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Supporting Information

The Importance of Asn52 in the Structure-Function Relationship of Human Cytochrome *c*

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1. Materials and Methods

1.1 Purification of human cytochrome c

The pBTR1 plasmid containing the coding sequence of wild-type human cytochrome *c* (H-Cyt *c*) gene was obtained from Addgene (No. 22468). The N52S and N52A mutations of H-Cyt *c* were constructed by using QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing. These vectors were transformed to *E. coli* BL21(DE3) cells. Cells were grown in 5 mL of LB medium supplemented with ampicillin (100 mg/L) overnight at 37 ℃. Then 0.5 mL culture was transferred to 500 mL rich medium (10 g/L Trptone, 8 g/L yeast extraction, 5 g/L NaCl) and incubated 36 hours at 37 ℃ and 220 rpm for protein expression. The reddish cells were collected by centrifugation and lysed by sonication on ice in 50 mM phosphate buffer (pH 7.0). The supernatant was loaded to CM-52 cation exchange beads and wash by 100 mM NaCl. Cyt *c* was eluted by 1 M NaCl and then concentrated by ultrafiltration. The sample was loaded into a HiPrep^{TM} 26/60 Sephacryl S-100 Gel filtration column (GE Healthcare) in 100 mM phosphate buffer (pH 7.0). Fractions containing Cyt *c* were oxidized by $K_3Fe(CN)_6$. Oxidized protein was further load into Mono S^{TM} 10/100 GL column (GE Healthcare) in 20 mM phosphate buffer (pH 7.0) and eluted with a linear gradient from 100 mM to 300 mM NaCl. Fractions with an $A_{Soret}/A_{280nm} > 4.2$ were collected. The purified protein was concentrated and stored in 100 mM phosphate buffer at pH 7.0.

1.2 UV-Vis spectroscopy

The UV-Visible spectra were measured on the Aligent 8453 diode array spectrometer. The molar extinction coefficient was measured by the standard hemochromogen method.[1] The reduced protein samples were prepared by adding a few of sodium bisulfate powders to the oxidized proteins in 100 mM phosphate buffer (pH 7.0). The degradation rate of heme was determined by adding an equal volume of H_2O_2 solution (100 mM) to protein samples (10 μM). The kinetic study of azide binding was carried out by adding an equal volume of NaN_3 solution (200 mM) to protein samples (10 μM). The spectra were recorded every 10 sec for 5 min. All the observed rate constants (k_{obs}) were calculated via single-exponential fits.

1.3 Circular dichroism spectroscopy

Circular dichroism spectra in the far-UV (190-250 nm) and visible (250-600 nm) regions were recorded using a JASCO 1500 instrument. The spectra were acquired with a scan rate of 100 nm/min. In the far-UV region experiment, samples containing 2 μM Cyt *c* in 10 mM phosphate buffer were used. In the visible region experiment, samples containing 20 μM Cyt *c* in 100 mM phosphate buffer were used.

1.4 Mass spectrometry

Protein mass spectra were carried out on the G2-XS QTQF mass spectrometry (Waters). The protein sample was dissolved in 1% formic acid water solution. In the heme degradation experiments, 100 μM H_2O_2 was added to 10 μM protein samples and then incubated for 10 min before measurement. The protein molecular weight was calculated by using MaxEnt1 software.

1.5 Stopped-flow spectroscopy

The peroxidase activities of WT, N52S, and N52A H-Cyt *c* were measured on a dual mixing stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM) by using guaiacol, 2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) and 2,4,6-trichlorophenol (TCP) as substrates and H_2O_2 as the oxidant under room temperature. Typically, one syringe contains the protein (10 μM in 100 mM potassium phosphate buffer, pH 7.0) and the substrate, while the second syringe contains H_2O_2 solution. The concentration of H_2O_2 was calculated according to the absorption of 240 nm with ε_{240nm} = 39.4 M^{-1} cm^{-1}. The reaction was stated with mixing of equal volume of solutions from the both syringes. The formation of tetraguaiacol and $ABTS^{\cdot+}$ were monitored at 470 nm and 660 nm, respectively. The dehalogenation of TCP was monitored at 272 nm. The curves of initial rates versus substrate concentrations were fitted to the Michaelis-Menten equation.

