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Supporting Information

Iron Chelators Inhibit the Heme-Degradation Reaction by HutZ from *Vibrio cholerae*

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Experimental Procedures

Materials. The chemicals used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich (St. Louis, MO), and used without further purification.

Expression and Purification of HutZ. Mutant HutZ proteins were expressed in *Escherichia coli* and purified as described previously[1]. Mutagenesis was conducted utilizing a PrimeSTAR mutagenesis basal kit from Takara Bio (Otsu, Japan). DNA oligonucleotides were purchased from Eurofins Genomics Inc. (Tokyo, Japan). The mutated genes were sequenced by Eurofins Genomics to ensure that only the desired mutations were introduced.

Spectroscopy. Optical spectra of the purified protein were recorded with UV-visible spectrophotometer (V-660, Jasco, Tokyo, Japan) at room temperature. Resonance Raman spectra for the carbon monoxide (CO)-bound form were obtained with a single monochromator (SPEX500M, Jobin Yvon, Edison, NJ, USA) equipped with a liquid nitrogen-cooled CCD detector (Spec-10:400B/LN, Roper Scientific, Princeton, NJ, USA). The excitation wavelength employed was 413.1 nm from a krypton ion laser (BeamLok 2060, Spectra Physics, MO, USA). The laser power at the sample point was adjusted to 0.1 mW to prevent photodissociation. Raman shifts were calibrated with indene, CCl_4, acetone, and an aqueous solution of ferrocyanide. The accuracy of the peak positions of well-defined Raman bands was ±1 cm^{-1}. Sample concentrations for resonance Raman experiments were about 10 μM in 50 mM MES/150 mM NaCl, pH 6.0. Circular dichroism (CD) spectra were observed by a JASCO J-1500 CD spectropolarimeter operating. The sample concentration was 5 mM in 50 mM sodium phosphate/100 mM NaCl (pH 6.0)

Isothermal Titration Calorimetry. Calorimetry measurements were performed with a NANO-ITC titration calorimeter instrument from TA Instruments (Delaware, USA). The sample cell was filled with 30 mM of heme-HutZ complex. The injection syringe contained 3

mM DFO, and this experiment was performed in triplicate at 25 °C. Experiments were performed using 25 injections, a stirring speed of 250 rpm, and a 180 seconds delay between injections. The injection volumes for the heme-HutZ complex consisted of 2 μL each. The heat values recorded were integrated, and the data were fit to a one-to-one binding mode.

Reduction Rate of Heme. The reduction rate of heme, k_1 (Figure S4), was determined by following changes in absorbance at 418 nm. 10 mM of ferric heme-HutZ complex was reduced by 1 mM ascorbic acid in the presence or absence of 0.1 mM DFO. Although an oxygen scavenging system composed of glucose, glucose oxidase, and catalase was added to the solution to anaerobic, the reaction was conducted under CO atmosphere to prevent from oxidation of heme and/or re-oxidation by contaminated O_2. The k_1 was obtained by fitting the time course of the absorbance change to a single exponential expression.

Cyanide Binding Rate Constants. Cyanide binding (CN) was measured by s stopped-flow apparatus (Unisoku, Osaka, Japan) by following the decrease of absorbance at 403 nm. In a typical cyanide binding experiment, one syringe contained 3 μM HutZ in 50 mM MES/150 mM NaCl (pH 6.0), and another syringe contained at least a 100-fold excess of CN. Three determinations were performed for each ligand concentration. The mean of the pseudo-first-order rate constants, k_{obs}, was used in the calculation of the second-order rate constants obtained from the slope of a plot of k_{obs} versus ligand concentration ($k_{obs} = k_{on}[CN] + k_{off}$).

