Neuroglobin is capable of self-oxidation of methionine64 introduced at the heme axial position

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Supporting Information

Contents

1. Experimental Section

1.1 Protein preparation

The pET3a plasmid DNA containing the gene of wild-type (WT) human Ngb was a gift from Prof. T. Burmester, Gutenberg University of Mainz, Germany. The H64M Ngb and H64M/C120S Ngb genes were constructed using the QuikChange Site Directed Mutagenesis Kit (Stratagene) with two primers: H64M, forward, 5'-GAG TTC CTG GAC ATG ATC AGG AAG GTG-3', and reverse, 5'-CAC CTT CCT GAT CAT GTC CAG GAA CTC-3'; C120S, 5'- ATG CTG GAG AAG TCT CTG GGC CCT GCC-3', and reverse, 5'-GGC AGG GCC CAG AGA CTT CTC CAG CAT-3'. The mutations were confirmed by DNA sequencing assay. Both mutants were expressed in BL21(DE3) and purified using a similar procedure for WT Ngb.[1] After eluted from DEAE column with 200 mM NaCl, the protein was concentrated by Amicon filtration (PM10) and oxidized with $K_3[Fe(CN)_6]$. It was then passed though a Sephacryl S-100 column, followed by further purification using a Mono Q column and eluted with a linear gradient of 1 M NaCl.

1.2 UV-Vis and stopped-flow studies

UV-Vis spectrum of ferric Ngbs were recorded in 100 mM potassium phosphate buffer (pH 7.0) on a Agilent 8453 diode array spectrometer. The ferrous protein samples were obtained by addition of a small amount of sodium dithionite. The protein concentration was determined with an extinction coefficient of $\varepsilon_{405} = 160 \pm 5$ mM^{-1} cm^{-1} for both H64M Ngb and H64M/C120S Ngb mutants, as calculated using the standard hemochromagen method.[2] In another study, after addition of DTT to a final concentration of 0.5 mM, the spectral changes of H64M/C120S Ngb (10 μM) were collected at different time courses at 25 ^{o}C.

Kinetic UV-Vis studies of H64M/C120S Ngb in reaction with dithiothreitol (DTT), *m*-chloroperbenzoic acid (*m*-CPBA) or H_2O_2, were performed with a SF-61DX2 Hi-Tech KinetAsyst^{TM} dual mixing stopped-flow spectrophotometer. Typically, one syringe contains 20 μM protein (100 mM potassium phosphate buffer, pH 7.0), and the second syringe contains 1 mM DTT, 0.2 mM *m*-CPBA or H_2O_2. The reaction was stated with mixing of equal volume of solutions from the both syringes. 500 (or 50) time-dependent spectra were collected over 30 s (or 0.5 s) from 350 to 700 nm at 25 ^{o}C.

1.3 EPR studies

Electron Paramagnetic Resonance (EPR) spectra of WT and H64M Ngb (0.3 mM) in 100 mM potassium phosphate buffer (pH 7.0), containing 10% glycerol, were collected at the high magnetic field laboratory of Chinese Academy of Science (Hefei, China). The samples were analyzed by X-band EPR on a Bruker EMX plus 10/12 spectrometer. A standard Bruker cavity (ER4119hs TE011) was used in conjunction with an Oxford Instrument EPR910 liquid helium continuous-flow cryostat for low-temperature analysis. The spectra were measured at 10 K, with frequency of 9.43 GHz, center field 2200 G and sweep width 3600 G, microwave power 2 mW and modulation amplitude 2.0 G. The EPR spectra H64M/C120S Ngb (0.5 mM) and in reaction with DTT (50 mM) for 15, 30, 45 min, 1, 2 and 12 h, or in reaction with H_2O_2 (0.5 mM) for 30 s, 1, 5 and 10 min, were recorded on a Bruker A300 spectrometer (X-band), equipped with Bruker ER4141VTM liquid nitrogen system, as available in the authors' lab. The spectra were measured at 100 K, with frequency of 9.43 GHz, center field 2200 G and sweep width 3600 G, microwave power 2.0 mW and modulation amplitude 3.0 G.

1.4 Mass spectrometry

Protein mass spectrum measurement was carried out on G2-XS QToF mass spectrometer (Waters). The desalted protein solution (~20 μM) was mixed with 1% formic acid and transferred into the mass spectrometer chamber for measurement under positive mode. The multiple m/z peaks were transformed to the protein molecular weight by using software MaxEnt1. For trypsin digestion studies, 50 μL H64M Ngb and H64M/C120S Ngb (~0.1 mM) in ammonium carbonate buffer (50 mM, pH 7.8) was mixed with the same volume of trypsin solution (1 mg/mL) in the same buffer, and the mixture was heated at 37 ^{o}C for 12 h, followed by addition of DTT (10 mM) and treatment at 37 ^{o}C for 3 h, before determining the MS of the digested protein fragments. The mass spectra of the protein after reaction with 10 eq. *m*-CPBA, H_2O_2 or $K_3[Fe(CN)_6]$ for 10 min were determined used the same procedure.

References:

1. S. Dewilde, L. Kiger, T. Burmester, et al., *J. Biol. Chem.*, 2001, 276, 38949-38955.
2. M. Morrison, S. Horie, *Anal. Biochem.*, 1965, 12, 77-82.