References:

1. M. Morrison and S. Horie, *Anal. Biochem.*, 1965, **12**, 77-82.

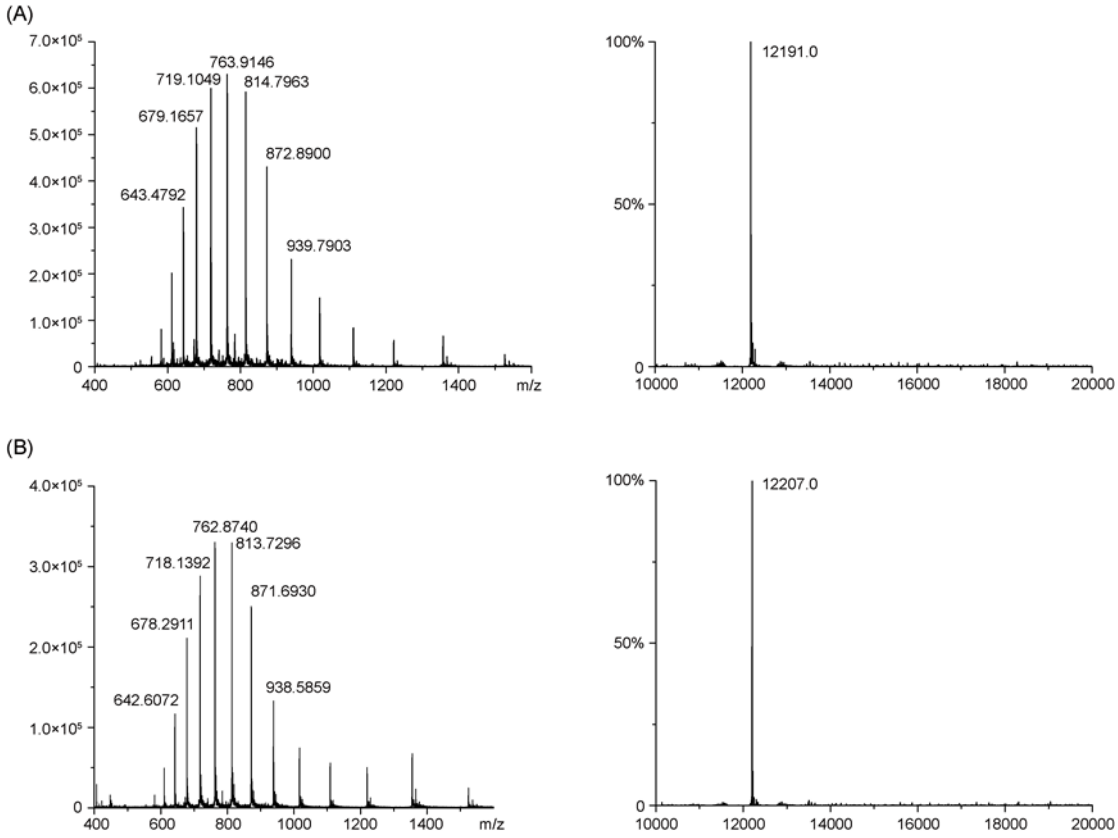

Figure S1. ESI-MS spectra of the purified N52A H-Cyt *c* (A) and N52S H-Cyt *c* (B). Molecular weight for N52A and N52S H-Cyt *c*, calculated 12190.9 Da and 12206.9 Da, observed 12191.0 Da and 12207.0 Da, respectively.