Supplementary Figures

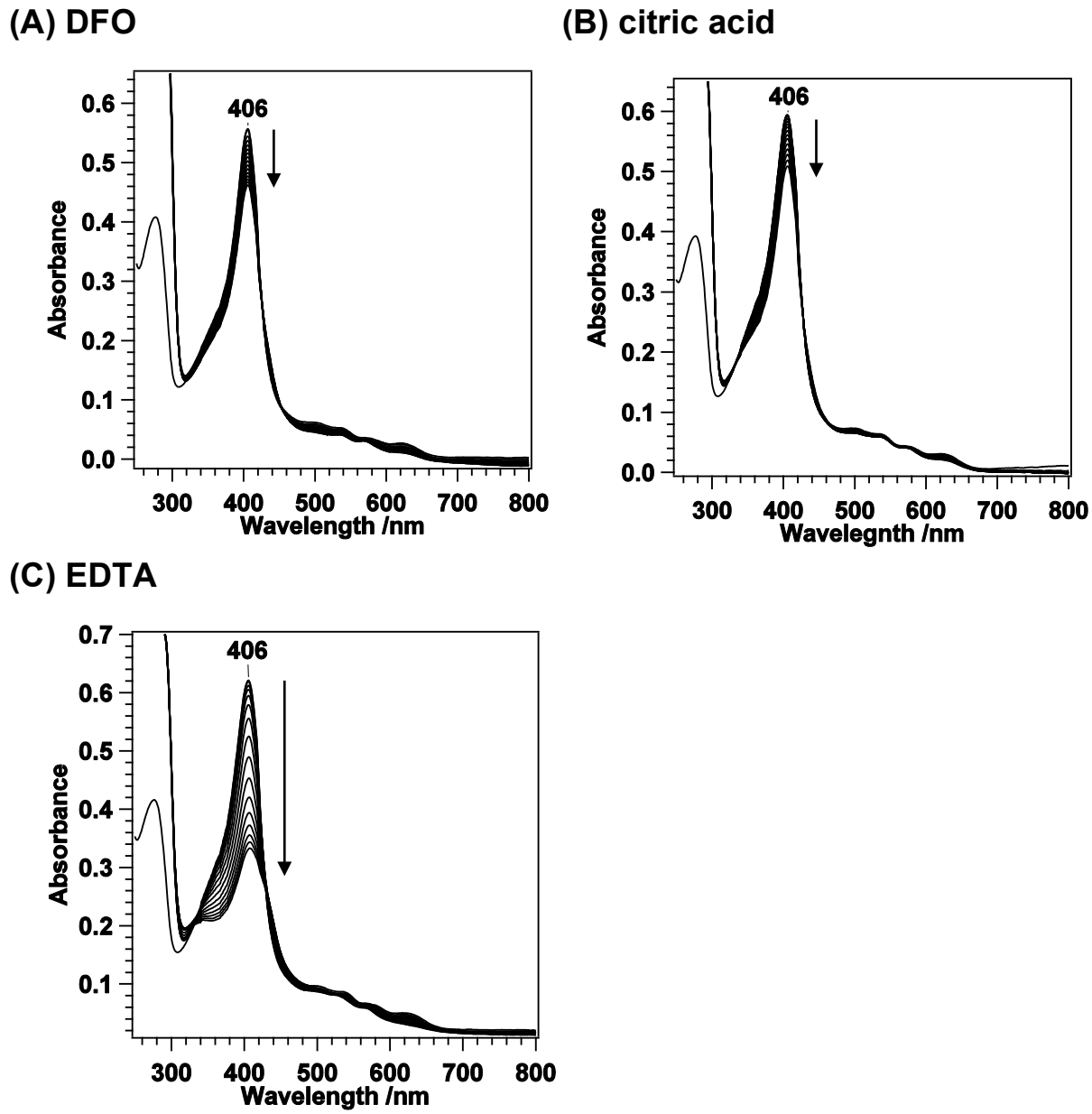

Fig. S1 Heme-degradation reaction of the heme-HutZ complex with ascorbic acid (1.0 mM) at pH 6.0. Spectra were measured before addition of ascorbic acid, and at 2-min intervals for 30 min after addition of ascorbic acid in the presence of (A) 0.10 mM DFO, (B) 1.0 mM citric acid, and (C) 0.10 mM EDTA.

