-3A-F and MtrF-3A-R primers and I5083 plasmid as a template (for creating I5087), with MtrF-3D-F and MtrF-3D-R primers and I5085 plasmid as a template (for creating I5088), and with MtrF-3K-F and MtrF-3K-R primers and I5086 plasmid as a template (for creating I5089), respectively.

The I5090, I5091 and I5092 plasmids that contain mutation(s) at heme10 region of MtrF were constructed by a further round of mutagenesis using MtrF-D609A-F and MtrF-AD608-R primers and I5077 plasmid as a template (for creating I5090), using MtrF-A608D-F and MtrF-AD608-R primers and

I5077 plasmid as a template (for creating I5091), and with using MtrF-KK608-F and MtrF-AD608-R primers and I5077 plasmid as a template (for creating I5092), respectively.

The I5095 plasmid contains, which contains five mutations, was constructed by subsequent rounds of mutagenesis using the MtrF-KK608-F and MtrF-AD608-R primers and I5092 plasmid as a template. All the resulting strains are listed in Table S3.

The conjugation method was referred from the Cornell iGEM 2012 Protocol (http://2012.igem.org/Team:Cornell/protocols). After the *E. coli* WM3064 transformants had been incubated in 2×YT containing 300 μM diaminopimelic acid (DAP) at 37 °C overnight, the cells were harvested from 500 or 1,000 μL of the culture by centrifugation and then washed the cells using 2×YT. The cells were resuspended in 150 μL of culture of *S. oneidensis* MR-1 that was incubated at 30 °C overnight and the resuspension was then incubated at 30 °C on a LB plate containing 300 μM DAP. The conjugated cells, *S. oneidensis* MR-1 harboring those plasmids, were isolated by incubation at 30 °C on a LB plate with 50 μg/mL kanamycin without DPA.

MtrF Expression, Purification, and Characterization:

S. oneidensis strains (MFm029 and MFm044-054) possessing a plasmid for expressing wild-type or its mutated MtrF were pre-incubated with 50 μg/mL kanamycin in 2×YT medium or on a LB plate at 30 °C overnight. The cells were inoculated in 1 L of terrific broth containing 50 μg/mL kanamycin and incubated at 30 °C. One mM arabinose was added to induce the wild-type or its point-mutated MtrF expression when the growth reached mid-log phase (normally after 5-6 hrs-incubation) and the cells were then incubated at 30 °C for 16 hrs. The culture was centrifuged and the supernatant was collected (Since the MtrF proteins are fused with the MtrB signal sequence in order to export the proteins in the culture, the expressed MtrF proteins should be in the culture). Ammonium sulfate (70% saturation) was added in

the culture and the proteins including MtrF were precipitated by centrifugation. The precipitant was solubilized in HEPES-NaOH buffer (20 mM HEPES-NaOH [pH 7.8], 150 or 300 mM NaCl, 2.5 mM β-mercaptoethanol) and the solution was dialyzed against the HEPES-NaOH buffer. The dialyzed solution containing MtrF was used for purification of the MtrF protein by affinity chromatography using a nickel-agarose resin column. After the purified protein had been concentrated using an Amicon Spin Filter (EMD Millipore), it was dialyzed against the HEPES-NaOH buffer containing 10% (v/v) glycerol or no salt buffer (5 mM MOPS-NaOH [pH 7.0], 10% [v/v] glycerol, 2.5 mM β-mercaptoethanol) and then stored at -20 °C. Purity of all the proteins was confirmed by SDS-PAGE (Fig. S1B and S6A).

Enhanced Chemiluminescence Assays were performed by first running SDS-PAGE using 10-40 pmol (1-3 μg) of the wild-type and its mutated MtrF protein solutions. The proteins in the SDS-gel were transferred onto a nitrocellulose membrane and the heme staining assay was then performed using ECL kit (Pierce Pico West Enhanced Chemiluminescence substrate [Thermo Scientific]) as described previously[1]. The single band stained by ECL kit was detected from the purified protein solutions (Fig. S1C and S6B).

Molecular weight measurements by ESI-MS were done performed on a wild-type MtrF solution that was dialyzed against 10 mM ammonium acetate buffer (pH 6.8). ESI-MS was performed using an Agilent 1200 series liquid chromatograph (Agilent Technologies, USA) connected in-line with an Agilent 6224 TOF LC-MS system with a Turbospray ion source[2].

Redox activity of wild-type MtrF and MtrF mutant proteins was measured by UV-Visible spectroscopy on a Perkin Elmer Lambda 850 spectrophotometer. Protein solutions (0.5-1 μM MtrF) under oxidizing and reducing conditions were analyzed as previously described[3]. The protein concentrations were determined using the value of 552 nm under reducing condition. The number of extinction coefficient (30,000 $M^{-1}cm^{-1}$ per heme)[4] was used for the determination.

Protein stability assessment at several pHs:

MtrF solution (final conc., 0.7 μM) is dissolved in several buffers, 50 mM sodium acetate (pH 3.0 and 4.0), 50 mM MES-NaOH (pH 6.0), 50 mM HEPES-NaOH (pH 8.0) and then kept at room temperature for 24 hrs. The protein solution was centrifuged (10,000 g, 10 min) to remove aggregated (insoluble) proteins. The supernatant was collected and spectrum of the supernatant was measured by UV-Visible absorption spectroscopy (Fig. S1E). As a control sample, spectrum of the MtrF solution (final conc., 0.7 μM) dissolved in 50 mM MOPS-NaOH buffer (pH 7.0) without any treatment was also measured (Fig. S1E).

Electron Microscopy:

Solutions of α-Fe_2O_3 were diluted by five-fold into Milli-Q water, and 8 μL of this dilution was immediately pipetted onto 400 Cu mesh grids, with a thin carbon film (Ted Pella Inc., Redding, CA). After incubating for 2 minutes at room temperature on the grid, the drop was removed by absorbing the liquid with filter paper. The grid was washed twice by pipetting Milli-Q water on the surface, then removing excess liquid with filter paper. Samples were imaged on a FESEM ULTRA 55 electron microscope (Carl Zeiss Microscopy, Thornwood, NY) in STEM mode at a voltage of 30 kV. Images were captured using SmartSEM software. Length measurements were performed using NIH ImageJ software (http://imagej.nih.gov/ij/index.html) v1.47a[5] on 90 single particles. The histogram of nanoparticle diameters was plotted and fit to a Gaussian distribution in Origin 8.5.0.

Fe_2O_3 and Al_2O_3 nanoparticles:

α-Fe_2O_3 and α-Al_2O_3 were obtained from Sigma-Aldrich (#544884 and #702129) and used for binding study of MtrF. The manufacturer reports the surface area per mass of the Fe_2O_3 nanoparticles as 5-245 m^2/g as measured by BET analysis. We use the average surface area per mass of 147.5 m^2/g in the

calculations of K_{abs} and $\Delta G^{o\prime}$. Given the wide range of reported surface area, using only the average value may introduce a systematic error in the calculations of K_{abs}, however it will not affect the relative changes in K_{abs}.We also found that using the upper and lower limits of the surface area per mass, i.e. 50 m^2/g and 245 m^2/g, affected the calculated value $\Delta G^{o\prime}$ by ±3kJ mol^{-1}, which is within the uncertainty of our experimental results.

The surface area of the α-Fe_2O_3 nanoparticles is 50-245 m^2/g. Thus, the average of surface area size, 147.5 m^2/g, was used for calculation of binding ability of MtrF. The average size of the α-Fe_2O_3 nanoparticles was determined from STEM images to be 27 ± 19 nm (Fig. S2A and B). Value of the surface area of the α-Al_2O_3 nanoparticles was calculated using the density (0.79 g/cm^3) and the diameter, 50 nm since the particle size is less than 50 nm. As a result the calculated surface area is 145 m^2/g. The molars of α-Fe_2O_3 and α-Al_2O_3 nanoparticles are calculated based on the molars of α-Fe_2O_3 (Molecular weight: 159.69) and α-Al_2O_3 (Molecular weight: 101.96). For example if 159.69 g of the α-Fe_2O_3 nanoparticles is added in 1 L of solution, the concentration of the nanoparticles is defined as 1 M.

Monitoring binding between MtrF and nanoparticles:

For fluorescence quenching (FQ) assay, we monitored intrinsic tryptophan and tyrosine fluorescence to probe MtrF binding to α-Fe_2O_3 and α-Al_2O_3. Two microliters of 100 nM wild-type or its point-mutated MtrF protein solution was dissolved in 50 mM sodium acetate buffer (pH 4.0 and 5.0), 50 mM MES-NaOH buffer (pH 6.0), or 50 mM MOPS-NaOH buffer (pH 7.0) and 3-6 mM α-Fe_2O_3 or 10 mM α-Al_2O_3 nanoparticles (Sigma-Aldrich, St. Louis, MO) were added in the protein solution and the resulting fluorescence from tyrosine and tryptophan residues was then measured via fluorimetry (Jobin Yvon Fluoromax, HORIBA Scientific, Kyoto, Japan). The excitation and emission wavelengths were set to 280 nm and 305-380 nm, respectively, each with 5 nm slit widths. The changes in tryptophan fluorescence

(360 nm) upon nanoparticle addition were identical to those in tyrosine fluorescence (310 nm) (data not shown). The FQ data was used for determining K_{ads} and $\Delta G^{o'}$.

We also performed sedimentation assays to monitor MtrF binding to α-Fe_2O_3. Several volumes (from 0 to 240 μL) of 5 mM α-Fe_2O_3 were added in 120 μL (at pH 4) or 200 μL (at pH 7) of 0.5 μM MtrF and the mixture was incubated for 5 min at room temperature to permit binding. The mixture was centrifuged at 10,000 g for 5 min, and then unbound MtrF in the supernatant was separated from MtrF bound to the nanoparticles in the pellet. The UV-visible spectrum of the supernatant was measured and the concentration of MtrF was determined using the Soret peak absorption at 410 nm. The sedimentation assay data was also used for determining K_{ads}.

Determination of the adsorption constant for MtrF with α-Fe_2O_3:

MtrF binding to the α-Fe_2O_3 nanoparticles to form a complex is described by the equilibrium: [MtrF] + [Fe_2O_3] ↔ [MtrF:Fe_2O_3], where [MtrF] is the concentration of unbound MtrF in solution in mol L^{-1}, [Fe_2O_3] is the surface area of Fe_2O_3 nanoparticles in mm^{-2}, and [MtrF:Fe_2O_3] is the concentration of MtrF in mol L^{-1}. Thus, the absorption constant, K_{ads}, is described by:

$K_{ads} = [MtrF:Fe_2O_3]/[MtrF][Fe_2O_3]$ (Equation 1) .