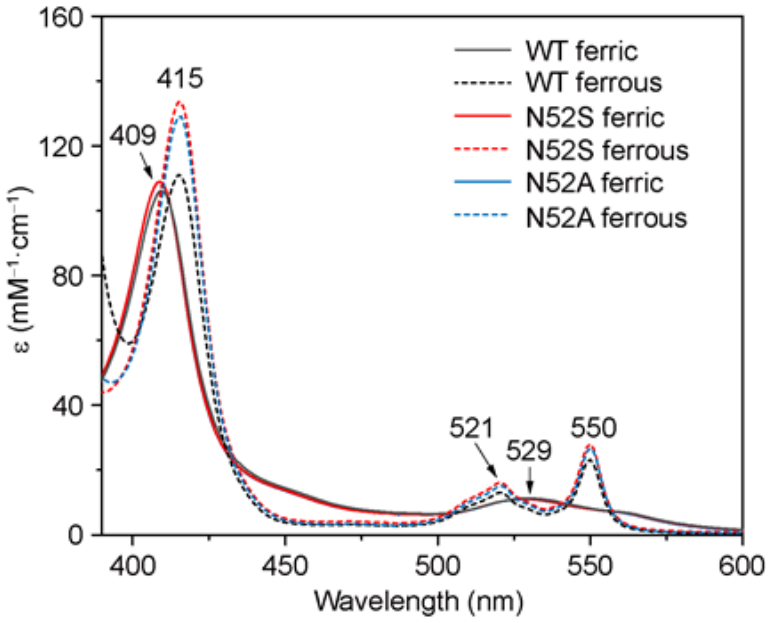

Figure S2. UV-Vis spectra of WT (black), N52S (red), and N52A (blue) H-Cyt *c*. The ferric and ferrous forms are indicated as solid and dash lines, respectively.

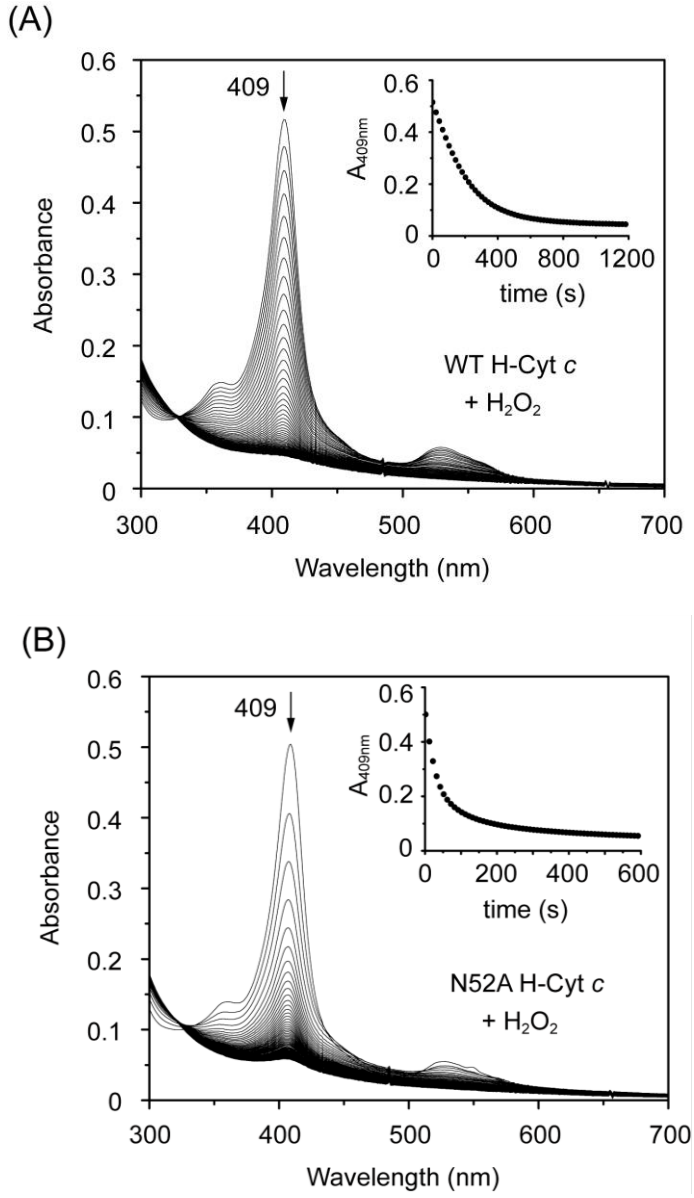

Figure S3. UV-Vis spectra of WT H-Cyt *c* (A) and N52A H-Cyt *c* (B) in reaction with 50 mM H_2O_2. Time-dependent change of the Soret band was shown as an inset.