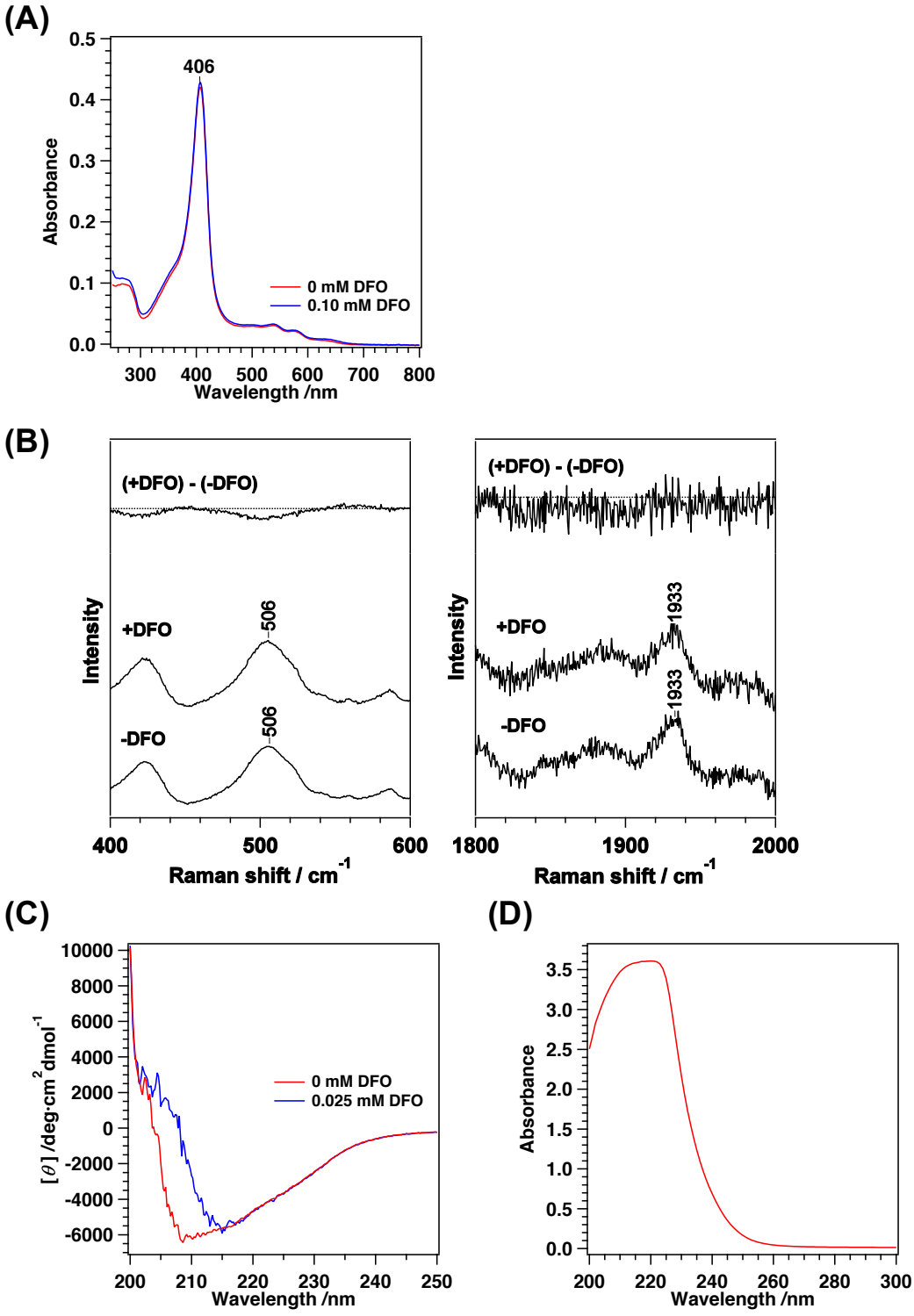

Fig. S2 (A) Absorption spectra of the heme-HutZ in the absence (red) and presence of 0.1 mM DFO (blue). (B) Resonance Raman spectra of the heme-HutZ in the CO-bound form. The protein concentration is 10 mM in 50 mM Tris-HCl/150 mM NaCl (pH 6.0). (C) CD spectra of heme-HutZ in the absence (red) and presence of DFO (blue). Protein concentration was 5.0 μM in 50 mM sodium phosphate/100 mM NaCl. (D) Absorption spectra of 1.0 mM DFO in 50 mM sodium phosphate/100 mM NaCl.