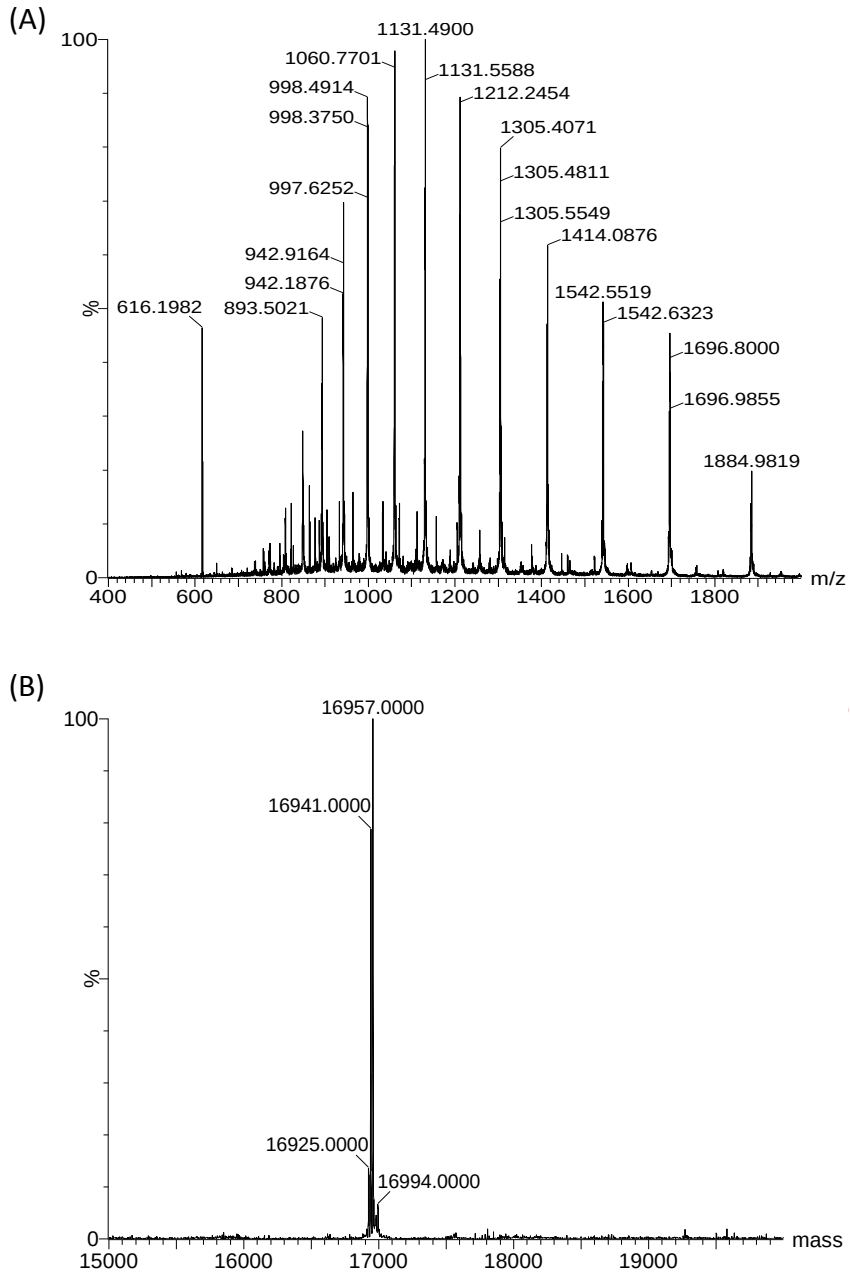

Fig. S1 ESI-MS spectra of H64M Ngb as purified. (A) Original multiply-charged series. (B) The MaxEnt survey spectrum showing the major components. Note that the calculated mass of H64M Ngb with an intramolecular disulfide bond of Cys46-Cys55 is 16925.5 Da.