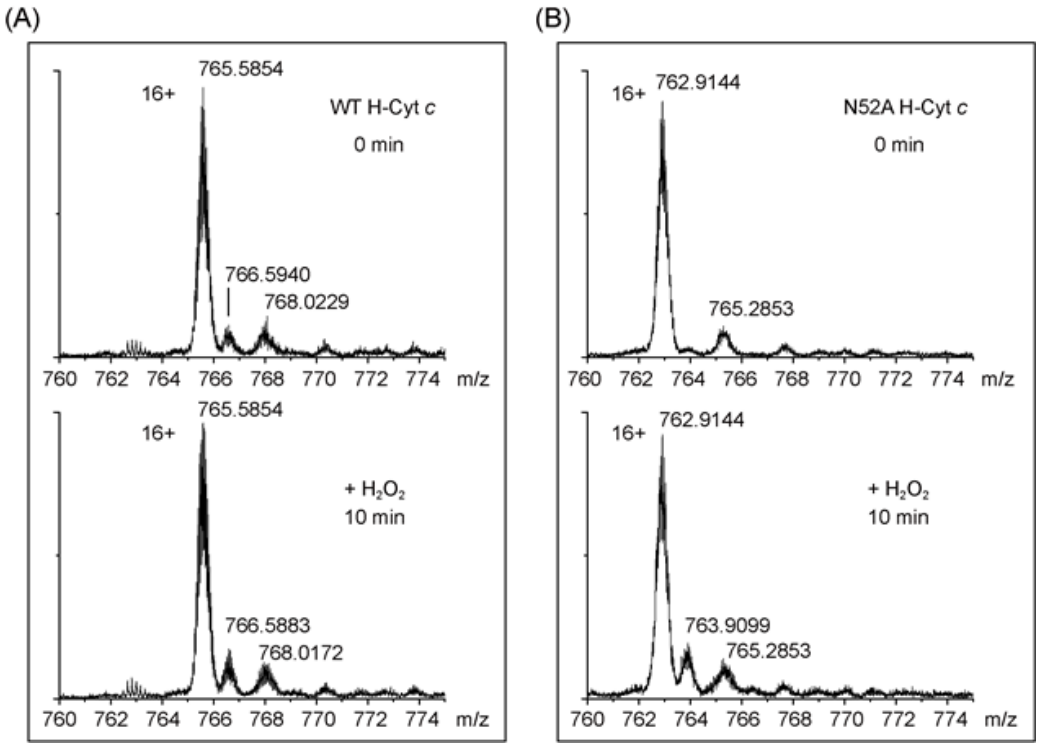

Figure S4. Mass spectra of WT H-Cyt *c* (A) and N52A H-Cyt *c* (B) acquired before and after incubation with 100 μM H_2O_2 for 10 min. The fragments with a maximum intensity (16+ charges) were shown for clarification.