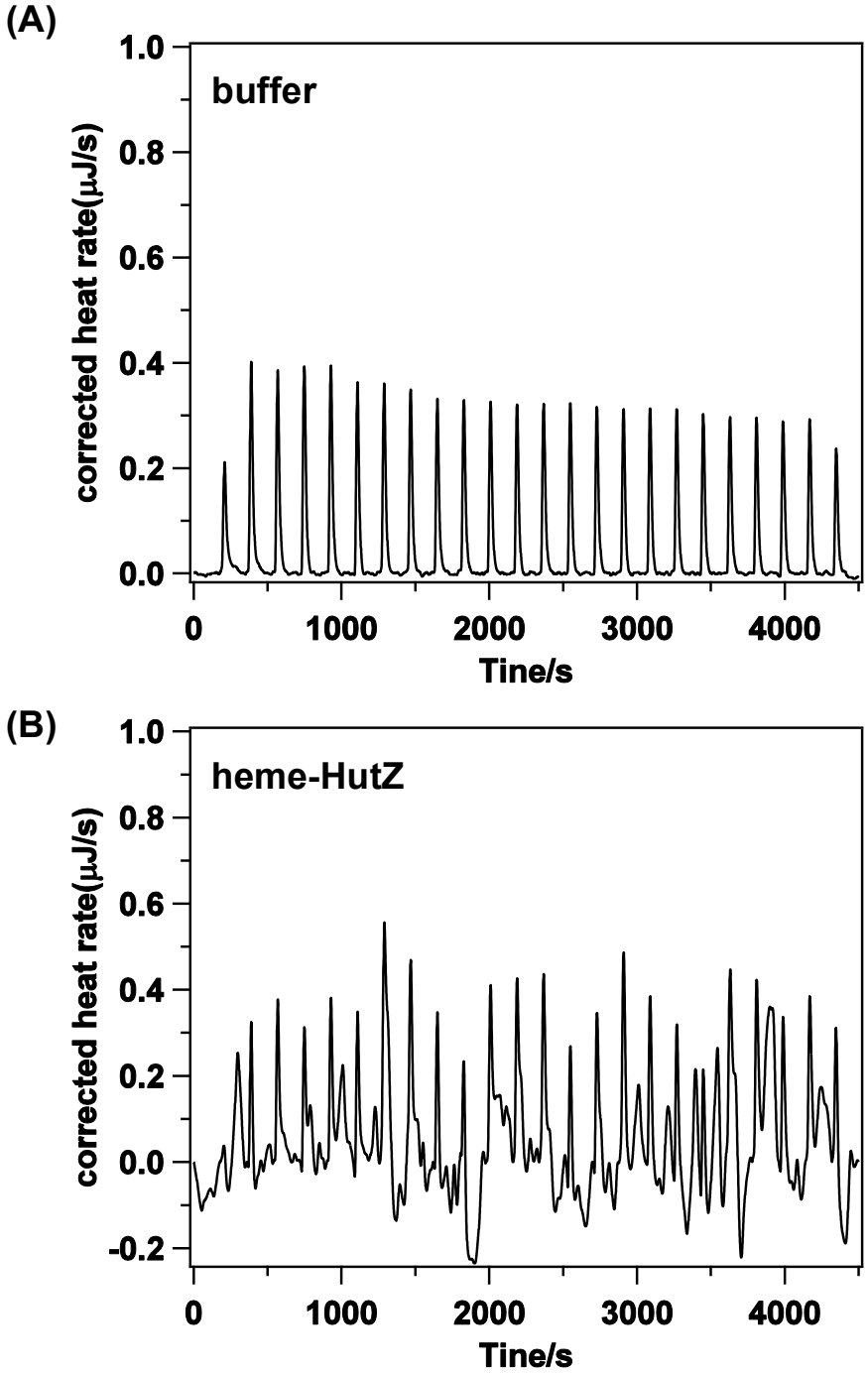

Fig. S3 ITC data for titration of 3 mM DFO into (A) buffer, and (B) 35 μM heme-HutZ. Injections (25 × 2 μL) were made at 180 sec intervals in 50 mM Tris-HCl/150 mM NaCl (pH 8.0) at 25 °C.

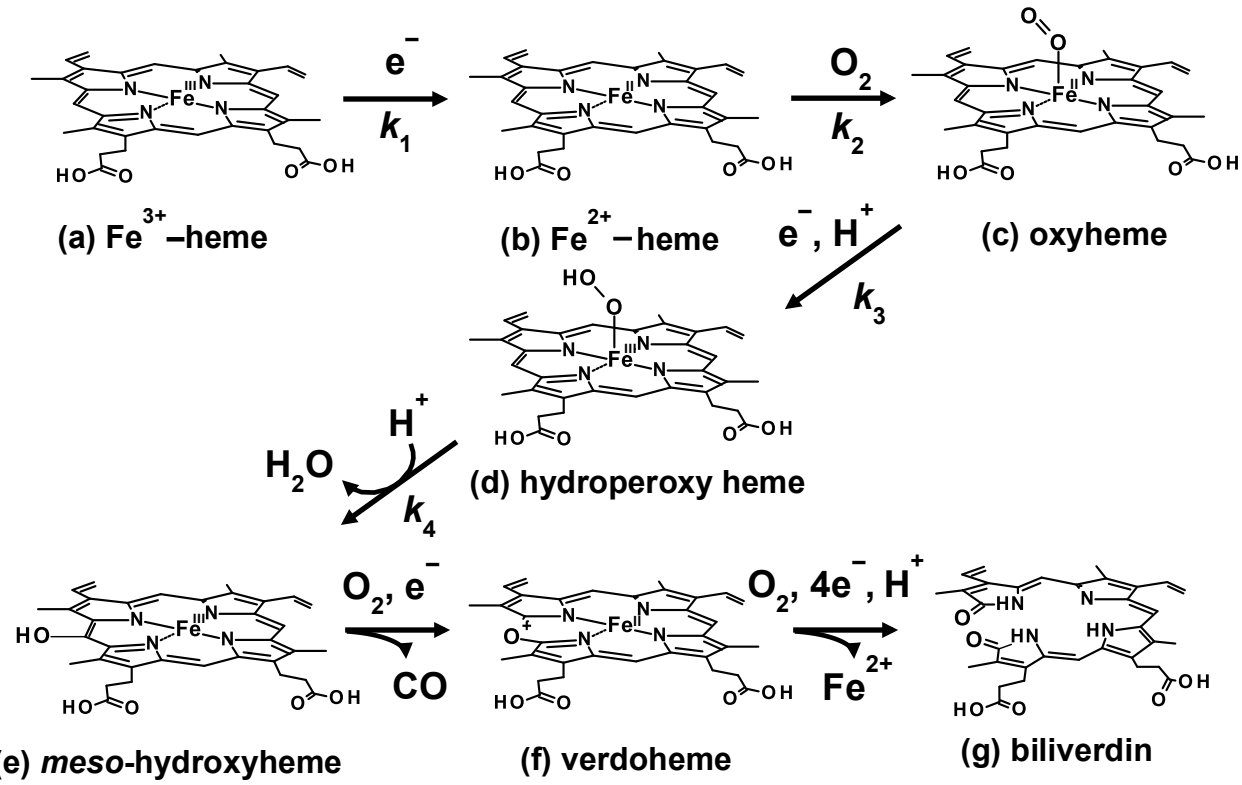

Fig. S4 Proposed heme degradation mechanism in HutZ[1].