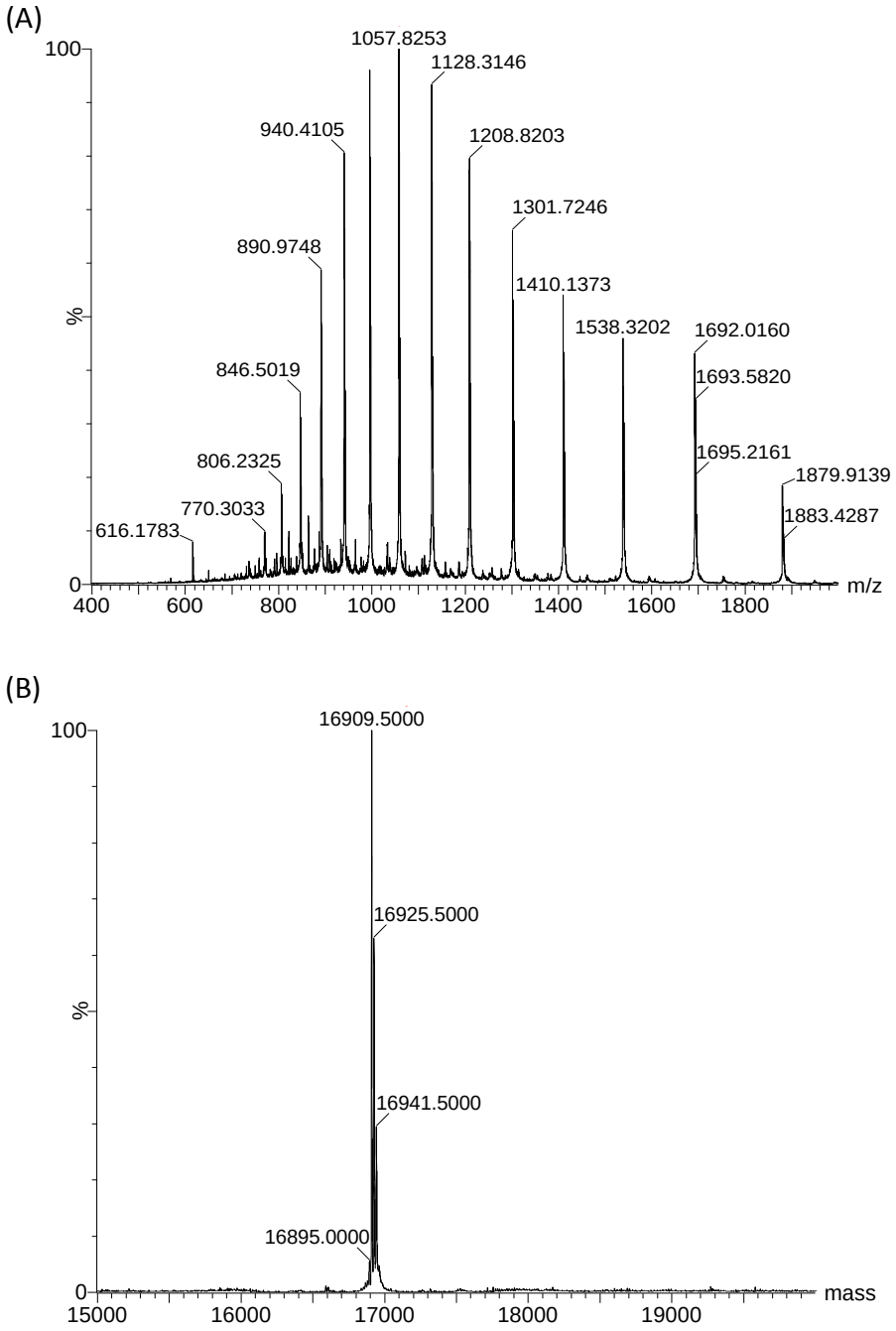

Fig. S2 ESI-MS spectra of H64M/C120S Ngb as purified. (A) Original multiply-charged series. (B) The MaxEnt survey spectrum showing the major components. Note that the calculated mass of H64M/C120S Ngb with an intramolecular disulfide bond of Cys46-Cys55 is 16909.4 Da.