This equation is readily re-arranged to:

$[MtrF:Fe_2O_3] = K_{ads}[MtrF][Fe_2O_3]$ (Equation 2) .

It is convenient to describe the equilibrium in terms of the fraction of MtrF that is bound to the Fe_2O_3,

$\theta_{MtrF} = [MtrF:Fe_2O_3]/[MtrF]_{total}$ (Equation 3),

when the total concentration of MtrF is notated as $[MtrF]_{total}$.

The fraction of unbound MtrF is thus $(1-\theta_{MtrF})$. By substituting equation 2 into equation 3, we arrive at:

$\theta_{MtrF} = K_{ads}[MtrF][Fe_2O_3]/[MtrF]_{total}$ (Equation 4).

Conversation of mass dictates that $[MtrF]_{total} = [MtrF{:}Fe_2O_3] + [MtrF]$, so substituting this expression for $[MtrF]_{total}$ into equation 4, we find that:

$\theta_{MtrF} = K_{ads}[MtrF][Fe_2O_3]/([MtrF] + [MtrF{:}Fe_2O_3])$ (Equation 5).

Substituting Equation 2 in for $[MtrF{:}Fe_2O_3]$ in Equation 5 and canceling out [MtrF] from the numerator and denominator, we arrive at:

$\theta_{MtrF} = K_{ads}[Fe_2O_3]/([1 + K_{ads}[Fe_2O_3])$ (Equation 6).

This equation can be re-written in linear form to give the Scatchard equation,

$\theta_{MtrF}/[Fe_2O_3] = -\theta_{MtrF} \cdot K_{ads} + K_{ads}$ (Equation 7).

The fluorescence quenching data and sedimentation data was used for the calculation of adsorption constants, K_{ads}.

Calculation of $\Delta G^{o'}$:

We also used the Langmuir adsorption isotherm to describe binding of MtrF to the Fe_2O_3 surface, so as to allow calculation of the Gibbs free energy of binding ($\Delta G^{o'}$). In this framework, equilibrium adsorption of a molecule (MtrF) to a solid Fe_2O_3 surface can be described by:

$\Gamma_m = \Gamma_m[MtrF]/([MtrF]+a)$ (Equation 8),

where Γ_m is the surface concentration of MtrF in $mol \cdot cm^{-2}$ and a is constant related to the free energy of adsorption. Since $\Gamma_m = [MtrF{:}Fe_2O_3]/A_{Fe2O3}$, where A_{Fe2O3} is the surface area of the Fe_2O_3 nanoparticles, we could readily calculate Γ_m from our data. Equation 8 can be linearized to give:

$[MtrF]/\Gamma_1 = [MtrF]/\Gamma_m + a / \Gamma_m$ (Equation 9).

By plotting [MtrF] versus Γ_m and fitting Eqn. 9 to the data, we determined a. We could readily calculate $\Delta G^{o'}$ by using that $-\log a = \Delta G^{o'}/2.3RT-1.74$.

Protease footprinting :

Twenty microliters of 2 µM MtrF (approximately 3 µg) was dialyzed against 10 mM ammonium acetate (approximately pH 7) or 10 mM sodium phosphate (pH 7.0) for trypsin and chymotrypsin

digestion and 1 mM acetic acid (~pH 4.0) for pepsin digestion. Two microliters of 50 mM α-Fe_2O_3 nanoparticles in Milli-Q water (the sample is α-Fe_2O_3:MtrF complex) or 2 μL of Milli-Q water (the sample is MtrF alone) was added to the protein solution (MtrF fully binds to the nanoparticles in the α-Fe_2O_3:MtrF complex solution). The samples with or without the nanoparticles were digested with 0.3 μg trypsin (Promega, trypsin:MtrF = 1:10, w/w) at pH 7 at 37 °C for 16 hrs, 0.3 μg chymotrypsin (Promega, chymotrypsin:MtrF = 1:10, w/w) at pH 7 at 37 °C for 16 hrs, or 0.3 μg pepsin (Sigma, pepsin:MtrF = 1:10, w/w) at pH 4 at 37 °C for 6 hrs.

After digestion, the samples were centrifuged at 10 k rcf for 5 min to pellet the nanoparticles, and the peptides in supernatant were subjected to LC-MS analysis. The peptides were analyzed using an Ascentis Peptides ES-C18 reverse phase column (2.1 mm × 100 mm, 2.7-μm particle size; Sigma-Aldrich) in an 1290 LC system coupled to 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies, San Jose, CA). Peptide mass identification and peptide MS/MS sequencing were carried out using Mascot (Matrix Science, Boston, MA) and MassHunter (Agilent Technologies) software.

XFMS analyses:

Before radiolysis of MtrF was performed, we confirmed that α-Fe_2O_3 nanoparticles did not interfere with the radiolysis. Zero, 0.5, 1, or 5 mM α-Fe_2O_3 nanoparticles were added in 10 mM sodium phosphate buffer (pH 6.8) with 5 μM Alexa fluoro 488 as a fluorophore probe and the samples were exposed at Beamline 5.3.1 at Advanced Light Source (ALS) of Lawrence Berkeley National Laboratory. The loss of fluorescence intensity by radiolysis was monitored and the rate constants of hydroxyl radical modification were calculated using OriginLab 7.5 software as described previously[6]. The calculated rate constants of the fluorophore modification in the solutions containing 0, 0.5, 1 and 5 mM the α-Fe_2O_3 nanoparticles

were 5.51×10^3 (s^{-1}), 5.17×10^3 (s^{-1}), 5.52×10^3 (s^{-1}), and 5.03×10^3 (s^{-1}), respectively, indicating that the nanoparticles did not affect the hydroxyl radical modification of the target molecules.

Five hundred μL of 2 μM MtrF was dialyzed against 10 mM sodium phosphate (pH 6.8) or 1 mM acetic acid (pH 4.0). After 500 μL of 10 mM sodium phosphate (pH 6.8) or 1 mM acetic acid (pH 4.0) buffer with or without α-Fe_2O_3 nanoparticles had been added and mixed in the protein solution (final concentrations of the solution are 1 μM MtrF with 0 or 2.5 mM the nanoparticles and the MtrF fully binds to the nanoparticles in this condition), the samples were radiolyzed using 0, 300, 500 and 800 μs of X-ray exposure at beamline 5.3.1 as previously described.

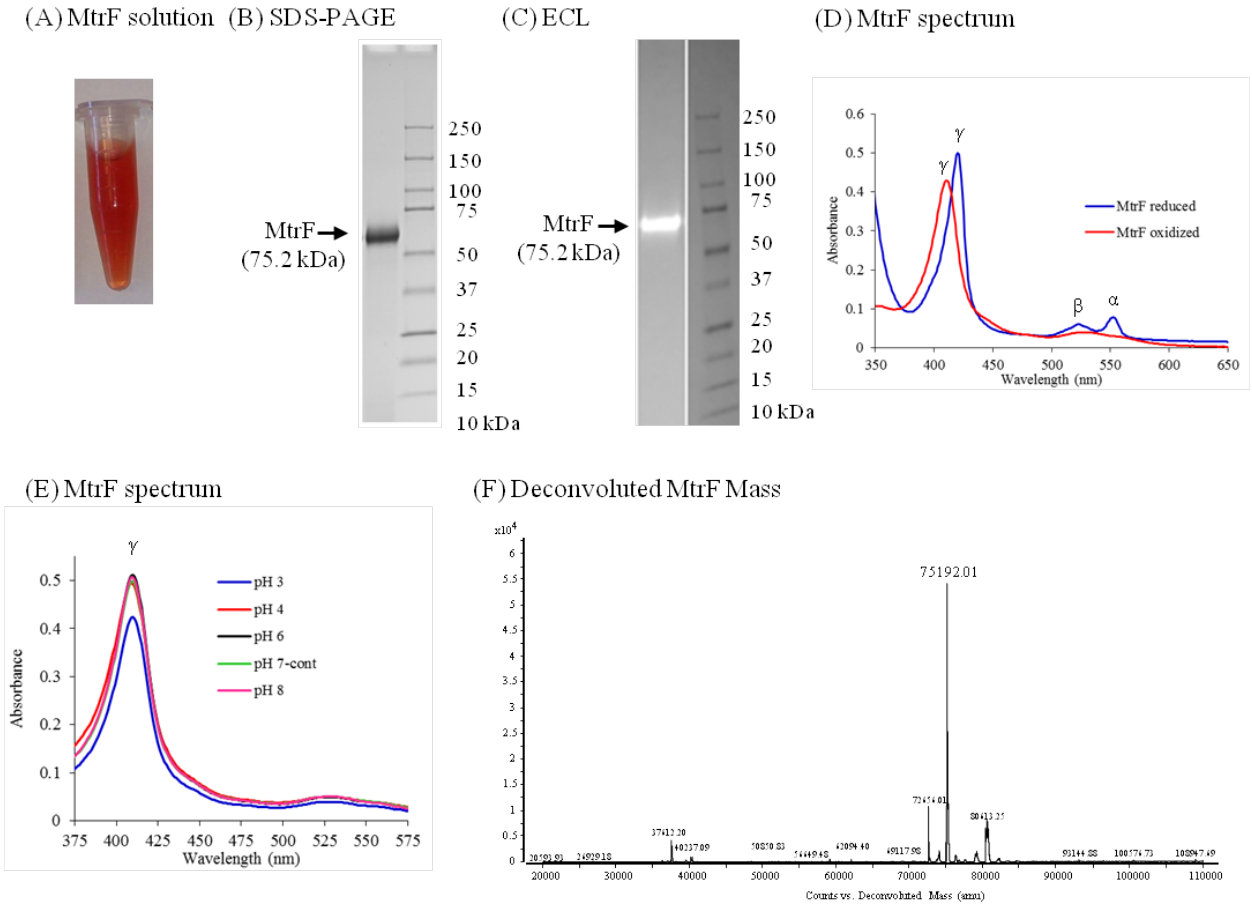
To facilitate precise mass determination, the thioether bonds linking heme *c* to cysteine residues were cleaved and the free cysteines were carbamidomethylated after radiolysis. The reduction of disulfide bonds was carried out by first adjusting the pH to 8.0 using ammonium bicarbonate to a final concentration of 50 mM followed by addition of dithiothreitol to a final concentration of 5 mM. The samples were kept at 55 °C for 30 min to eliminate hemes from cysteine residues of the protein. The carbamidomethylation of reduced Cys in the radiolyzed MtrF samples was performed by addition of iodoacetamide to a final conc., 20 mM followed by incubation at room temperature in dark.