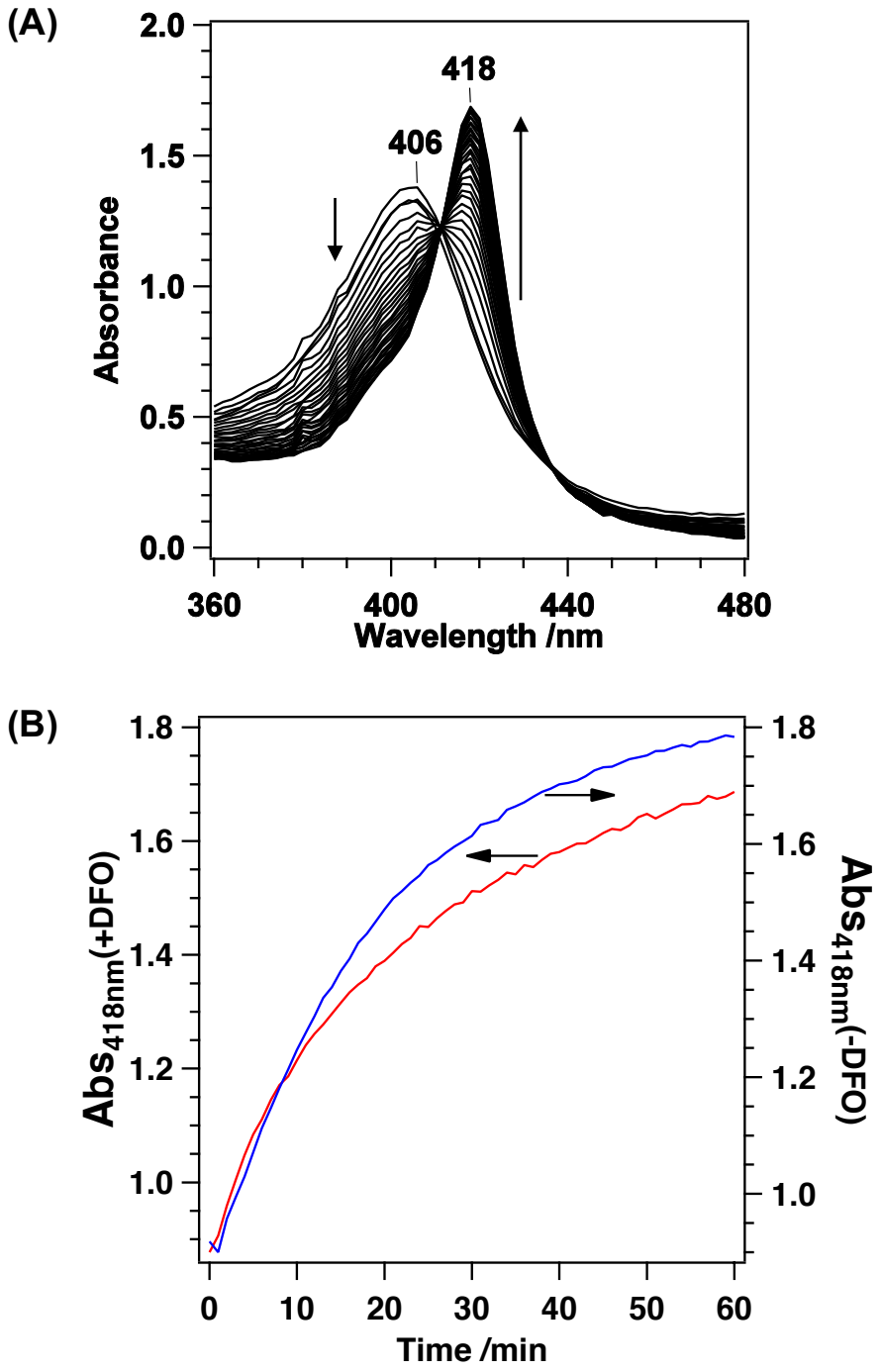

Fig. S5 Determination of reduction rate of heme. (A) UV-vis spectra of heme-HutZ after addition of ascorbic acid (1 mM) in the presence of DFO at pH 6.0 under CO atmosphere. (B) Time course of absorbance change at 418 nm in the presence (red, left axis) and absence (blue, right axis) of DFO.