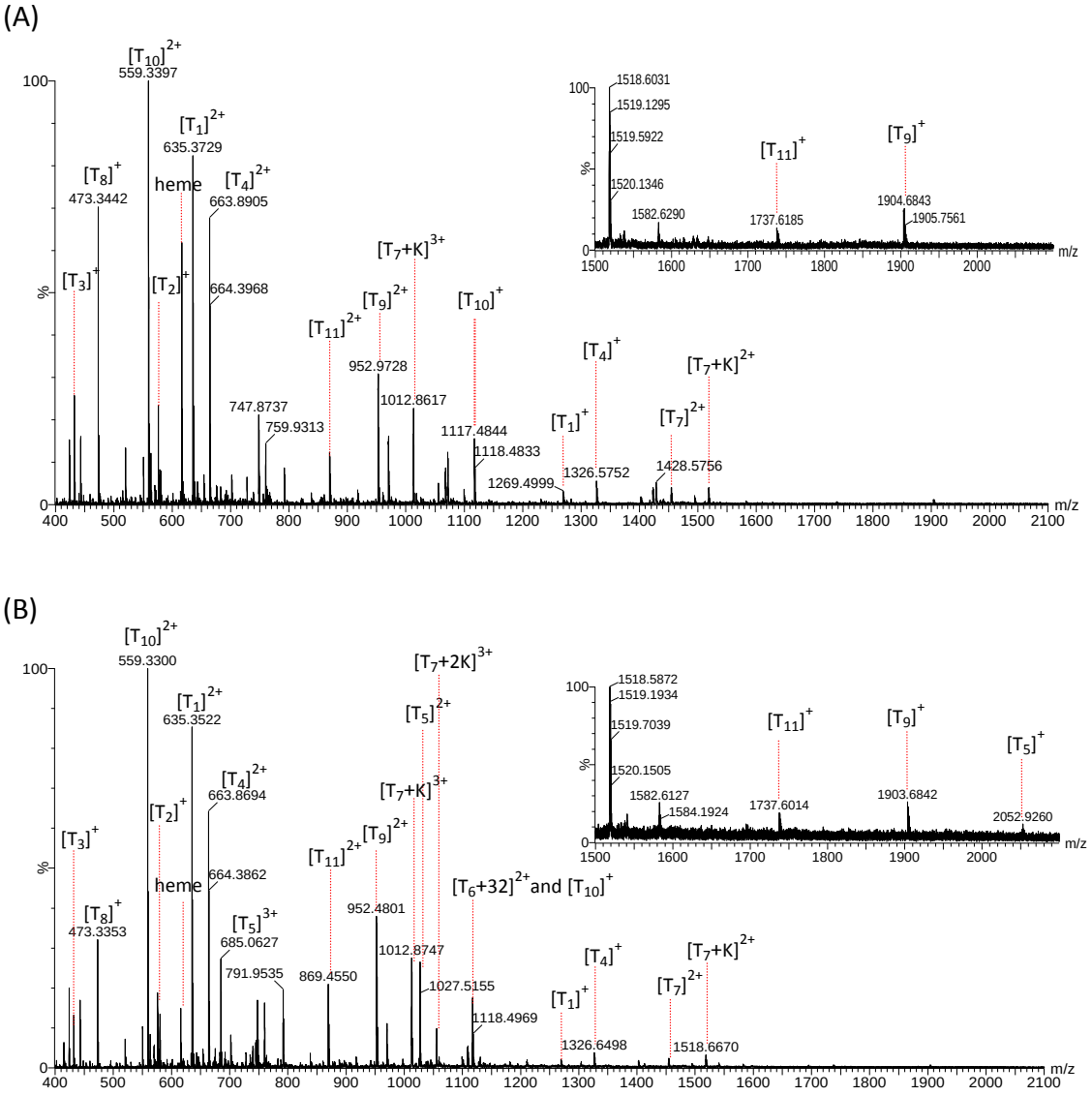

Fig. S3 ESI-MS spectra of trypsin-digested (12 h) H64M/C120S Ngb without (A) and with (B) treated by DTT at 37 ^{o}C for 3 h. The mass spectra in the 1500–2100 m/z region are shown as insets.

(A)

MERPEPELIR | QSWR | AVSR | SPLEHGTVLFAR | LFALEPDLLPLFQYN[46]CR |

T1	T2	T3	T4	T5
1269.65	576.28	432.25	1326.71	2052.05

QFSSPED[55]CLSSPEFLD[64]**M**IR | K | V[69]**M**LVIDAAVTNVEDLSSLEEYLASLGR | K | HR |

T6	T7
2200.98	2906.49

AVGVK | LSSFSTVGESLLY[116]**M** LEK | [120]**C**LGPAFTPATR | AAWSQLYGAVVQA[144]**M** SR | GWDGE

T8	T9	T10	T11	T12
473.30	1903.96	1133.57	1737.87	563.20

(B)

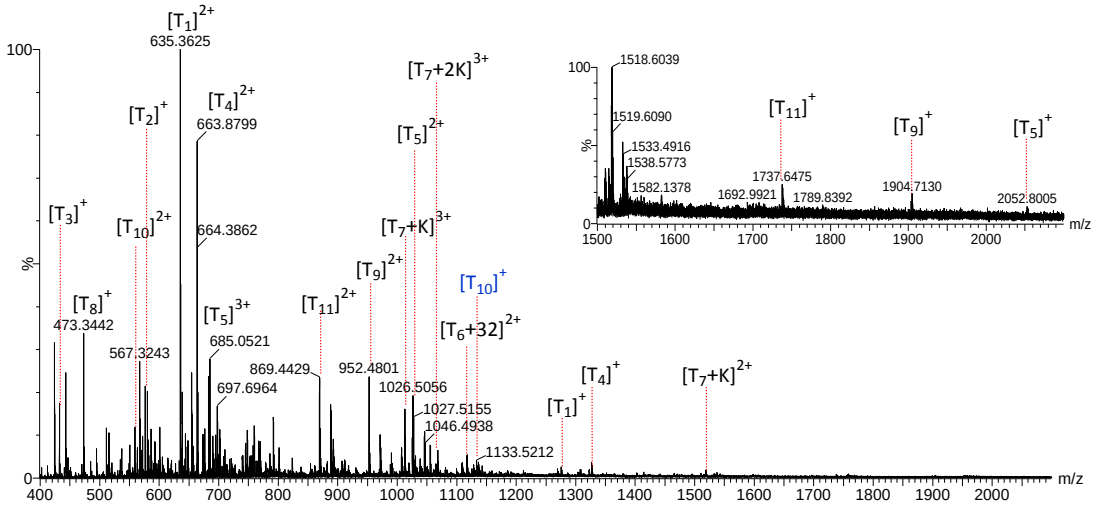

Fig. S4 (A) Amino acid sequence of H64M Ngb and the expected peptide fragments with molecular weight larger than 400 Da obtained by trypsin digestion. (B) ESI-MS spectrum of trypsin-digested (12 h) H64M Ngb treated by DTT (10 mM) at 37 ^{o}C for 3 h. The mass spectrum in the 1500–2100 m/z region is shown as an inset.