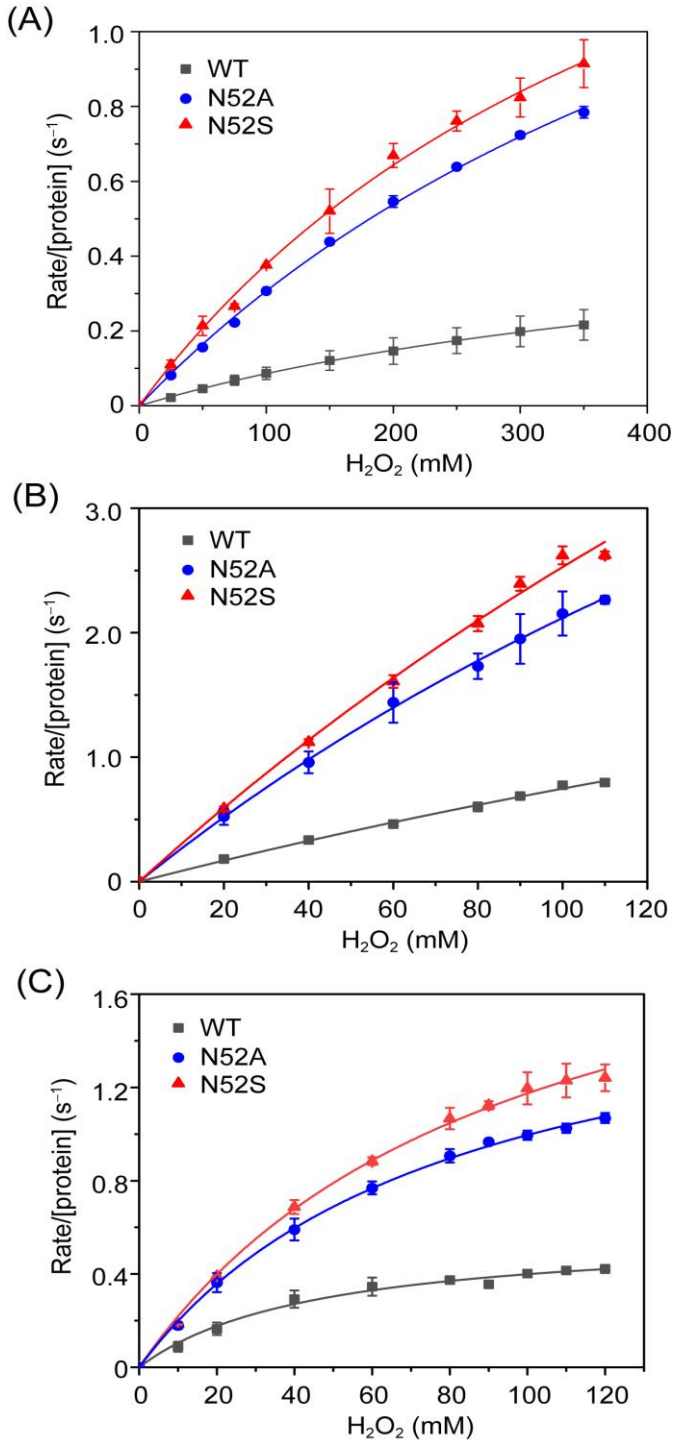

Figure S5. Initial oxidation rates of guaiacol (A, 0.5 mM), ABTS (B, 0.1 mM) and TCP (C, 0.1 mM) catalyzed by N52S, N52A and WT H-Cyt *c* at various concentrations of H_2O_2. Absorptions were monitored at 470, 660 and 272 nm, respectively. Reaction conditions: 5 μM protein, 50 mM potassium phosphate buffer at pH 7.0, 25 ℃.