For protease digestion, first, pepsin digestion was performed at pH < 4 (pepsin:MtrF = 1:10, w/w) at 37 °C for 6 hrs. Second, a double proteases digestion using Asp-N (Promega) and chymotrypsin (Sigma) was also performed. The sample was first digested with Asp-N (Asp-N:MtrF = 1:20, w/w) at pH 7 at 37 °C for 16 hrs and then with chymotrypsin (chymotrypsin:MtrF = 1:10, w/w) at 37 °C for another 12 hrs. Third, another double protease digestion, MtrF was digested using trypsin (Promega) and chymotrypsin. The sample was first digested with trypsin (trypsin:MtrF = 1:20, w/w) at 37 °C for 16 hrs at pH 7 and then with chymotrypsin (chymotrypsin:MtrF = 1:10, w/w) at 37 °C for another 12 hrs.

MtrF Charge Maps:

Electrostatic surface potentials of MtrF and the mutants of the protein were calculated using Poisson-Boltzmann equation[7]. However, to calculate the electrostatic field of the protein, the heme cofactors had to be parameterized. First, models of the heme ligand were create using the CHARMM-GUI ligand reader and modeler (http://www.charmm-gui.org[8]. The heme ligand models were then parameterized using PRODRG, without the iron atom, generating a .mol2 file[9]. The resulting parameterized model was then uploaded along with the MtrF crystal structure file (pdb entry: 3PMQ) onto the PDB2PQR server (http://nbcr-222.ucsd.edu/opal2/dashboard[10], which directly calculated the Poisson-Boltzmann charges of the atoms in the MtrF structure. The surface charge maps were loaded and colored using the UCSF Chimera package[11].

To generate surface potentials of the mutant MtrF proteins, the indicated amino acids were swapped in the UCSF Chimera package, and the resulting file was saved as a pdb file type. This file with the mutated residues was then uploaded into the PDB2PQR server to calculate the surface potential of each one.

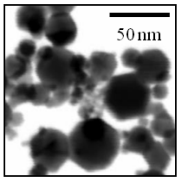


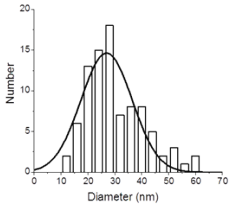
(G) MtrF protein sequence

MKFKLNLITLALLANTGLAVAADGGSDGDDGSPGEPGKPPAMTISSLNISVDKVAISDGI
AQVDYQVSNQENQAVVGIPSATFIAAQLLPQGATGAGNSSEWQHFTSETCAASCPGTFVD
HKNGHYSYRFSATFNGMNGVTFLSDATQRLVIKIGGDALADGTVLPITNQHYDWQSSGNM
LAYTRNLVSIDTCNSCHSNLAFHGGRYNQVETCVTCHNSKKVSNAADIFPQMIHSKHLTG
FPQSISNCQTCHADNPDLADRQNWYRVPTMEACGACHTQINFPAGQGHPAQTDNSNCVAC
HNADWTANVHSNAAQTSALAQFNASISSASMDANGTITVAVSLTNPTTGTAYADSADKLK
FISDLRIYANWGTSFDYSSRSARSIRLPESTPIAGSNGTYSYNISGLTVPAGTESDRGGL
AIQGRVCAKDSVLVDCSTELAEVLVIKSSHSYFNMSALTTTGRREVISNAKCASCHGDQQ
LNIHGARNDLAGQCQLCHNPNMLADATATNPSMTSFDFKQLIHGLHSSQFAGFEDLNYPG
NIGNCAQCHINDSTGISTVALPLNAAVQPLALNNGTFTSPIAAVCSNCHSSDATQNHMRQ
QGAVFAGTKADATAGTETCAFCHGQGTVADVLKVHPINDDDDKLKGELKLEGKPIPNPLL
GLDSTRTGHHHHHH

Figure S1.

(A) α-Fe_2O_3 nanoparticles (B) Histogram of the nanoparticles





(C) FQ for α-Al_2O_3 nanoparticles

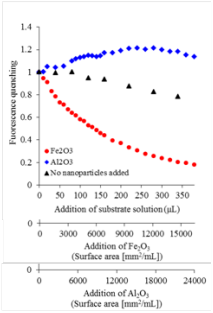
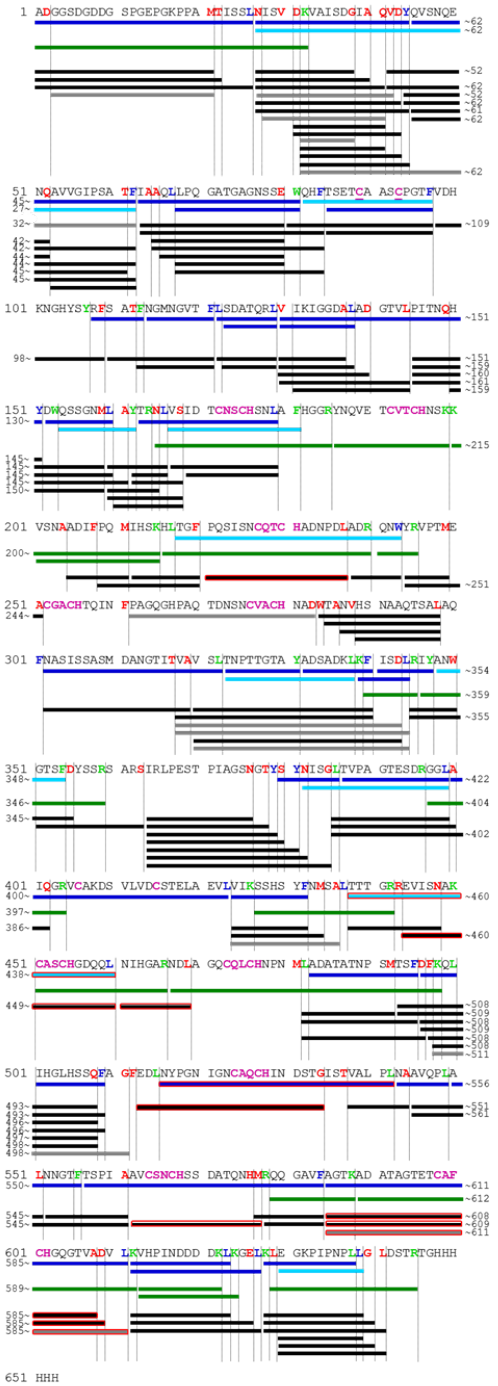


Figure S2.

A)



B)

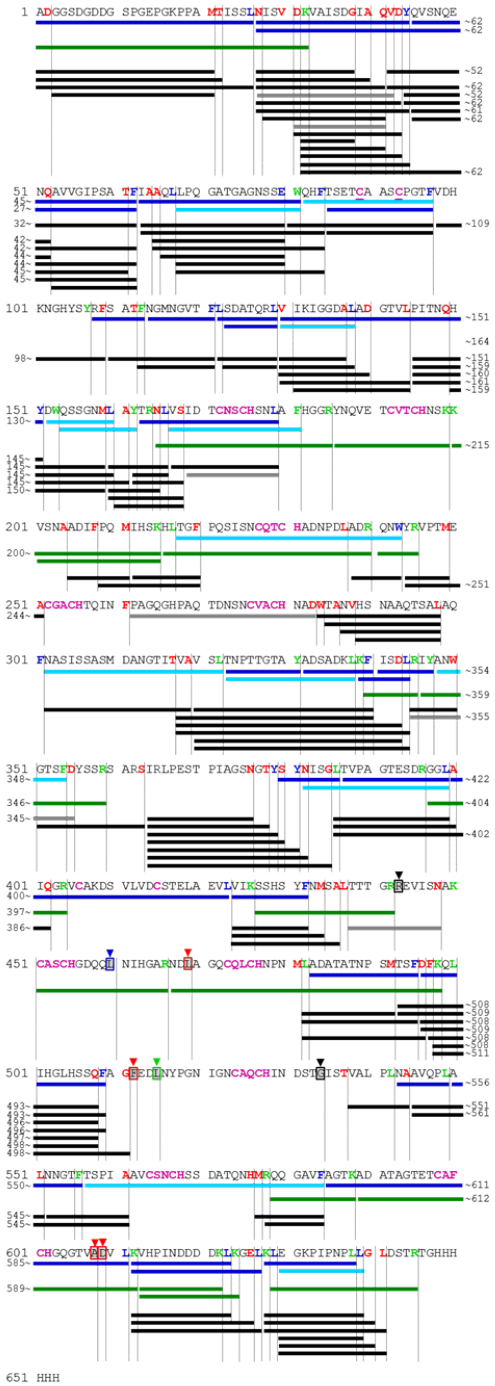


Figure S3.

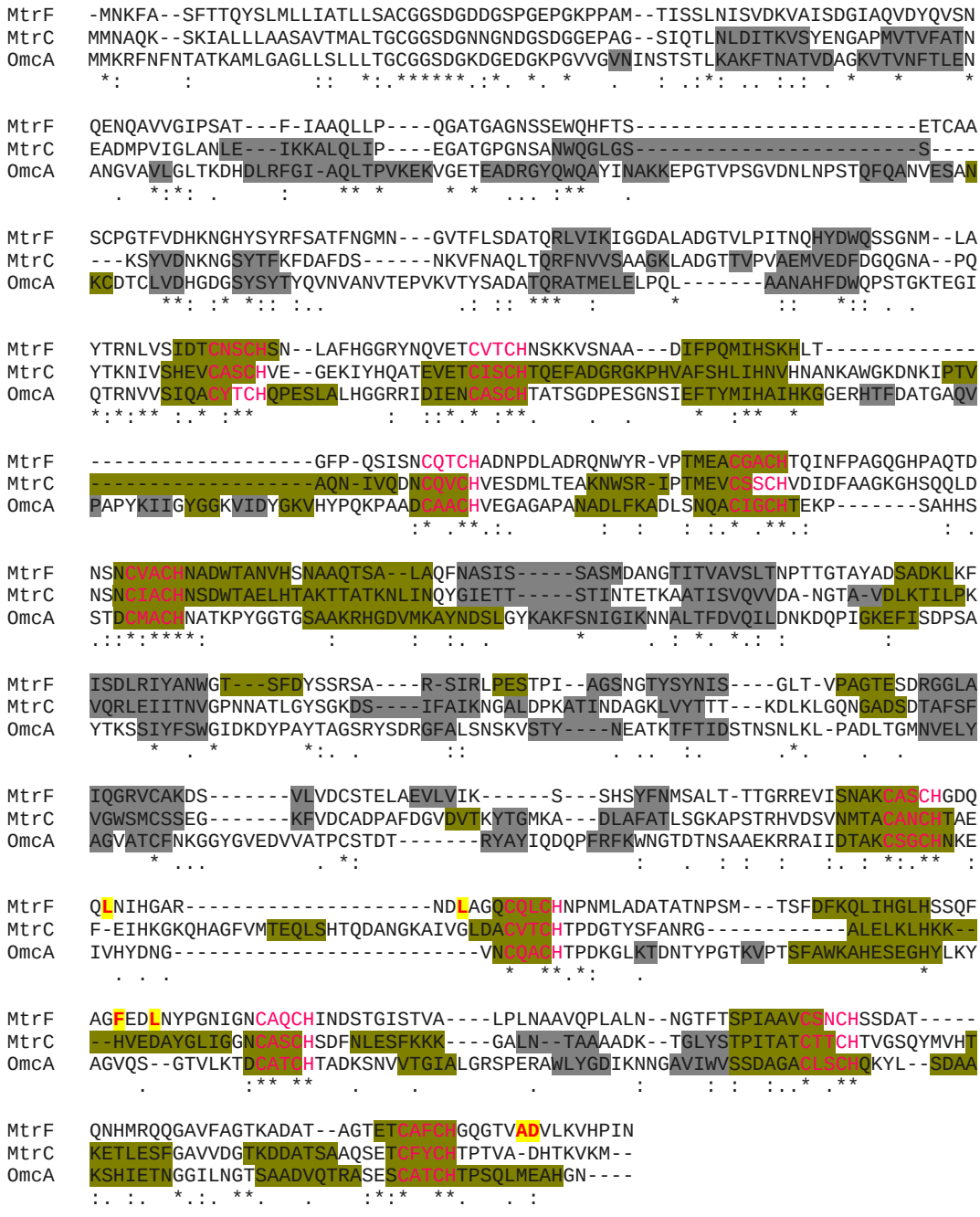
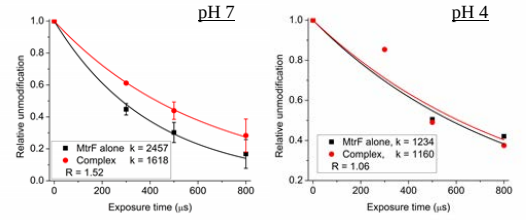
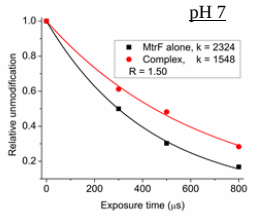