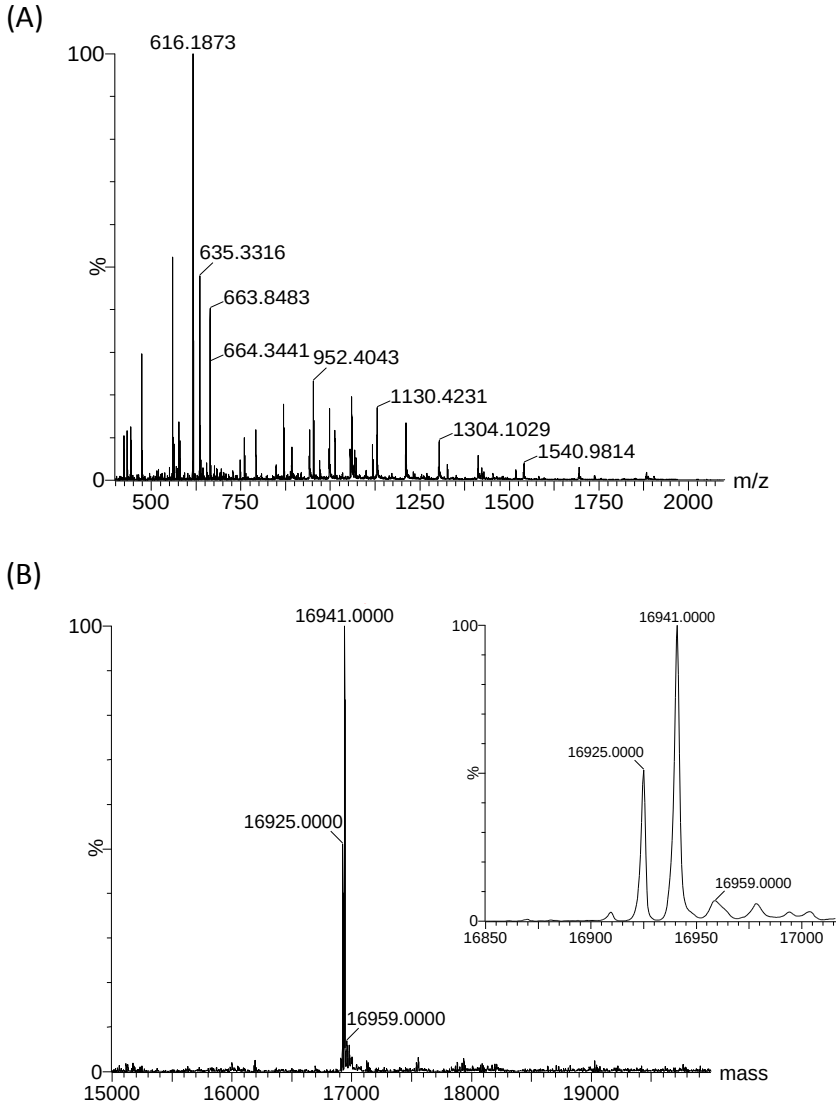

Fig. S5 ESI-MS spectra of H64M/C120S Ngb after digestion by trypsin with DTT (10 mM) at 37 ^{o}C for 5 h before fully digested. (A) Original multiply-charged series. (B) The MaxEnt survey spectrum showing the major components. Note that the calculated mass of H64M/C120S Ngb with an intramolecular disulfide bond of Cys46-Cys55 is 16909.4 Da, which was almost converted to SO-Met (+16 Da) and SO_2-Met (+32 Da) species (inset).