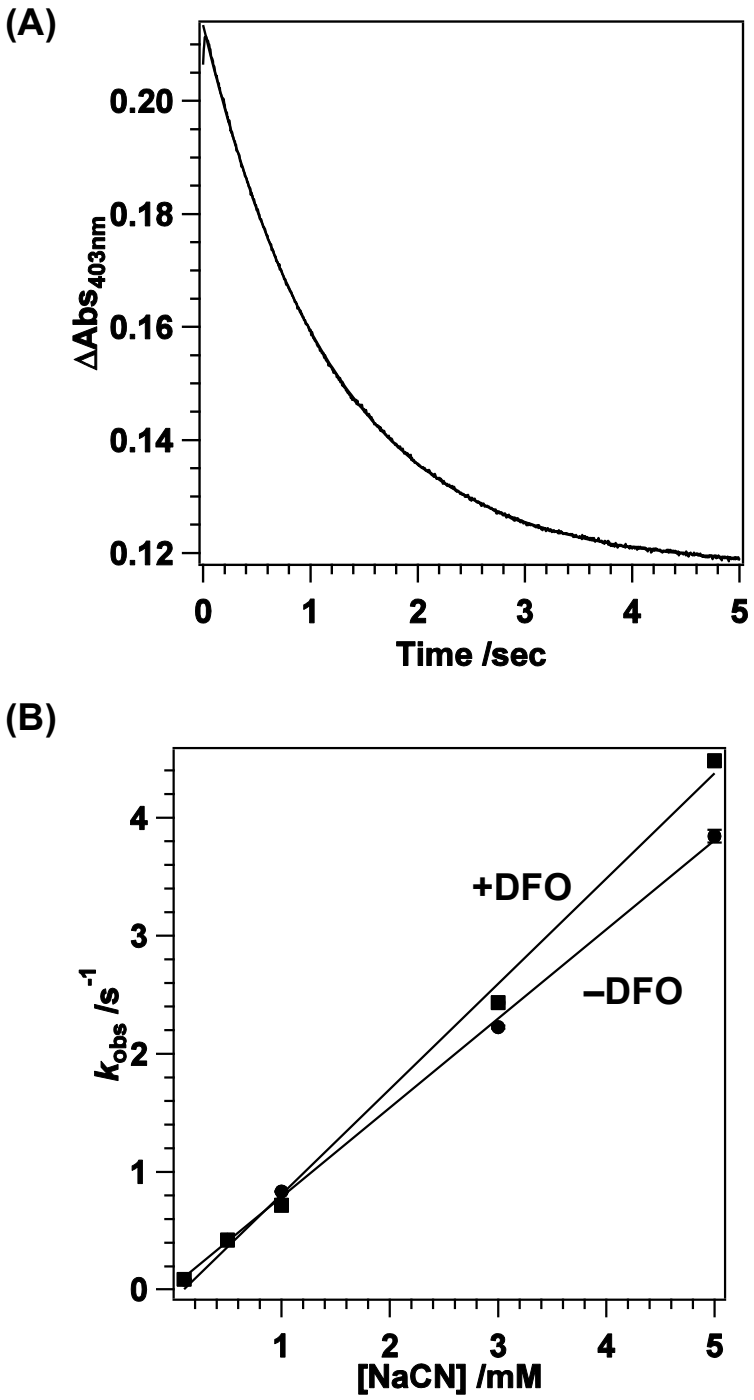

Fig. S6 Cyanide binding to heme-HutZ. (A) typical time trace of the absorbance change at 403 nm upon mixing 3.0 μM of heme-HutZ with 1.0 mM CN^- in 50 mM MES-NaOH/150 mM NaCl (pH 6.0). (B) Plots of k_{obs} versus CN^- concentrations.