MtrF -MNKFA--SFTTQYSLMLLIATLLSACGGSDGDDGSPGEPGKPPAM--TISSLNISVDKVAISDGIAQVDYQVSN
MtrC MMNAQK--SKIALLLAASAVTMALTGCGGSDGNNGNDGSDGGEPAG--SIQTLNLDITKVSYENGAPMVTVFATN
OmcA MMKRFNFNTATKAMLGAGLLSLLLTGCGGSDGKDGEDGKPGVVGVNINSTSTLKAKFTNATVDAGKVTVNFTLEN
MtrF QENQAVVGIPSAT---F-IAAQLLP----QGATGAGNSSEWQHFTS----------ETCAA
MtrC EADMPVIGLANLE---IKKALQLIP----EGATGPGNSANWQGLGS----------S----
OmcA ANGVAVLGLTKDHDLRFGI-AQLTPVKEKVGETEADRGYQWQAYINAKKEPGTVPSGVDNLNPSTQFQANVESAN
MtrF SCPGTFVDHKNGHYSYRFSATFNGMN---GVTFLSDATQRLVIKIGGDALADGTVLPITNQHYDWQSSGNM--LA
MtrC ---KSYVDNKNGSYTFKFDAFDS------NKVFNAQLTQRFNVVSAAGKLADGTTVPVAEMVEDFDGQGNA--PQ
OmcA KCDTCLVDHGDGSYSYTYQVNVANVTEPVKVTYSADATQRATMELELPQL-------AANAHFDWQPSTGKTEGI
MtrF YTRNLVSIDTCNSCHSN--LAFHGGRYNQVETCVTCHNSKKVSNAA---DIFPQMIHSKHLT----------
MtrC YTKNIVSHEVCASCHVE--GEKIYHQATEVETCISCHTQEFADGRGKPHVAFSHLIHNVHNANKAWGKDNKIPTV
OmcA QTRNVVSIQACYTCHQPESLALHGGRRIDIENCASCHTATSGDPESGNSIEFTYMIHAIHKGGERHTFDATGAQV
MtrF ------------------GFP-QSISNCQTCHADNPDLADRQNWYR-VPTMEACGACHTQINFPAGQGHPAQTD
MtrC ------------------AQN-IVQDNCQVCHVESDMLTEAKNWSR-IPTMEVCSSCHVDIDFAAGKGHSQQLD
OmcA PAPYKIIGYGGKVIDYGKVHYPQKPAADCAACHVEGAGAPANADLFKADLSNQACIGCHTEKP-------SAHHS
MtrF NSNCVACHNADWTANVHSNAAQTSA--LAQFNASIS-----SASMDANGTITVAVSLTNPTTGTAYADSADKLKF
MtrC NSNCIACHNSDWTAELHTAKTTATKNLINQYGIETT-----STINTETKAATISVQVVDA-NGTA-VDLKTILPK
OmcA STDCMACHNATKPYGGTGSAAKRHGDVMKAYNDSLGYKAKFSNIGIKNNALTFDVQILDNKDQPIGKEFISDPSA
MtrF ISDLRIYANWGT---SFDYSSRSA----R-SIRLPESTPI--AGSNGTYSYNIS----GLT-VPAGTESDRGGLA
MtrC VQRLEIITNVGPNNATLGYSGKDS----IFAIKNGALDPKATINDAGKLVYTTT---KDLKLGQNGADSDTAFSF
OmcA YTKSSIYFSWGIDKDYPAYTAGSRYSDRGFALSNSKVSTY----NEATKTFTIDSTNSNLKL-PADLTGMNVELY
MtrF IQGRVCAKDS-------VLVDCSTELAEVLVIK------S---SHSYFNMSALT-TTGRREVISNAKCASCHGDQ
MtrC VGWSMCSSEG-------KFVDCADPAFDGVDVTKYTGMKA---DLAFATLSGKAPSTRHVDSVNMTACANCHTAE
OmcA AGVATCFNKGGYGVEDVVATPCSTDT-------RYAYIQDQPFRFKWNGTDTNSAAEKRRAIIDTAKCSGCHNKE
MtrF QLNIHGAR----------NDLAGQCQLCHNPNMLADATATNPSM---TSFDFKQLIHGLHSSQF
MtrC F-EIHKGKQHAGFVMTEQLSHTQDANGKAIVGLDACVTCHTPDGTYSFANRG----------ALELKLHKK--
OmcA IVHYDNG----------VNCQACHTPDKGLKTDNTYPGTKVPTSFAWKAHESEGHYLKY
MtrF AGFEDLNYPGNIGNCAQCHINDSTGISTVA----LPLNAAVQPLALN--NGTFTSPIAAVCSNCHSSDAT-----
MtrC --HVEDAYGLIGGNCASCHSDFNLESFKKK----GALN--TAAAADK--TGLYSTPITATCTTCHTVGSQYMVHT
OmcA AGVQS--GTVLKTDCATCHTADKSNVVTGIALGRSPERAWLYGDIKNNGAVIWVSSDAGACLSCTQKYL--SDAA
MtrF QNHMRQQGAVFAGTKADAT--AGTETCAFCHGQGTVADVLKVHPIN
MtrC KETLESFGAVVDGTKDDATSAAQSETCFYCHTPTVA-DHTKVKM--
OmcA KSHIETNGGILNGTSAADVQTRASESCATCHTPSQLMEAHGN----

Figure S4.

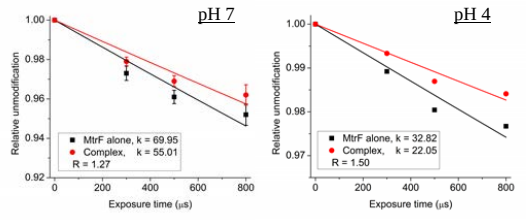
(A) 65-84 AQLLPQGATGAGNSSEWQHF



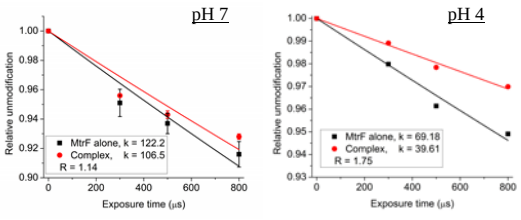
(B) 67-84 LLPQGATGAGNSSEWQHF



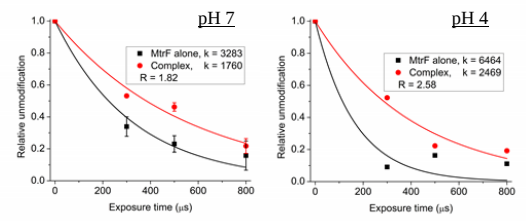
(C) 130-144 VIKIGGDALADGTVL



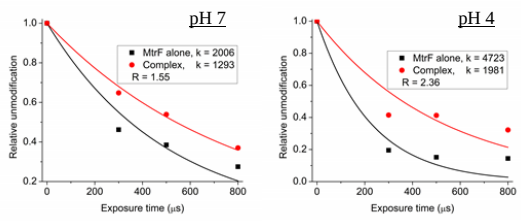
(D) 131-144 IKIGGDALADGTVL



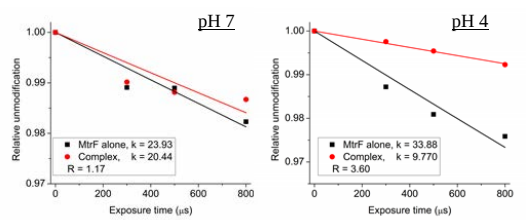
(E) 205-220 ADIFPQMIHSKHLTGF



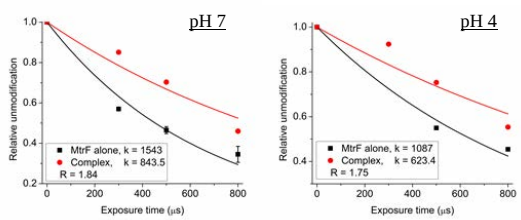
(F) 206-211 DIFPQM



(G) 216-222 HLTGFPQ



(H) 482-496 LADATATNPSMTSFD



(I) 572-584 ATQNHMRQQGAVF

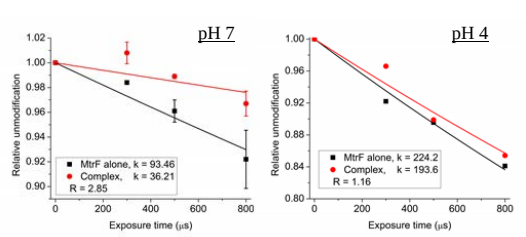


Figure S5.