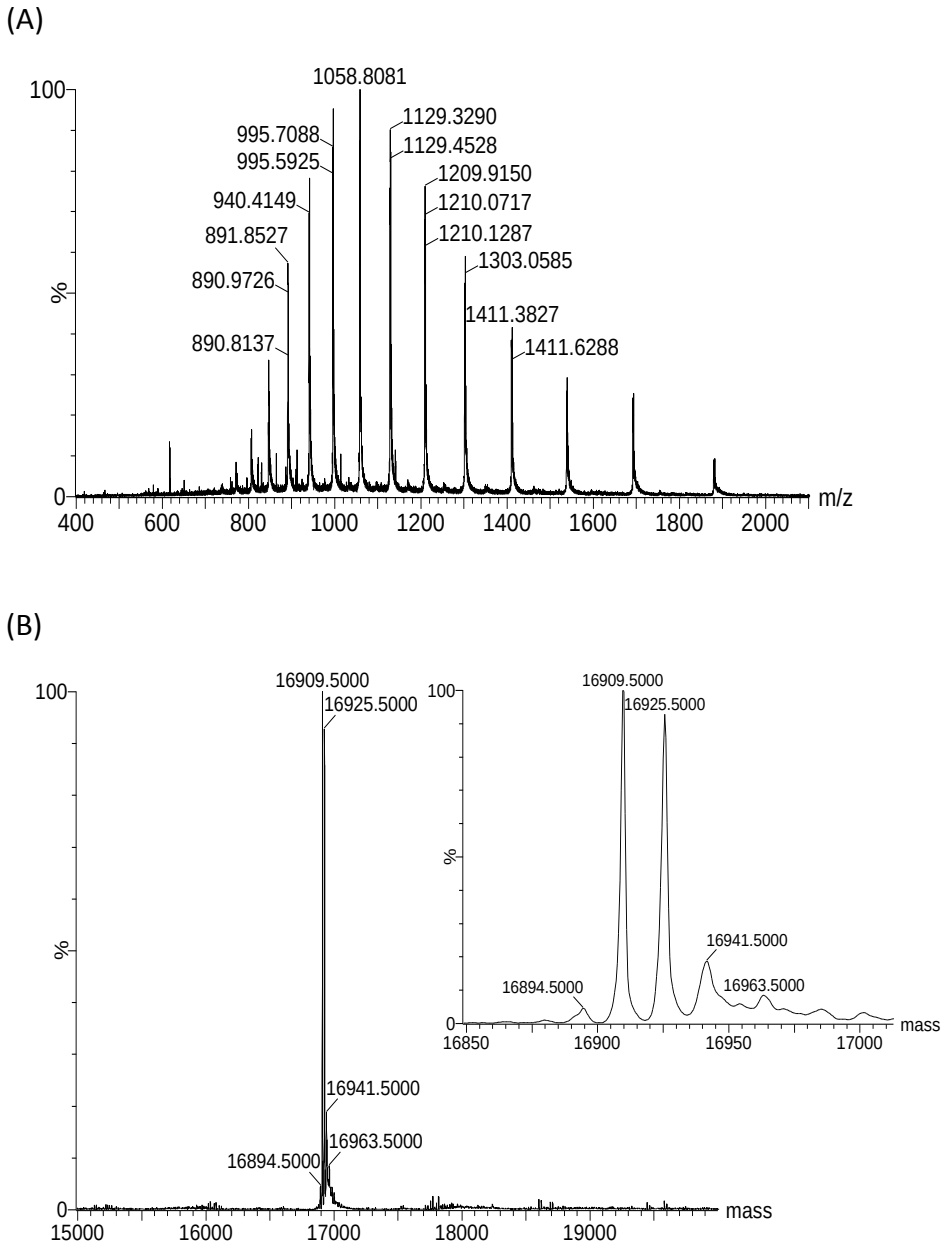

Fig. S6 ESI-MS spectra of H64M/C120S Ngb (10 μM) after incubation with 1 mM DTT in air for 10 min. (A) Original multiply-charged series and (B) the MaxEnt survey spectrum showing the major components.

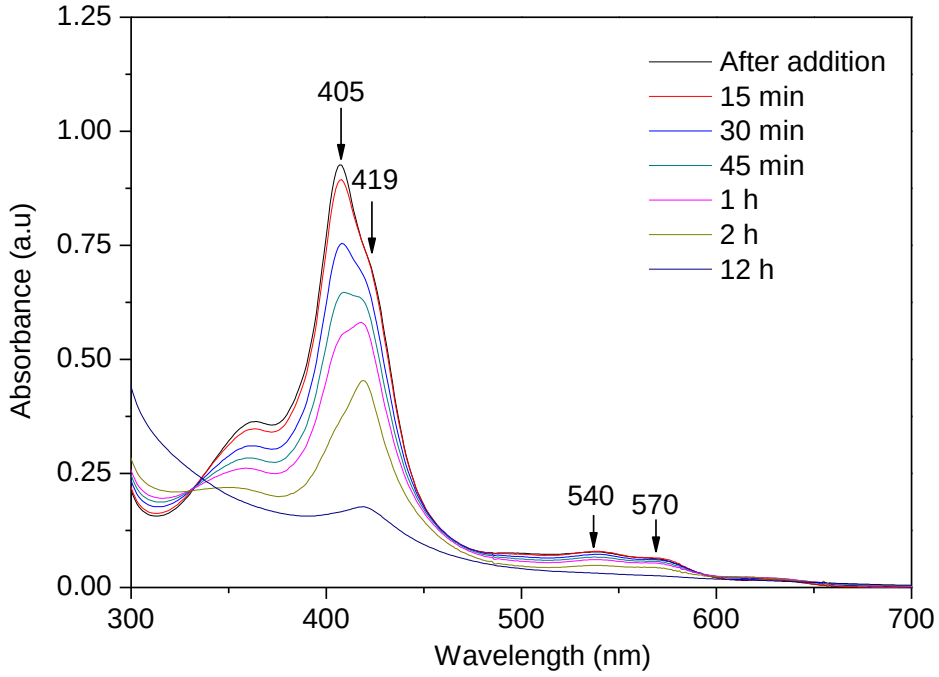

Fig. S7 UV-Vis spectral changes of H64M/C120S Ngb (10 μM) in presence of DTT (0.5 mM) and in 100 mM potassium phosphate buffer (pH 7.0).

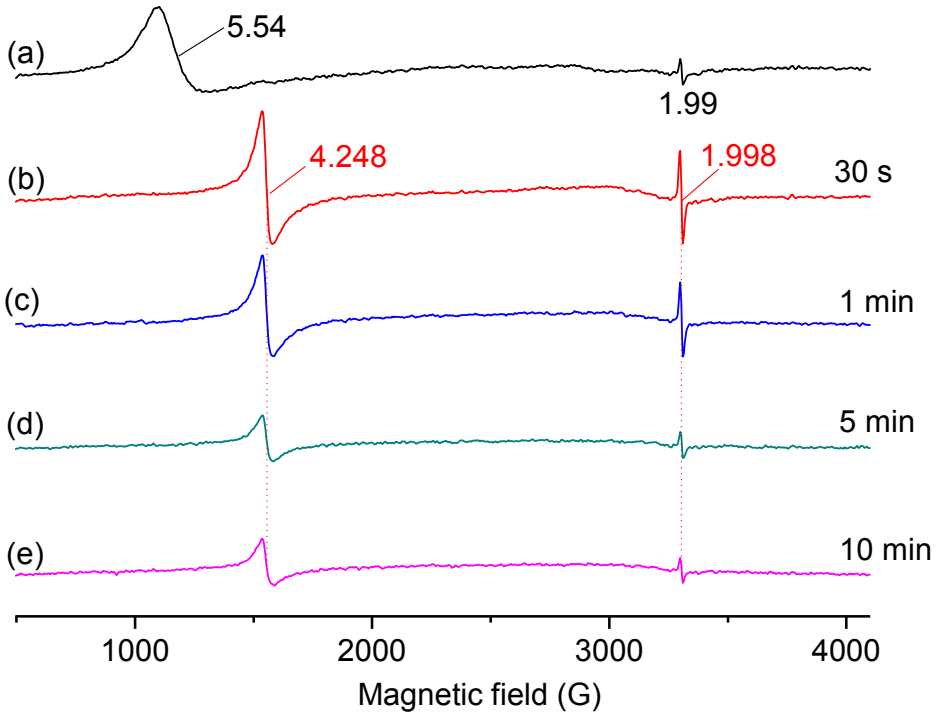

Fig. S8 EPR spectra of ferric H64M/C120S Ngb (0.5 mM) and in reaction with H_2O_2 (0.5 mM) in air for 30 sec, 1, 5 and 10 min.