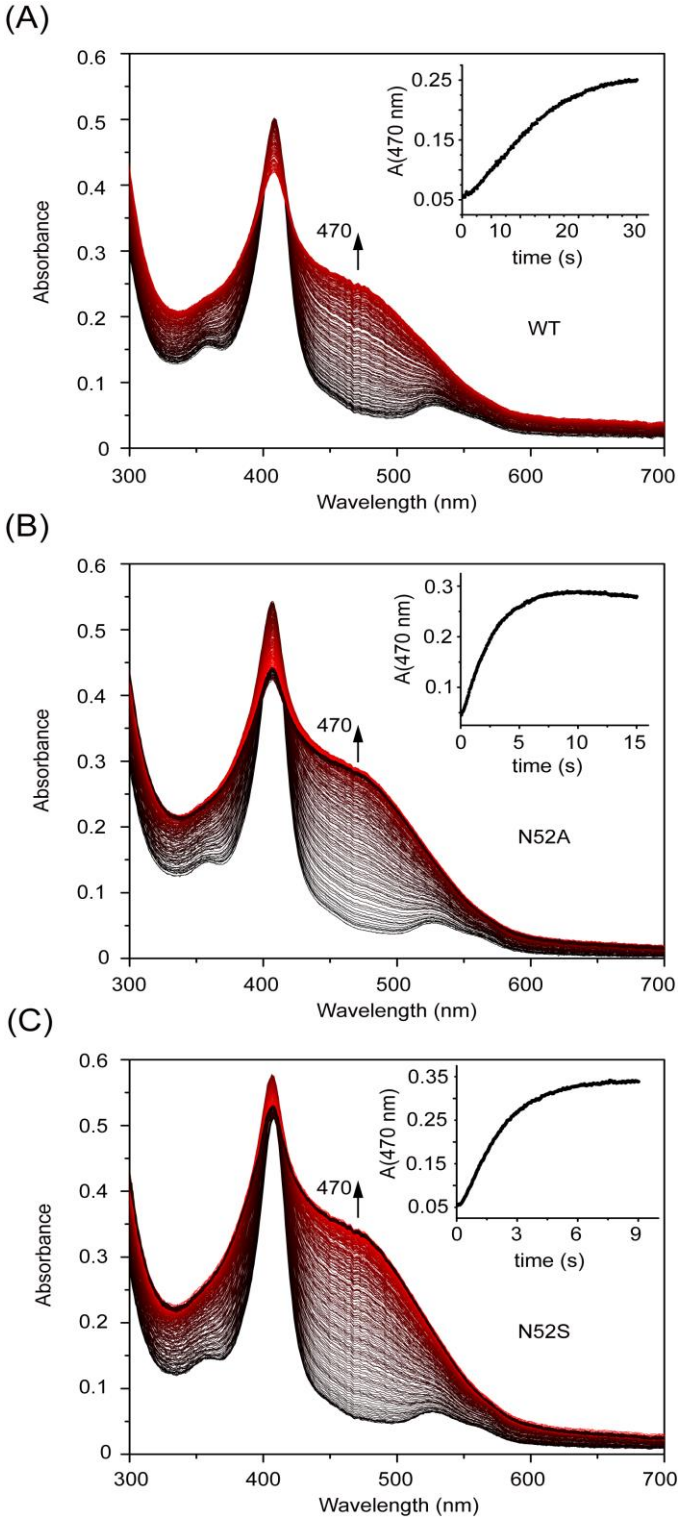

Figure S6. Stopped-flow spectra of guaiacol (0.1 mM) oxidation catalyzed by WT (A), N52A (B), and N52S (C) H-Cyt *c* (5 μM) in presence of H_2O_2 (200 mM) in 50 mM potassium phosphate buffer at pH 7.0, 25 ℃.