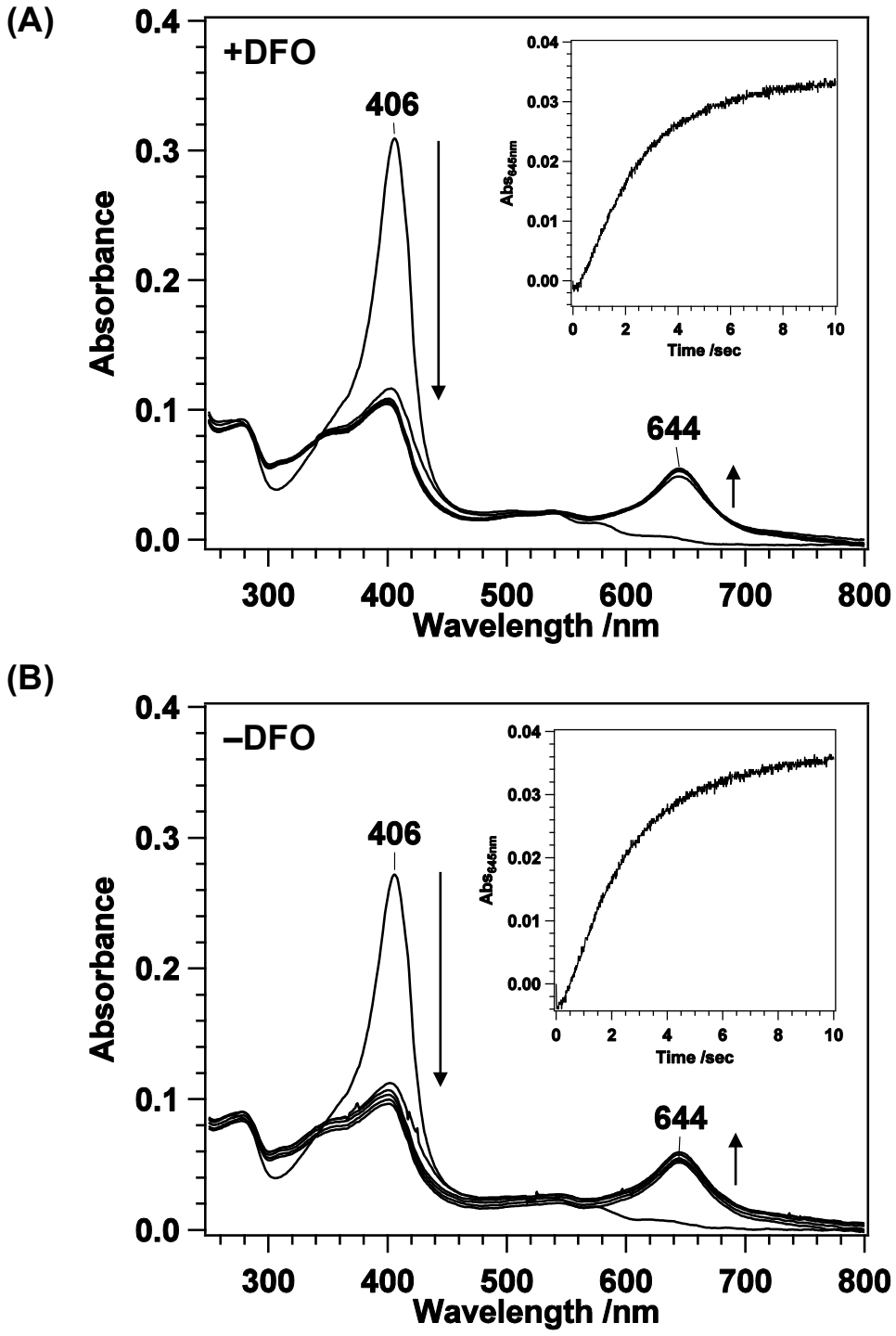

Fig. S7 Heme-degradation reaction of heme-HutZ with H_2O_2 (0.10 mM) at pH 6.0 in the presence (A), and absence of DFO (B). Spectra were measured before addition of H_2O_2, and at 1-min intervals for 5 min after addition of H_2O_2. Inset: Time course of absorbance at 644 nm.

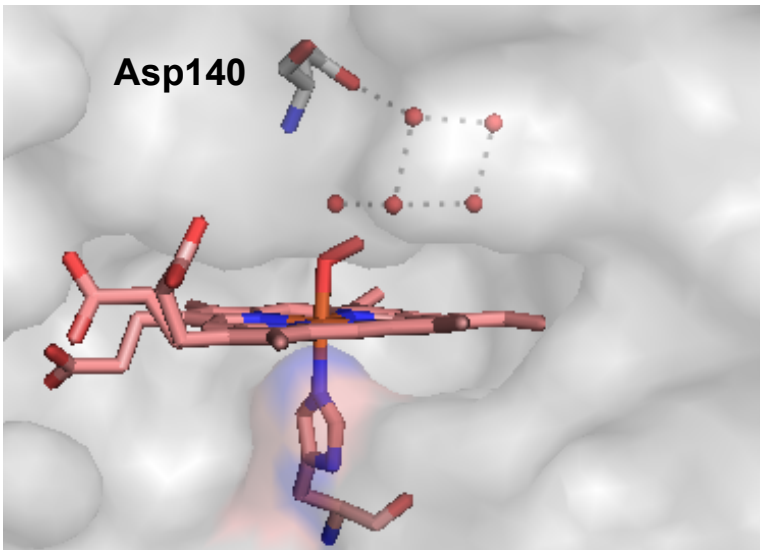

Fig. S8 Crystal structure of HO-1 form rat in the O_2-bound form (PDB ID code 4G7L).