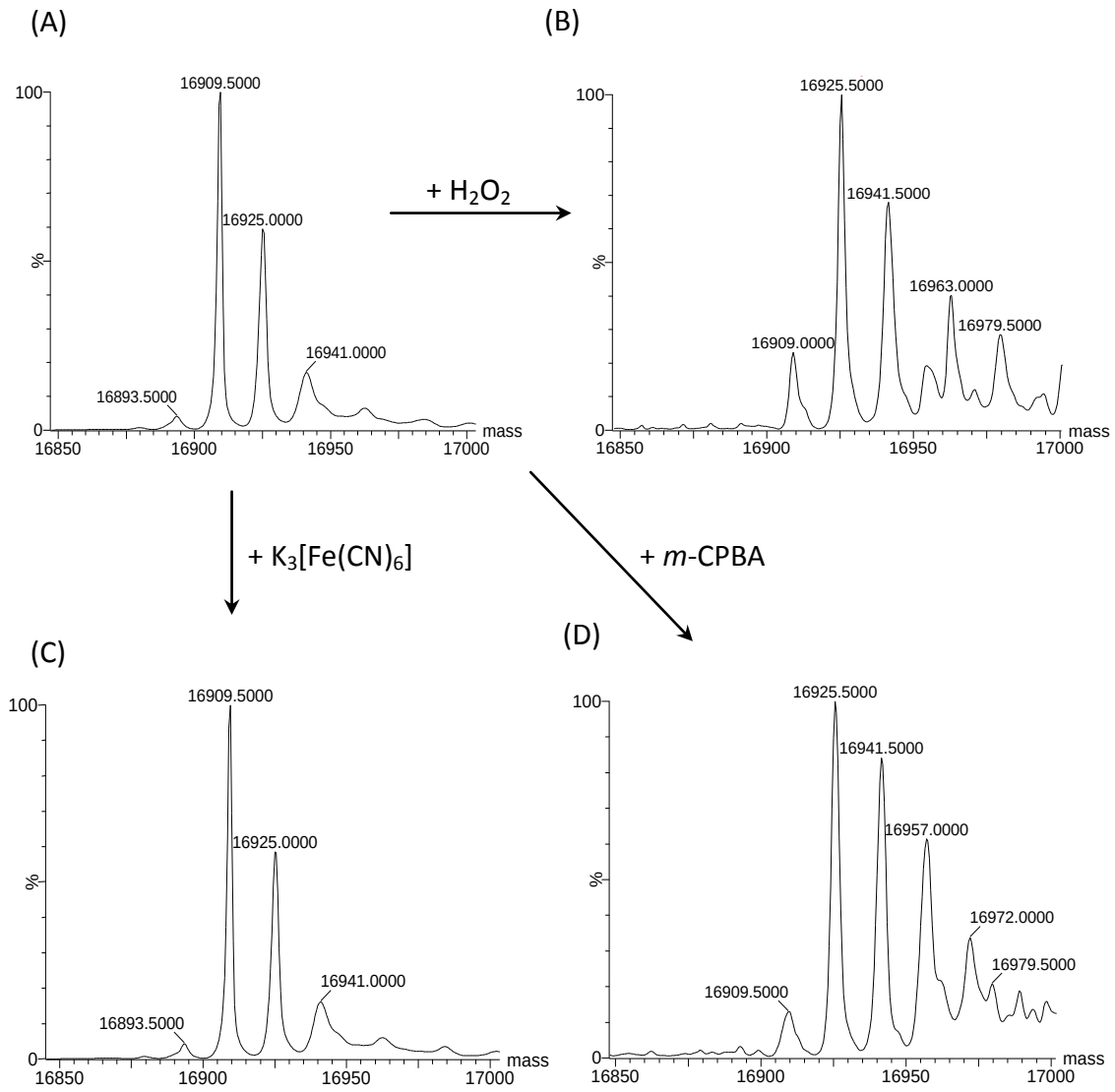

Fig. S9 ESI-MS spectra of H64M/C120S Ngb (A) and after addition of 10 eq. H_2O_2 (B), $K_3[Fe(CN)_6]$ (C), and *m*-CPBA (D), respectively, for 10 min at room temperature.