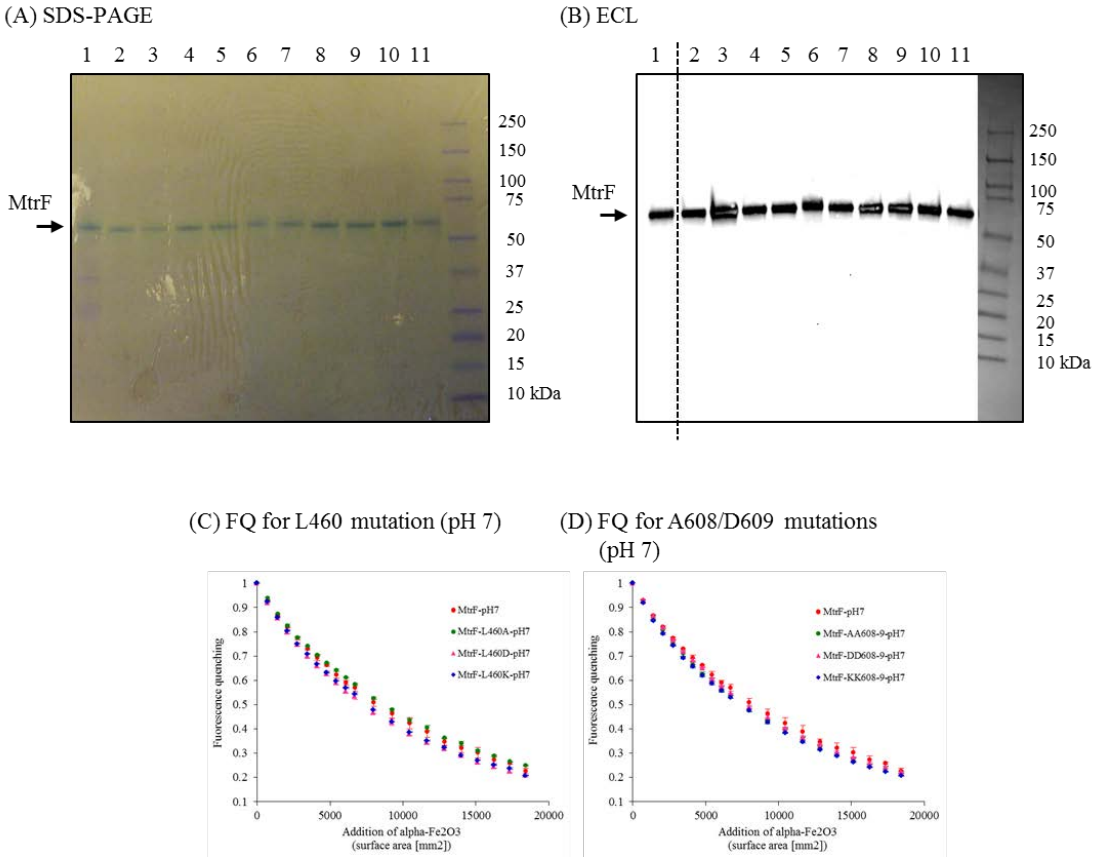

(A) SDS-PAGE
1 2 3 4 5 6 7 8 9 10 11
MtrF
250
150
100
75
50
37
25
20
15
10 kDa
(B) ECL
1 2 3 4 5 6 7 8 9 10 11
MtrF
250
150
100
75
50
37
25
20
15
10 kDa
(C) FQ for L460 mutation (pH 7)
Fluorescence quenching
Addition of alpha-Fe2O3
(surface area [mm2])
MtrF-pH7
MtrF-L460A-pH7
MtrF-L460D-pH7
MtrF-L460K-pH7
(D) FQ for A608/D609 mutations
(pH 7)
Fluorescence quenching
Addition of alpha-Fe2O3
(surface area [mm2])
MtrF-pH7
MtrF-AA608-9-pH7
MtrF-DD608-9-pH7
MtrF-KK608-9-pH7

Figure S6.

Table S1. Rate constants of hydroxyl radical modification for amino acid residues of MtrF at pH 7

Peptide position	Major peptide sequence[a]	Protease used[b]	Rate constant of the modification[c] (s^{-1})		Ratio of rate constants (MtrF/the complex)
			MtrF alone	α-Fe_2O_3:MtrF complex	
1-26	**ADGGSDGDDGSPGEPGKPPAMTISSL (Not detected)**	**-**			
27-32	NISVDK (No oxidation)	Chy/Tryp			
27-38	NISVDKVAISDG (No oxidation)	Pepsin			
27-40	NISVDKVAISDGIA (No oxidation)	Pepsin			
27-41	NISVDKVAISDGIAQ (No oxidation)	Pepsin			
27-42	NISVDKVAISDGIAQV (No oxidation)	Pepsin			
27-44	NISVDKVAISDGIAQVDY (No oxidation)	Pepsin			
31-36	DKVAIS (No oxidation)	Chy/AspN			
31-38	DKVAISDG (No oxidation)	Pepsin			
31-41	DKVAISDGIAQ (No oxidation)	Pepsin			
32-38	KVAISDG (No oxidation)	Pepsin			
32-40	KVAISDGIA (No oxidation)	Pepsin			
32-41	KVAISDGIAQ (No oxidation)	Pepsin			
32-43	KVAISDGIAQVD (No oxidation)	Pepsin			
33-44	VAISDGIAQVDY (No oxidation)	Chy/Tryp			
37-42	DGIAQV (No oxidation)	Chy/AspN			
42-52	VDYQVSNQENQ (No oxidation)	Pepsin			
43-52	DYQVSNQENQ (No oxidation)	Pepsin			
44-52	YQVSNQENQ (No oxidation)	Pepsin			
45-62	QVSNQENQAVVGIPSATF (No oxidation)	Pepsin Chy/Tryp			
53-61	AVVGIPSAT (No oxidation)	Pepsin			
53-62	AVVGIPSATF (No oxidation)	Pepsin Chy/AspN			
63-70	IAAQLLPQ (No oxidation)	Chy/Tryp			
63-80	IAAQLLPQGATGAGNSSE (No oxidation)	Pepsin			
63-81	IAAQLLPQGATGAGNSSEW (No oxidation)	Chy/Tryp			
65-80	AQLLPQGATGAGNSSE (No oxidation)	Pepsin			
65-84	**AQLLPQGATGAGNSSE<u>W</u>QHF (W81 is modified [+16])**	**Pepsin**	**2,541 ± 345**	**1,602 ± 220**	**1.59 ± 0.004**
67-77	LLPQGATGAGN (No oxidation)	Chy/Tryp Chy/AspN			
67-81	LLPQGATGAGNSSEW (No oxidation)	Chy/Tryp Chy/AspN			
67-84	**LLPQGATGAGNSSE<u>W</u>QHF (W81 is modified [+32])**	**Chy/Tryp**	**2,324 ± 37.0**	**1,548 ± 24.5**	**1.50 ± 0.001**
68-78	LPQGATGAGNS (No oxidation)	Pepsin			
68-80	LPQGATGAGNSSE (No oxidation)	Pepsin			
81-87	WQHFTSE (No oxidation)	Pepsin			
85-97	TSETC*AASC*PGTF (No oxidation)	Chy/Tryp			
88-97	TC*AASC*PGTF (No oxidation)	Pepsin			
98-109	**VDHKNGHYSYRF (Not detected)**	**-**			
110-121	**SATFNG<u>MN</u>GVTF (M116 or N117 is modified)**	**Pepsin**	**38.43 ± 2.95**	**42.85 ± 3.22**	**0.90 ± 0.001**
122-129	**<u>L</u>SDATQRL (L122 is modified)**	**Pepsin**	**57.67 ± 10.9**	**42.40 ± 6.01**	**1.32 ± 0.089**
123-129	SDATQRL (No oxidation)	Pepsin			
124-129	DATQRL (No oxidation)	Chy/AspN			
129-135	LVIKIGG (No oxidation)	Chy/AspN			
130-137	VIKIGGDA (No oxidation)	Pepsin			
130-138	VIKIGGDAL (No oxidation)	Pepsin			
130-140	VIKIGGDALAD (No oxidation)	Pepsin			
130-141	VIKIGGDALADG (No oxidation)	Pepsin			

130-143	VIKIGGDALADGTV (No oxidation)	Pepsin			
130-144	**VIKIGGDALADGTVL (V143 is modified)**	**Pepsin**	**69.95 ± 6.07**	**55.01 ± 5.41**	**1.27 ± 0.013**
131-138	IKIGGDAL (No oxidation)	Pepsin			
131-144	**IKIGGDALADGTVL (V143 is modified)**	**Pepsin**	**122.2 ± 12.0**	**106.5 ± 6.12**	**1.14 ± 0.101**
133-148	IGGDALADGTVLPITN (No oxidation)	Chy/Tryp			
133-151	IGGDALADGTVLPITNQHY (No oxidation)	Chy/Tryp			
140-151	DGTVLPITNQHY (No oxidation)	Chy/AspN			
145-151	PITNQHY (No oxidation)	Pepsin			
152-159	**DWQSSGNM (Not detected)**				
160-165	LAYTRN (No oxidation)	Pepsin			
162-168	YTRNLVS (No oxidation)	Pepsin			
163-168	TRNLVS (No oxidation)	Pepsin			
169-179	**IDTCNSCHSNL (Not detected)**	**-**			
180-186	AFHGGRY (No oxidation)	Pepsin			
187-199	**NQVETCVTCHNSK (Not detected)**	**-**			
200-210	KVSNAADIFPQ (No oxidation)	Chy/Tryp			
201-210	VSNAADIFPQ (No oxidation)	Chy/Tryp			
205-211	ADIFPQM (No oxidation)	Pepsin			
205-220	**ADIFPQMIHSKHLTGF (M211 is modified)**	**Pepsin**	**3,283 ± 523**	**1,760 ± 151**	**1.82 ± 0.218**
206-210	DIFPQ (No oxidation)	Chy/AspN			
206-211	**DIFPQM (M211 is modified)**	**Chy/Tryp**	**2,006 ± 118**	**1,293 ± 56.4**	**1.55 ± 0.004**
212-220	IHSKHLTGF (No oxidation)	Pepsin			
216-222	**HLTGFPQ (H216 or L217 is modified)**	**Chy/Tryp**	**23.93 ± 1.94**	**20.44 ± 1.37**	**1.17 ± 0.017**
217-222	LTGFPQ (No oxidation)	Chy/AspN Chy/Tryp			
223-231	**SISNCQTCH (Not detected)**	**-**			
232-240	ADNPDLADR (No oxidation)	Chy/Tryp			
238-243	ADRQNW (No oxidation)	Pepsin			
244-258	**YRVPTMEACGACHTQ (Not detected)**	**-**			
259-271	INFPAGQGHPAQT (No oxidation)	Chy/AspN			
272-283	**DNSNCVACHNAD (Not detected)**	**-**			
284-292	WTANVHSNA (No oxidation)	Pepsin			
284-295	WTANVHSNAAQT (No oxidation)	Pepsin			
285-298	TANVHSNAAQTSAL (No oxidation)	Pepsin			
288-298	VHSNAAQTSAL (No oxidation)	Chy/AspN			
289-298	HSNAAQTSAL (No oxidation)	Pepsin			
290-298	SNAAQTSAL (No oxidation)	Chy/Tryp Chy/AspN			
290-301	SNAAQTSALAQF (No oxidation)	Chy/Tryp			
292-298	AAQTSAL (No oxidation)	Chy/Tryp			
302-319	**NASISSASMDANGTITVA (M311 is modified)**	**Pepsin**	**991.2± 114**	**938.0 ± 44.7**	**1.06 ± 0.002**
320-330	VSLTNPTTGTA (No oxidation)	Pepsin			
320-340	**VSLTNPTTGTAYADSADKLKF (Y331 is modified)**	**Pepsin**	**35.65 ± 1.84**	**38.77 ± 1.81**	**0.92 ± 0.005**
323-331	TNPTTGTAY (No oxidation)	Chy/Tryp			
323-332	TNPTTGTAYA (No oxidation)	Chy/AspN			
327-340	TGTAYADSADKLKF (No oxidation)	Pepsin			
331-340	YADSADKLKF (No oxidation)	Pepsin			
340-345	**FISDLR (F340 is modified)**	**Chy/Tryp**	**26.29 ± 2.42**	**33.36 ± 1.71**	**0.79 ± 0.016**
341-345	ISDLR (No oxidation)	Chy/Tryp			
344-350	LRIYANW (No oxidation)	Pepsin			
345-350	RIYANW (No oxidation)	Pepsin			
351-363	**GTSFDYSSRSARS (Not detected)**				
364-370	IRLPEST (No oxidation)	Pepsin			