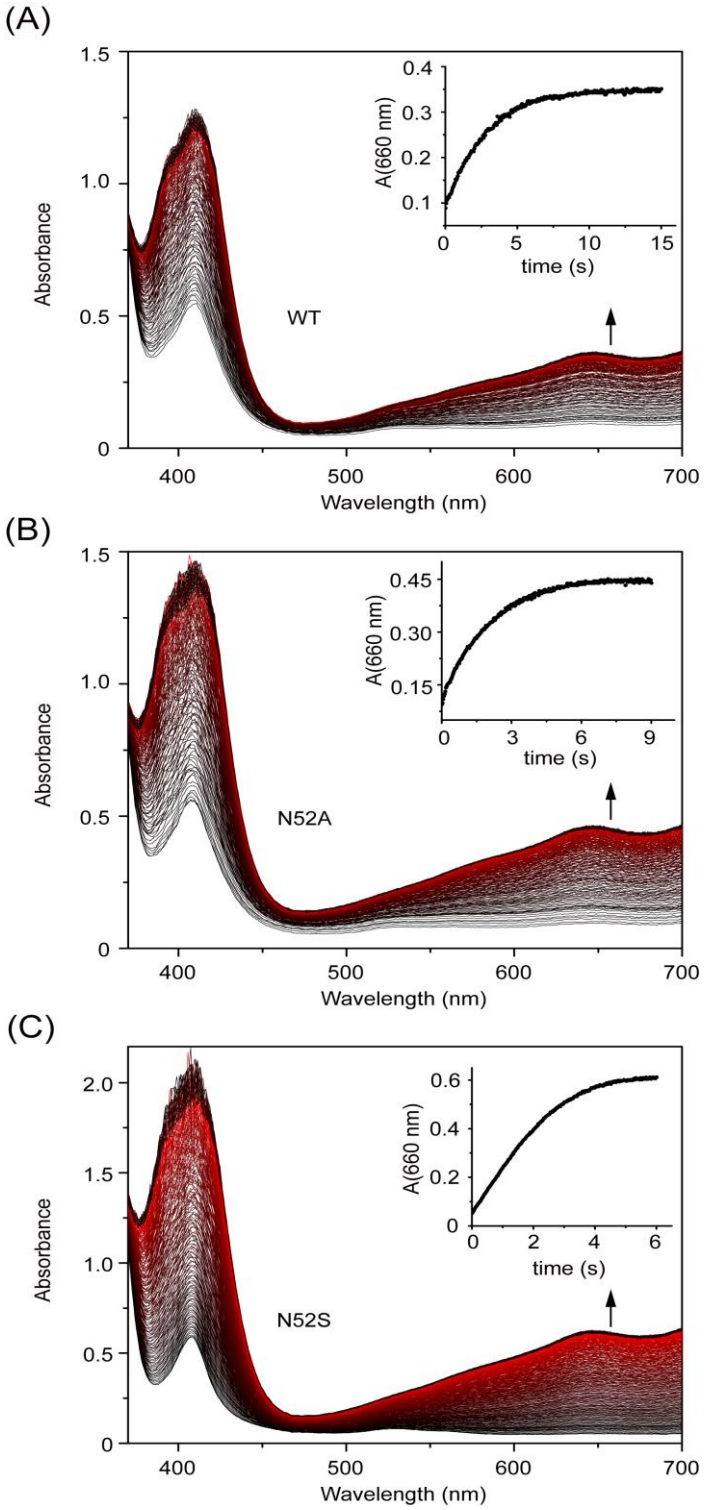

Figure S7. Stopped-flow spectra of ABTS (0.1 mM) oxidation catalyzed by WT (A), N52A (B), and N52S (C) H-Cyt *c* (5 μM) in presence of H_2O_2 (100 mM) in 50 mM potassium phosphate buffer at pH 7.0, 25 ℃.

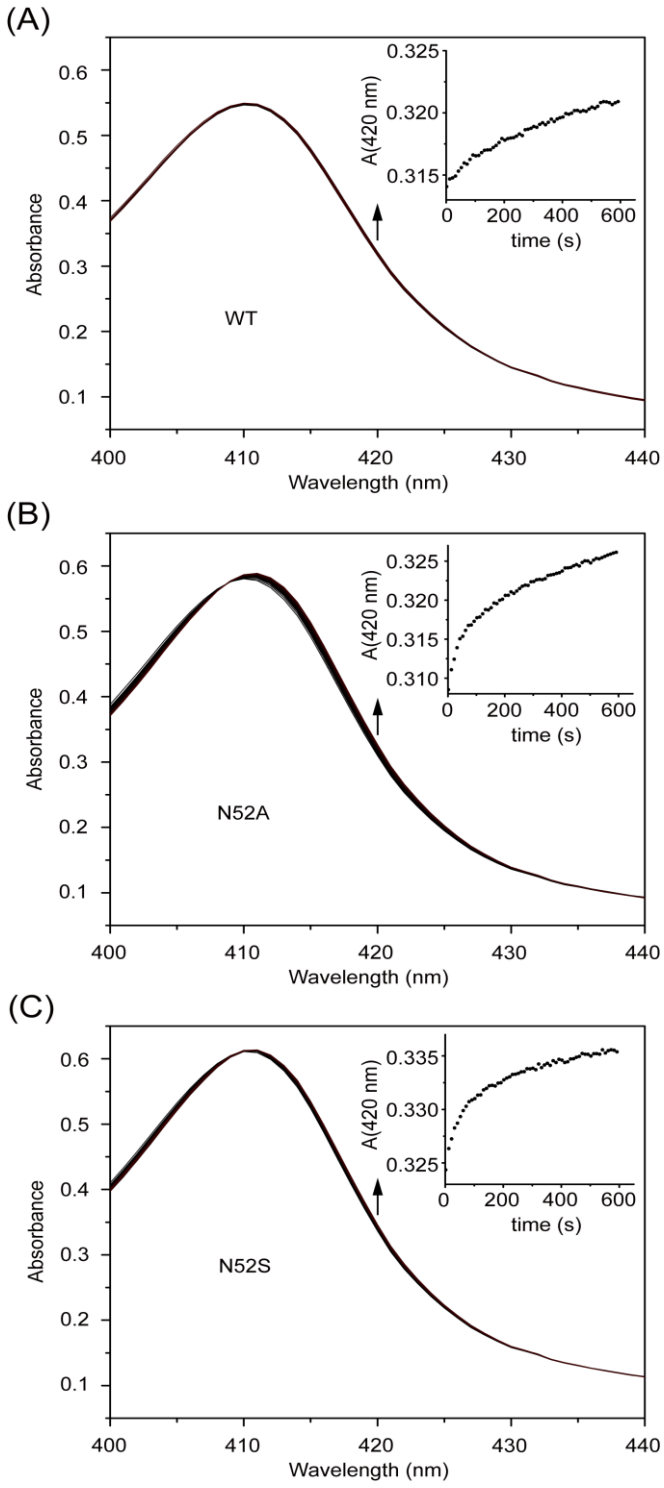

Figure S8. UV-Vis spectra of WT (A), N52A (B), and N52S (C) H-Cyt *c* (10 μM) upon mixing with NaN_3 (200 mM) in 50 mM potassium phosphate buffer at pH 7.0, 25 ℃. The absorption at 420 nm was recorded to calculate the k_{obs}.