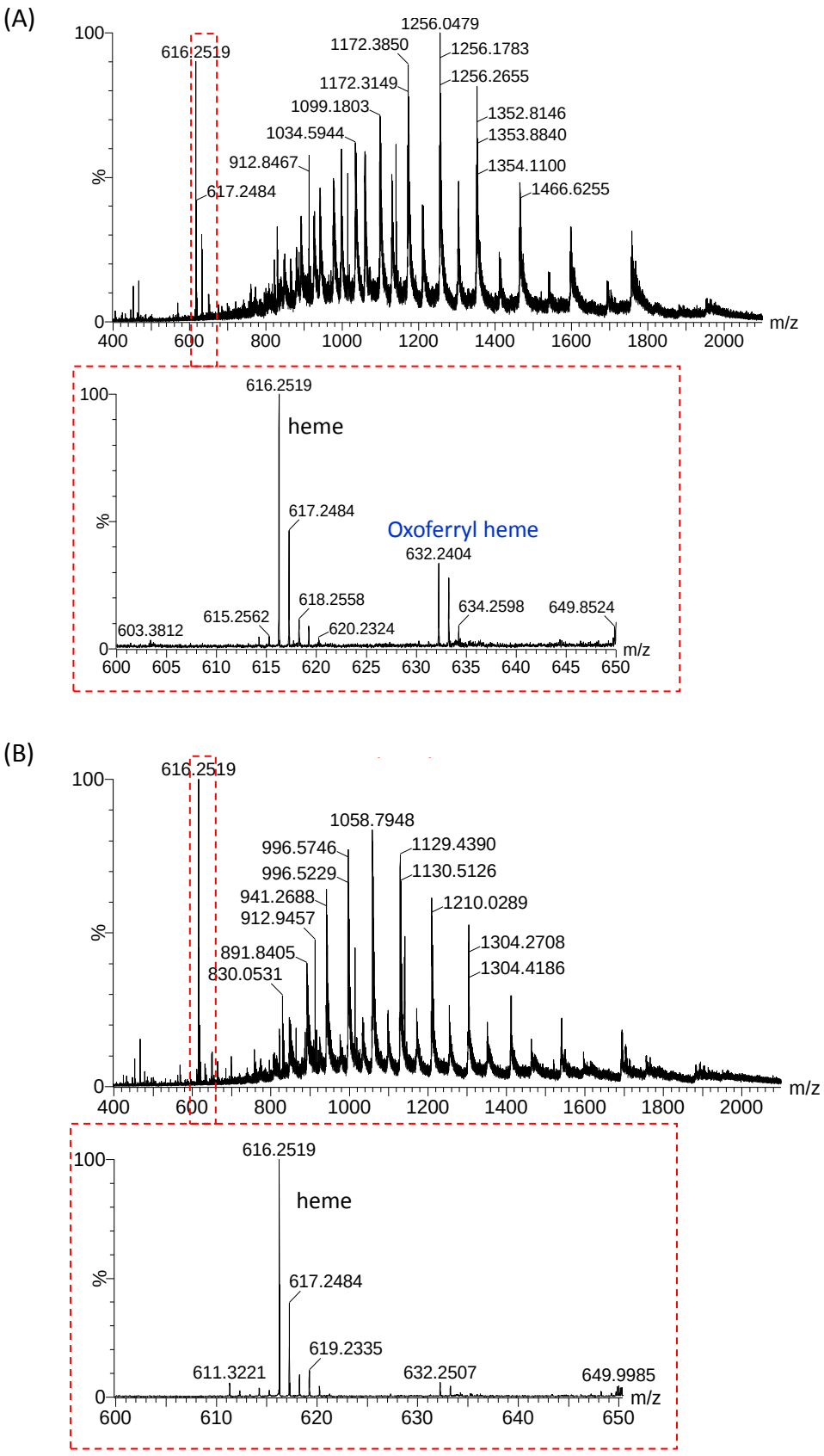

Fig. S10 Original multiply-charged series in ESI-MS spectra of H64M/C120S Ngb after addition of *m*-CPBA (A) or H_2O_2 (B) or for 10 min at room temperature.

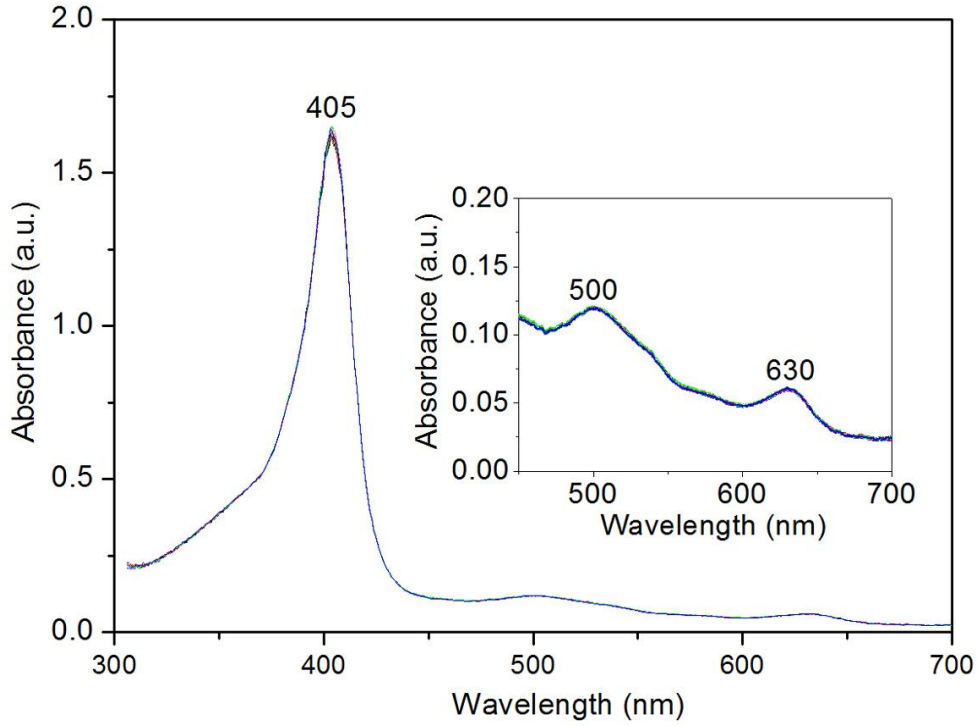

Fig. S11 Stopped-flow UV-Vis spectra of H64M/C120S Ngb (10 μM) in reaction with H_2O_2 (0.1 mM) for 0.5 sec at 25 ^{o}C.