364-374	IRLPESTPIAG (No oxidation)	Pepsin			
364-376	IRLPESTPIAGSN (No oxidation)	Pepsin			
364-381	IRLPESTPIAGSNGTYSY (No oxidation)	Pepsin			
382-385	**NISG (Not detected)**				
386-399	**LTVPAGTESDRGGL (R396 or G397 is modified)**	**Pepsin**	**46.43 ± 3.78**	**37.85 ± 2.47**	**1.23 ± 0.015**
386-400	LTVPAGTESDRGGLA (No oxidation)	Pepsin			
387-394	TVPAGTES (No oxidation)	Chy/AspN			
387-396	TVPAGTESDR (No oxidation)	Chy/Tryp			
397-404	GGLAIQGR (No oxidation)	Chy/Tryp			
400-406	AIQGRVC* (No oxidation)	Pepsin			
400-407	AIQGRVC*A (No oxidation)	Pepsin			
400-408	AIQGRVC*AK (No oxidation)	Chy/AspN			
400-412	AIQGRVC*AKDSVL (No oxidation)	Pepsin			
403-412	GRVC*AKDSVL (No oxidation)	Pepsin			
404-412	RVC*AKDSVL (No oxidation)	Pepsin			
409-423	DSVLVDC*STELAEVL (No oxidation)	Chy/Tryp			
424-432	VIKSSHSYF (No oxidation)	Pepsin			
427-432	SSHSYF (No oxidation)	Chy/Tryp			
433-434	**NM (Not detected)**				
435-442	SALTTTGR (No oxidation)	Chy/Tryp			
437-448	LTTTGRREVISN (No oxidation)	Pepsin			
438-448	TTTGRREVISN (No oxidation)	Pepsin			
449-460	**AKCASCHGDQQL (Not detected)**				
461-469	NIHGARNDL (No oxidation)	Pepsin			
467-475	NDLAGQC*QL (No oxidation)	Chy/Tryp			
468-475	DLAGQCQL (No oxidation)	Chy/AspN			
476-481	**CHNPNM (Not detected)**				
482-492	LADATATNPSM (No oxidation)	Pepsin			
482-496	**LADATATNPSMTSFD (M492 is modified)**	**Pepsin**	**1,543 ± 91.2**	**843.5 ± 51.6**	**1.84 ±0.036**
496-504	DFKQLIHGL (No oxidation)	Pepsin			
496-508	DFKQLIHGLHSSQ (No oxidation)	Pepsin			
496-509	DFKQLIHGLHSSQF (No oxidation)	Pepsin			
497-506	FKQLIHGLHS (No oxidation)	Pepsin			
499-504	QLIHGL (No oxidation)	Chy/Tryp			
503-509	GLHSSQF (No oxidation)	Chy/AspN			
505-509	HSSQF (No oxidation)	Chy/Tryp			
510-528	**AGFEDLNYPGNIGNCAQCH (Not detected)**				
529-542	INDSTGISTVALPL (No oxidation)	Chy/Tryp			
537-544	TVALPLNA (No oxidation)	Pepsin			
538-544	VALPLNA (No oxidation)	Pepsin			
543-549	NAAVQPL (No oxidation)	Chy/Tryp Chy/AspN			
545-551	AVQPLAL (No oxidation)	Pepsin			
545-555	AVQPLALNNGT (No oxidation)	Pepsin			
545-561	AVQPLALNNGTFTSPIA (No oxidation)	Pepsin			
552-561	NNGTFTSPIA (No oxidation)	Pepsin			
562-571	**AVCSNCHSSD (Not detected)**				
572-584	**ATQNHMRQQGAVF (N575, H576 or M577 is modified)**	**Pepsin**	**93.46 ± 19.8**	**36.21 ± 12.7**	**2.85 ± 0.515**
578-584	RQQGAVF (No oxidation)	Chy/AspN			
585-600	**AGTKADATAGTETCAF (Not detected)**				
601-612	C*HGQGTVADVLK (No oxidation)	Chy/Tryp			
612-623	KVHPINDDDDKL (No oxidation)	Pepsin			

Letters with underline or red color indicate modified amino acid residues by hydroxyl radicals or the CXXCH cytochrome *c* binding motif, respectively.

Carbamidomethylated Cys is shown as C with an asterisk.

[a]Modified amino acid residues are identified by MS/MS.

[b]Peptidases, pepsin, chymotrypsin and trypsin (Chy/Tryp) or chymotrypsin and Asp-N (Chy/AspN), were used for MtrF digestion.

[c]Rate constants were calculated with Origin software by using a non-linear fit of hydroxyl radical modification data to a first order decay and errors in the rate constants are calculated from the non-linear fit (see Fig. S5).

Table S2. Rate constants of hydroxyl radical modification for amino acid residues of MtrF at pH 4

Peptide position	Major peptide sequence[a]	Protease used[b]	Rate constant of the modification[c] (s^{-1})		Ratio of rate constants (MtrF/the complex)
			MtrF alone	α-Fe_2O_3:MtrF complex	
1-26	**ADGGSDGDDGSPGEPGKPPAMTISSL (Not detected)**	**-**			
27-32	NISVDK (No modification)	Chy/Tryp			
27-38	NISVDKVAISDG (No modification)	Pepsin			
27-40	NISVDKVAISDGIA (No modification)	Pepsin			
27-41	NISVDKVAISDGIAQ (No modification)	Pepsin			
27-42	NISVDKVAISDGIAQV (No modification)	Pepsin			
27-43	NISVDKVAISDGIAQVD (No modification)	Pepsin			
27-44	NISVDKVAISDGIAQVDY (No modification)	Pepsin			
28-38	ISVDKVAISDG (No modification)	Pepsin			
31-36	DKVAIS (No modification)	Chy/AspN			
31-38	DKVAISDG (No modification)	Pepsin			
32-38	KVAISDG (No modification)	Pepsin			
32-40	KVAISDGIA (No modification)	Pepsin			
32-41	KVAISDGIAQ (No modification)	Pepsin			
32-43	KVAISDGIAQVD (No modification)	Pepsin			
32-44	KVAISDGIAQVDY (No modification)	Pepsin			
33-44	VAISDGIAQVDY (No modification)	Chy/Tryp			
37-42	DGIAQV (No modification)	Chy/AspN			
42-52	VDYQVSNQENQ (No modification)	Pepsin			
43-52	DYQVSNQENQ (No modification)	Pepsin			
44-52	YQVSNQENQ (No modification)	Pepsin			
44-62	YQVSNQENQAVVGIPSATF (No modification)	Pepsin			
45-62	QVSNQENQAVVGIPSATF (No modification)	Pepsin			
		Chy/Tryp			
51-62	NQAVVGIPSATF (No modification)	Pepsin			
52-62	QAVVGIPSATF (No modification)	Chy/Tryp			
53-61	AVVGIPSAT (No modification)	Pepsin			
53-62	AVVGIPSATF (No modification)	Pepsin			
		Chy/Tryp			
		Chy/AspN			
53-63	AVVGIPSATFI (No modification)	Pepsin			
54-62	VVGIPSATF (No modification)	Pepsin			
63-80	IAAQLLPQGATGAGNSSE (No modification)	Pepsin			
65-80	AQLLPQGATGAGNSSE (No modification)	Pepsin			
65-84	**AQLLPQGATGAGNSSE<u>W</u>QHF (W81 is modified [+16])**	**Pepsin**	**1,234 ± 137**	**1,160 ± 117**	**1.06 ± 0.011**
67-77	LLPQGATGAGN (No modification)	Chy/Tryp			
		Chy/AspN			
67-81	LLPQGATGAGNSSEW (No modification)	Chy/Tryp			
		Chy/AspN			
68-80	LPQGATGAGNSSE (No modification)	Pepsin			
81-87	WQHFTSE (No modification)	Pepsin			
88-97	TC*AASC*PGTF (No modification)	Chy/Tryp			
98-109	**VDHKNGHYSYRF (Not detected)**	**-**			
110-121	**SATFNG<u>MN</u>GVTF (M116 or N117 is modified)**	**Pepsin**	**307.0 ± 24.5**	**278.7 ± 24.1**	**1.10 ± 0.007**
122-129	**<u>L</u>SDATQRL (L122 is modified)**	**Pepsin**	**22.67 ± 1.17**	**17.77 ± 1.17**	**1.28 ± 0.018**
123-129	SDATQRL (No modification)	Pepsin			