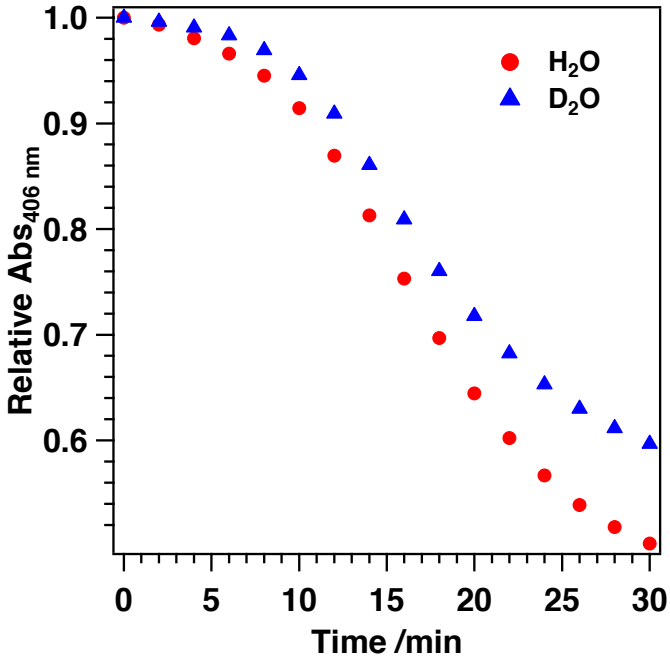

Fig. S9 Isotope effect of the heme-degradation reaction by HutZ. Time course of absorbance change at 406 nm in the reaction with ascorbic acid in H_2O/D_2O buffer at pH 6.0.

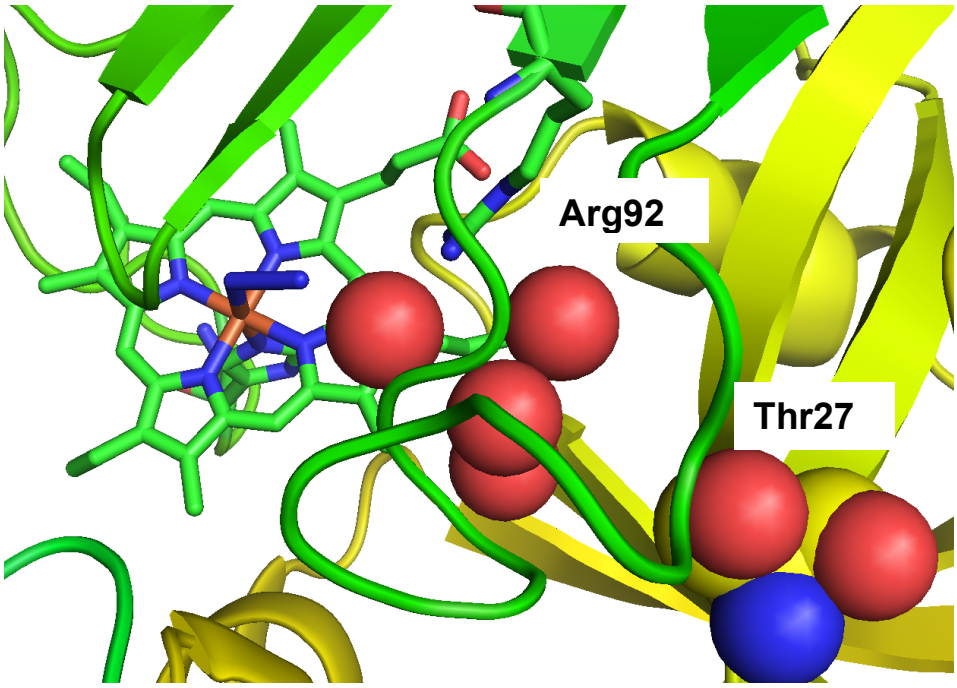

Fig. S10 Crystal structure of HugZ from *Helicobacter pylori* (PDB ID code 3GAS).

Supplementary Tables

Table S1 Heme-degradation ratio determined from the amount of ferrous iron in heme-HutZ wild-type (WT) and heme-HutZ T27V and T27S mutants.

Protein	Iron yield (%)
WT	86 ± 7
WT + ferrozine	32 ± 7
T27V	61 ± 5
T27S	83 ± 3
WT + tetracycline	49 ± 4
WT + chloramphenicol	83 ± 3

Table S2 Rate constants of heme reduction (k_1), CN binding (k_2'), and H_2O_2 reaction (k_4) for heme-HutZ

Protein	k_1 (min^{-1})	k_2' ($mM^{-1}s^{-1}$)	k_4 (sec^{-1})
WT + DFO	0.045 ± 0.008	0.76 ± 0.01	0.392 ± 0.002
WT – DFO	0.052 ± 0.005	0.88 ± 0.01	0.389 ± 0.001
T27V	0.045 ± 0.000	1.09 ± 0.01	0.396 ± 0.001
T27S	0.048 ± 0.000	0.93 ± 0.00	0.401 ± 0.001

References

1 T. Uchida, Y. Sekine, T. Matsui, M. Ikeda-Saito and K. Ishimori, *Chem. Commun.*, 2012, **48**, 6741–6743.