124-129	DATQRL (No modification)	Chy/AspN			
129-135	LVIKIGG (No modification)	Chy/AspN			
129-144	LVIKIGGDALADGTVL (No modification)	Pepsin			
130-137	VIKIGGDA (No modification)	Pepsin			
130-138	VIKIGGDAL (No modification)	Pepsin			
130-140	VIKIGGDALAD (No modification)	Pepsin			
130-141	VIKIGGDALADG (No modification)	Pepsin			
130-143	VIKIGGDALADGTV (No modification)	Pepsin			
130-144	**VIKIGGDALADGTVL (V143 is modified)**	**Pepsin**	**32.82 ± 1.51**	**22.05 ± 1.51**	**1.50 ± 0.034**
131-138	IKIGGDAL (No modification)	Pepsin			
131-144	**IKIGGDALADGTVL (V143 is modified)**	**Pepsin**	**69.18 ± 1.82**	**39.61 ± 1.79**	**1.75 ± 0.033**
133-148	IGGDALADGTVLPITN (No modification)	Chy/Tryp			
133-151	IGGDALADGTVLPITNQHY (No modification)	Chy/Tryp			
145-151	PITNQHY (No modification)	Pepsin			
152-159	**DWQSSGNM (Not detected)**				
160-165	LAYTRN (No modification)	Pepsin			
162-168	YTRNLVS (No modification)	Pepsin			
163-168	TRNLVS (No modification)	Pepsin			
169-179	**IDTCNSCHSNL (Not detected)**	**-**			
180-186	AFHGGRY (No modification)	Pepsin			
187-199	**NQVETCVTCHNSK (Not detected)**	**-**			
200-210	KVSNAADIFPQ (No modification)	Chy/Tryp			
201-210	VSNAADIFPQ (No modification)	Chy/Tryp			
205-211	ADIFPQM (No modification)	Pepsin			
205-220	**ADIFPQMIHSKHLTGF (M211 is modified)**	**Pepsin**	**6,464 ± 1,107**	**2,469 ± 243**	**2.58 ± 0.195**
206-210	DIFPQ (No modification)	Chy/AspN			
206-211	**DIFPQM (M211 is modified)**	**Chy/AspN**	**4,723 ± 728**	**1,981 ± 224**	**2.36 ± 0.100**
212-220	IHSKHLTGF (No modification)	Pepsin			
216-222	**HLTGFPQ (H216 or L217 is modified)**	**Chy/Tryp**	**33.88 ± 1.32**	**9.770 ± 1.35**	**3.57 ± 0.356**
217-222	LTGFPQ (No modification)	Chy/AspN			
223-231	**SISNCQTCH (Not detected)**	**-**			
232-240	ADNPDLADR (No modification)	Chy/Tryp			
238-243	ADRQNW (No modification)	Pepsin			
244-256	**YRVPTMEACGACH (Not detected)**	**-**			
257-271	TQINFPAGQGHPAQT (No modification)	Chy/AspN			
259-271	INFPAGQGHPAQT (No modification)	Chy/AspN			
272-283	**DNSNCVACHNAD (Not detected)**	**-**			
284-292	WTANVHSNA (No modification)	Pepsin			
284-295	WTANVHSNAAQT (No modification)	Pepsin			
285-298	TANVHSNAAQTSAL (No modification)	Pepsin			
288-298	VHSNAAQTSAL (No modification)	Chy/AspN			
289-298	HSNAAQTSAL (No modification)	Pepsin			
290-298	SNAAQTSAL (No modification)	Chy/Tryp			
		Chy/AspN			
299-301	**AQF (Not detected)**				
302-319	**NASISSASMDANGTITVA (M311 is modified)**	**Pepsin**	**726.6 ± 32.0**	**581.6 ± 29.3**	**1.25 ± 0.008**
311-319	DANGTITVA (No modification)	Pepsin			
318-340	VAVSLTNPTTGTAYADSADKLKF (No modification)	Pepsin			
320-330	VSLTNPTTGTA (No modification)	Pepsin			
320-340	**VSLTNPTTGTAYADSADKLKF (Y331 is modified)**	**Pepsin**	**31.44 ± 1.62**	**31.39 ± 1.62**	**1.00 ± 0.001**
323-332	TNPTTGTAYA (No modification)	Chy/AspN			
331-340	YADSADKLKF (No modification)	Pepsin			
332-340	ADSADKLKF (No modification)	Pepsin			

340-345	**FISDLR (F340 is modified)**	**Chy/Tryp**	**25.11 ± 0.87**	**19.03 ± 0.86**	**1.32 ± 0.014**
341-345	ISDLR (No modification)	Chy/Tryp			
344-350	LRIYANW (No modification)	Pepsin			
345-350	RIYANW (No modification)	Pepsin			
351-363	**GTSFDYSSRSARS (Not detected)**				
364-370	IRLPEST (No modification)	Pepsin			
364-376	IRLPESTPIAGSN (No modification)	Pepsin			
364-378	IRLPESTPIAGSNGT (No modification)	Pepsin			
364-379	IRLPESTPIAGSNGTY (No modification)	Pepsin			
364-380	IRLPESTPIAGSNGTYS (No modification)	Pepsin			
364-381	IRLPESTPIAGSNGTYSY (No modification)	Pepsin			
364-382	IRLPESTPIAGSNGTYSYN (No modification)	Pepsin			
364-385	IRLPESTPIAGSNGTYSYNISG (No modification)	Pepsin			
386-399	**LTVPAGTESDRGGL (R396 or G397 is modified)**	**Pepsin**	**23.94 ± 1.50**	**17.59 ± 1.50**	**1.37 ± 0.032**
386-400	LTVPAGTESDRGGLA (No modification)	Pepsin			
387-399	TVPAGTESDRGGL (No modification)	Pepsin			
389-399	PAGTESDRGGL (No modification)	Pepsin			
397-404	GGLAIQGR (No modification)	Chy/Tryp			
400-407	AIQGRVC*A (No modification)	Pepsin			
400-408	AIQGRVC*AK (No modification)	Chy/AspN			
400-412	AIQGRVC*AKDSVL (No modification)	Pepsin			
404-412	RVC*AKDSVL (No modification)	Pepsin			
409-423	DSVLVDC*STELAEVL (No modification)	Chy/Tryp			
423-430	LVIKSSHS (No modification)	Pepsin			
423-432	LVIKSSHSYF (No modification)	Pepsin			
424-432	VIKSSHSYF (No modification)	Pepsin			
427-432	**SSHSYF (H429 is modified)**	**Chy/Tryp**	**24.14 ± 4.54**	**19.94 ± 4.75**	**1.24 ± 0.068**
433-434	**NM (Not detected)**				
435-442	SALTTTGR (No modification)	Chy/Tryp			
437-448	LTTTGRREVISN (No modification)	Pepsin			
438-447	TTTGRREVIS (No modification)	Pepsin			
449-460	**AKCASCHGDQQL (Not detected)**				
461-469	NIHGARNDL (No modification)	Pepsin			
461-471	NIHGARNDLAG (No modification)	Pepsin			
467-475	NDLAGQC*QL (No modification)	Chy/Tryp			
468-475	DLAGQCQL (No modification)	Chy/AspN			
476-481	**CHNPNM (Not detected)**				
482-495	LADATATNPSMTSF (No modification)	Chy/Tryp			
482-496	**LADATATNPSMTSFD (M492 is modified)**	**Pepsin**	**1,087 ± 85.5**	**623.4 ± 58.1**	**1.75 ±0.026**
493-508	TSFDFKQLIHGLHSSQ (No modification)	Pepsin			
496-508	DFKQLIHGLHSSQ (No modification)	Pepsin			
496-509	DFKQLIHGLHSSQF (No modification)	Pepsin			
497-504	FKQLIHGL (No modification)	Pepsin			
497-508	FKQLIHGLHSSQ (No modification)	Pepsin			
498-508	KQLIHGLHSSQ (No modification)	Pepsin			
499-504	QLIHGL (No modification)	Chy/Tryp			
503-509	GLHSSQF (No modification)	Chy/AspN			
505-509	HSSQF (No modification)	Chy/Tryp			
510-528	**AGFEDLNYPGNIGNCAQCH (Not detected)**				
529-542	INDSTGISTVALPL (No modification)	Chy/Tryp			
538-544	VALPLNA (No modification)	Pepsin			
543-549	NAAVQPL (No modification)	Chy/Tryp Chy/AspN			

545-551	AVQPLAL (No modification)	Pepsin			
545-555	AVQPLALNNGT (No modification)	Pepsin			
545-556	AVQPLALNNGTF (No modification)	Pepsin			
545-561	AVQPLALNNGTFTSPIA (No modification)	Pepsin			
545-562	AVQPLALNNGTFTSPIAA (No modification)	Pepsin			
552-561	NNGTFTSPIA (No modification)	Pepsin			
562-571	**AVCSNCHSSD (Not detected)**				
572-584	**ATQNHMRQQGAVF (N575, H576 or M577 is modified)**	**Pepsin**	**224.2 ± 9.52**	**193.6 ± 9.33**	**1.16 ± 0.007**
585-599	AGTKADATAGTETC*A (No modification)	Pepsin			
600-608	**FCHGQGTVA (Not detected)**				
601-612	C*HGQGTVADVLK (No modification)	Chy/Tryp			
609-627	DVLKVHPINDDDDKLKGEL (No modification)	Pepsin			
610-627	VLKVHPINDDDDKLKGEL (No modification)	Pepsin			
612-623	KVHPINDDDDKL (No modification)	Pepsin			

Letters with underline or red color indicate modified amino acid residues by hydroxyl radicals or the CXXCH cytochrome *c* binding motif, respectively.

Carbamidomethylated Cys is shown as C with an asterisk.

[a]Modified amino acid residues are identified by MS/MS ~~(see Fig. S5)~~.

[b]Peptidases, pepsin, chymotrypsin and trypsin (Chy/Tryp) or chymotrypsin and Asp-N (Chy/AspN), were used for MtrF digestion.

[c]Rate constants were calculated with Origin software by using a non-linear fit of hydroxyl radical modification data to a first order decay and errors in the rate constants are calculated from the non-linear fit (see Fig. S5).

Table S3. Strains and plasmids used in this study

Strain or plasmid name	Genotype or description
Plasmids	
pBAD202D	F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA*1 *nupG*
LS271	MtrF in pBAD202D, with C-terminus 6xHis and V5 epitope. Arabinose inducible (Gift from Liang Shi)
I5049	pSB1ET2 containing *S. oneidensis* CymA and MtrCAB[12]
I5077	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF[a]
I5083	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-L460A[a]
I5085	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-L460D[a]
I5086	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-L460K[a]
I5087	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-3A (MtrF-L460A/F512A/L515A)[a]
I5088	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-3D (MtrF-L460D/F512D/L515D)[a]
I5089	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-3K (MtrF-L460K/F512K/L515K)[a]
I5090	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-AA608-9 (MtrF-D609A)[a]
I5091	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-DD608-9 (MtrF-A608D)[a]
I5092	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-KK608-9 (MtrF-A608K/D609K)[a]
I5095	pBAD202D containing *S. oneidensis* $MtrB_{signal}$-MtrF-5K (MtrF-L460K/F512K/L515K/A608K/D609K)[a]
Strains	
E. coli Mach1	*lacZ*ΔM15 *hsdR lacX*74 *recA endA tonA* (Invitrogen)
E. coli DH5α	F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17(rk-, mk+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ-
MFe699	*E. coli* Mach1 possessing I5077
MFe775	*E. coli* DH5α possessing I5083
MFe777	*E. coli* DH5α possessing I5085
MFe778	*E. coli* DH5α possessing I5086
MFe779	*E. coli* DH5α possessing I5087
MFe780	*E. coli* DH5α possessing I5088
MFe781	*E. coli* DH5α possessing I5089
MFe782	*E. coli* DH5α possessing I5090
MFe783	*E. coli* DH5α possessing I5091
MFe784	*E. coli* DH5α possessing I5092
MFe787	*E. coli* DH5α possessing I5095
E. coli WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*(wt)] (Saltikov and Newman-2003)
MFe774	*E. coli* WM3064 possessing I5077
MFe788	*E. coli* WM3064 possessing I5083
MFe790	*E. coli* WM3064 possessing I5085
MFe791	*E. coli* WM3064 possessing I5086
MFe792	*E. coli* WM3064 possessing I5087
MFe793	*E. coli* WM3064 possessing I5088
MFe794	*E. coli* WM3064 possessing I5089
MFe795	*E. coli* WM3064 possessing I5090
MFe796	*E. coli* WM3064 possessing I5091
MFe797	*E. coli* WM3064 possessing I5092
MFe798	*E. coli* WM3064 possessing I5095

S. oneidensis MR-1

MFm029	*S. oneidensis* MR-1 possessing I5077 for expressing MtrF
MFm044	*S. oneidensis* MR-1 possessing I5090 for expressing MtrF-AA608-9 (MtrF-D609A)
MFm045	*S. oneidensis* MR-1 possessing I5091 for expressing MtrF-DD608-9 (MtrF-A608D)
MFm046	*S. oneidensis* MR-1 possessing I5092 for expressing MtrF-KK608-9 (MtrF-A608K/D609K)
MFm047	*S. oneidensis* MR-1 possessing I5083 for expressing MtrF-L460A
MFm049	*S. oneidensis* MR-1 possessing I5085 for expressing MtrF-L460D
MFm050	*S. oneidensis* MR-1 possessing I5086 for expressing MtrF-L460K
MFm051	*S. oneidensis* MR-1 possessing I5087 for expressing MtrF-3A (MtrF-L460A/F512A/L515A)
MFm052	*S. oneidensis* MR-1 possessing I5088 for expressing MtrF-3D (MtrF-L460D/F512D/L515D)
MFm053	*S. oneidensis* MR-1 possessing I5089 for expressing MtrF-3K (MtrF-L460K/F512K/L515K)
MFm054	*S. oneidensis* MR-1 possessing I5095 for expressing MtrF-5K (MtrF-L460D/F512D/L515D/A608K/D609K)

[a]These plasmids contain a signal sequence of MtrB in *S. oneidensis* MR-1.

Table S4. Primers used in this study

Primer name	Sequence (5'→3')	Constructed plasmids for this primer
MtrF no lipidation Fwd	GGAGGCAGTGATGGTGATGA	I5077
LS271 upstream-reverse	GGGATGTATATCTCCTTAGGT	I5077
MtrB Nterm Fwd	AAGGAGATATACATCCCATGAAATTTAAACTCA ATTTGATC	I5077
MtrB Nterm Rev	CACCATCACTGCCTCCATCAGCAGCGACGG	I5077
MtrF-wt-R (5'-phosphated)	ATGGCAGCTAGCACATTTTGCATTA	I5085
MtrF-L460D-F (5'-phosphated)	GGCGATCAGCAAgatAACATCCATG	I5085
MtrF-L460A-F	CGATCAGCAAgcgAACATCCATGGC	I5083
MtrF-L460K-F	CGATCAGCAAaaaAACATCCATGGC	I5086
MtrF-L460-R	CCATGGCAGCTAGCACAT	I5083, I5086
MtrF-3A-F	GACgccAATTACCCTGGGAATATCGG	I5087
MtrF-3A-R	TTCggcACCTGCAAATTGGCTGCT	I5087
MtrF-3D-F	GACgatAATTACCCTGGGAATATCGG	I5088
MtrF-3D-R	TTCatcACCTGCAAATTGGCTGCT	I5088
MtrF-3K-F	GACaaaAATTACCCTGGGAATATCGG	I5089
MtrF-3K-R	TTCtttACCTGCAAATTGGCTGCT	I5089
MtrF-AD608-R	GACAGTGCCTTGTCCGTGGCAAAATG	I5090, I5091, I5092, I5095
MtrF-D609A-F	GCCgccGTACTCAAAGTCCATCCAATAAACGATG	I5090
MtrF-A608D-F	gacGACGTACTCAAAGTCCATCCAATAAACGATG	I5091
MtrF-KK608-F	aagaaggtactcaaagtccatccaataaacgatg	I5092, I5095

*Bases with underlines or double underlines are homologous regions for Gibson assembly to construct I5077.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. *S. oneidensis* MtrF with a signal sequence from MtrB, can be purified and is redox active. (A) Purified MtrF protein solution. (B) SDS-PAGE of purified MtrF. (C) Enhanced Chemiluminescence (ECL) assay of purified the MtrF solution. (D) MtrF spectrum under reduced condition (blue line) and non-reduced condition (red line). The peaks α, β and γ indicate the cyt *c* specific peaks. (E) MtrF spectrum after 24 hrs-incubation at room temperature at pH 3 (blue line), pH 4 (red line), pH 6 (black line), or pH 8 (pink line). The spectrum shown by the green line is a control sample at pH 7 without incubation. (F) ESI-MS analysis of MtrF. The many charged mass peaks were detected and deconvoluted to measure a value of 75,192.0 kDa for the molecular weight. (The theoretical molecular weight of MtrF

is 75,180.5 Da). (G) The primary sequence of recombinant MtrF. The signal sequence of MtrB (underline) is cleaved when the protein is secreted in the culture. Red letters are the position of heme insertion and blue letters indicate the identified or predicted disulfide bonds[13].

Fig. S2. (A) STEM image of α-Fe_2O_3 nanoparticles used in this study. (B) Histogram of size of the α-Fe_2O_3 nanoparticles measured based on the STEM data. The average of the nanoparticles diameter is 27 ± 19 nm. (C) FQ assay of MtrF for α-Al_2O_3 nanoparticles shows that MtrF does not bind to the nanoparticles. The assay was performed at pH 6 and the buffer sample (black triangles) was added 50 mM MES-NaOH (pH 6) without any nanoparticles.

Fig. S3. Peptidase footprinting (FP) mapping shows that heme 6-7 and 10 regions are protected by α-Fe_2O_3 nanoparticles from the peptidase digestion. MtrF alone (panel A) and the α-Fe_2O_3:MtrF complex (panel B) were digested with chymotrypsin (pH 7), trypsin (pH 7), or pepsin (pH 4). The peptidase FP was performed in three independent experiments. Gray and black bars indicate the identified peptide fragments digested by pepsin in two and three independent experiments, respectively. Light blue and blue bars are the identified peptide fragments digested by chymotrypsin in two and three independent experiments, respectively. Green bars are the identified peptide fragments digested by trypsin. Peptide fragments identified in only the MtrF alone sample are shown in bars with red frames. Regions in which the peptidase(s) cannot digest the specific amino acid residues due to protection by the nanoparticles are shown in red boxes with arrowheads, no digestion by pepsin; green box with an arrowhead, no digestion by chymotrypsin; blue boxes with arrowheads, no digestion by pepsin and chymotrypsin; black boxes with arrowhead, amino acid residues, R443 and G534, possibly not to be protected due to non-digestion of the other amino acid residues [L460 and F512, respectively] of the peptides). Red, green, and blue letters are amino acid residues digested by pepsin (pH 4), chymotrypsin or trypsin (pH 7), and

pepsin/chymotrypsin or pepsin/trypsin (pH4 and 7), respectively, in both MtrF alone and α-Fe_2O_3:MtrF samples.

Fig. S4. Amino acid residues protected from protease digestions by Fe_2O_3 nanoparticles binding are not clustered in primary sequence or secondary structure. Light brown and gray highlights indicate α-helices and β-sheets, respectively. Pink letters are heme-insertion motifs. Red letters with yellow highlight are amino acid residues protected from protease digestions by the nanoparticle binding. These residues are located in less conserved regions among MtrF, MtrC and OmcA.

Fig. S5. XFMS analysis shows that modification of several peptides in MtrF by hydroxyl radicals is prevented within the α-Fe_2O_3:MtrF complex. Graphs are shown as decay of non-oxidation peptides. Black and red graphs are the decay of MtrF alone and α-Fe_2O_3:MtrF samples, respectively. Red letters of peptide sequences indicate amino acid residues modified by hydroxyl radicals and the modified residues are identified by LC-MS/MS. Rate constants (*k*) were calculated with Origin software by using a non-linear fit of hydroxyl radical modification data to a first order decay and R values are the ratio of rate constants (MtrF alone/the complex, α-Fe_2O_3:MtrF) (see Table S1 and S2).

Fig. S6. (A and B) SDS-PAGE (A) and ECL assay for detecting heme proteins (B) of purified point-mutated MtrF. Lane 1, MtrF-5K (L460K, F512K, L515K, A608K, D609K); lane 2, MtrF-L460A (L460A); lane 3, MtrF-L460D (L460D); lane 4, MtrF-L460K (L460K); lane 5, MtrF-3A (L460A, F512A, L515A); lane 6, MtrF-3D (L460D, F512D, L515D); lane 7, MtrF-3K (L460K, F512K, L515K); lane 8, MtrF-AA608-9 (D609A); lane 9, MtrF-DD608-9 (A608D); lane 10, MtrF-KK608-9 (A608K, D609K); lane 11, wild-type MtrF. ECL assay was performed using a membrane which proteins are blotted on after the purified proteins in the solution had been separated by SDS-PAGE. (C and D) FQ assays of MtrF

point-mutations for α-Fe_2O_3 nanoparticles at pH 7. Panel C, FQ of MtrF-L460A (green circles), MtrF-L460D (pink triangles), MtrF-L460K (blue diamonds), and the wild-type, MtrF (red circles); panel D, FQ of MtrF-AA608-9 (green circles), MtrF-DD608-9 (pink triangles), MtrF-KK608-9 (blue diamonds), and the wild-type, MtrF (red circles).

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