A hydrogenase model system based on the sequence of cytochrome *c*: photochemical hydrogen evolution in aqueous media

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Instruments.

The UV-vis spectra were recorded by Multi Channel Photo Deterctor MCPD-3000 and UV-vis source MC-2530 system (Otsuka Electronics Co., Ltd., Japan) using optical fibers in a glove box (Unilab, MBraun, Germany). The IR spectra were recorded by JASCO FT/IR-4000 spectrometer using the SL-2 demountable cell unit (optical path-length = 0.1 mm, Pier Optics Co., Ltd., Japan) with CaF_2 windows. The circular dichroism (CD) spectra were recorded by JASCO J720S spectrometer using a quartz cell (optical path-length = 0.1 cm, GL Science Inc., Japan). The ESI-TOF MS analyses were carried out on an Applied Biosystems Mariner API-TOF Workstation and a Brucker micrOTOF. High-performance liquid chromatography (HPLC) analyses were performed using a Shimadzu SCL-10AVp HPLC system with a reversed-phase analytical column. The continuous Xe lamp irradiation was carried out by USHIO Optical Modulex SX-U1501XQ (500 W). The gas chromatographic (GC) experiments were performed using a Shimadzu GC-2014 gas chromatograph with a TCD. The pH values were monitored by HORIBA F-52 pH meter. The irradiation power of light was determined by Ophir 30A-P-SH power meter.

Materials.

Cytochrome *c* (cyt *c*) from equine heart was purchased from Sigma (C2506) and apocytochrome *c* (apocyt *c*) was prepared by the silver sulfate method reported by Fisher *et al*.[S1] Apocyt *c* concentration was determined by molar absorption coefficient at 280 nm (ε_{280} = 18600 M^{-1} cm^{-1}). The quantity of cysteine residues in apocyt *c* and apominicyt *c* peptide were determined by reaction of them with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent).[S2] H-His(Trt)-2Cl-Trt resin was purchased from Novabiochem. Fmoc-protected amino acids and hydroxybenzotriazole (HOBt) were purchased from Peptide Institute, Inc. (Osaka, Japan). All other reagents and chemicals were obtained from commercial sources and used as received unless otherwise noted. Distilled water demineralized using a Barnstead NANOpure Diamond^{TM} apparatus.

Preparation and characterization of H-apocyt *c*

$Fe_2(CO)_9$ (2.92 μmol) and apocyt *c* (0.370 μmol) were combined in 30 mL of 50 mM Tris/HCl buffer solution at pH 8 and the solution was then irradiated by Xe lamp under nitrogen atmosphere for 12 h resulting in a color change from purple to pink. The reaction solution was concentrated by Amicon stirred cell equipped with Ultracel PL-3 (Millipore), and the resulting solution was then purified with HiTrap Desalting column (GE Healthcare) for the removal of excess $Fe_2(CO)_9$.

H-apocyt *c* was identified by ESI-TOF MS as shown in Fig. S1. The folding of H-apocyt *c* in

Tris/HCl buffer solution (50 mM, pH 8) was determined by CD spectroscopy. The carbonyl stretching bands of the iron carbonyl in H-apocyt *c* were detected by IR spectroscopy at a 1.0 cm^{-1} resolution at room temperature.

Preparation and characterization of H-apominicyt *c*

Apominicyt *c* (partial sequence of cyt *c*; YKCAQCH) was synthesized according to a standard solid-phase method using *N,N'*-diisopropylcarbodiimide (DIPCI) and HOBt as coupling reagents in *N,N'*-dimethylformamide (DMF), where a preloaded H-His(Trt)-2Cl-Trt resin was used for the peptide syntheses. A tyrosine residue was attached at the *N*-terminus of each peptide to quantify the concentration of the peptide using characteristic absorbance at 280 nm. The resin was washed with DMF and dichloromethane (DCM) after each coupling cycle. The peptide was cleaved from the resins by treatment with trifluoroacetic acid (TFA)/1,2-ethanedithiol (EDT)/H_2O/triisopropylsilane (TIS) = 94/2.5/2.5/1 for 30 min in an ice bath, with standing for an additional 2 h at room temperature. A solution containing the cleaved peptides was concentrated and the crude peptide was precipitated by the addition of diethyl ether. The peptide was purified on a reverse-phase HPLC column (YMC, YMC-Pack Pro C18, 250 mm x 20 mm I.D., *S* = 20 μm) with acetonitrile (MeCN) and water as eluents (gradient containing 0.1% TFA), and the purities of the peptides were confirmed by analytical HPLC (> 95%). The purified solutions of the peptides were lyophilized to give a white powder. The peptides were identified by ESI-TOF MS with 50% MeCN aqueous solution containing 1% acetic acid. The molar absorption coefficient was determined using the tyrosine band in the peptide (apominicyt *c*; ε_{280} = 1240 M^{-1} cm^{-1}). Preparation and characterization of H-apominicyt *c* was carried out according to the same preparative method described above.

Quantitative analysis of H-apocyt *c*

The optical absorption of the diiron carbonyl cluster overlaps with that of the aromatic amino acids appeared at 280 nm. Thus, the concentration of apocyt *c* containing the diiron carbonyl cluster (H-apocyt *c*) was calculated by monitoring the formation of 5-mercapto-2-nitrobenzoic acid (ε_{412} = 15500 M^{-1} cm^{-1}) as an Ellman's test. Furthermore, the Fe^{+} ion of H-apocyt *c* was determined via reaction with 1,10-phenanthroline (phen) by the modified method of Treffry,[S3] because the ferrous ion was found to be released from H-apocyt *c* by adding a drop of 10% H_2SO_4. The $[Fe(phen)_3]^{2+}$ complex was measured at 512 nm (ε_{512} = 11100 M^{-1} cm^{-1}). The quantity of the cysteine residues was completely consistent with that of the Fe^{+} ions, supporting the fact that the

diiron carbonyl cluster is bound to apocyt c with the molecular ratio of 1:1.

Hydrogen generation catalyzed by H-apocyt *c*

The continuous irradiations of the H-apocyt *c* solutions were carried out using a 500 W Xe lamp with a UV and IR cut filter (Super Cold Filter, Asahi Spectra; < 390 nm, > 770 nm) and a UV cut filter (< 410 nm) to eliminate UV and IR light. A degassed mixture of H-apocyt *c* (14 μM), $[Ru(bpy)_3]^{2+}$ (140 μM), and ascorbate (100 mM) dissolved in 100 μL of Tris/HCl (50 mM) at a quarts cell equipped septum screw (optical path-length = 0.1 cm, cell volume = 1.0 mL) was irradiated with 0.189 W cm^{-2}, keeping a distance of 3.5 cm between the sample cell and the light source, under nitrogen atmosphere at 25 ºC. The headspace gas (100 μL) was sampled with a gas tight syringe equipped with a stopper valve, which was applied to GC equipped with an activated charcoal column and a TCD to determine the amount of generated hydrogen gas.

Reference

(S1) Fisher, W. R.; Taniuchi, H.; Anfinsen, C. B. *J. Biol. Chem.,* 1973, **248**, 3188.

(S2) Ellman, G. L. *Arch. Biochem. Biophys.,* 1959, **82**, 70.

(S3) Treffry, A.; Zhao, Z.; Quail, M. A.; Guest, J. R.; Harrison, P. M. *Biochemistry* 1995, **34**, 15204.

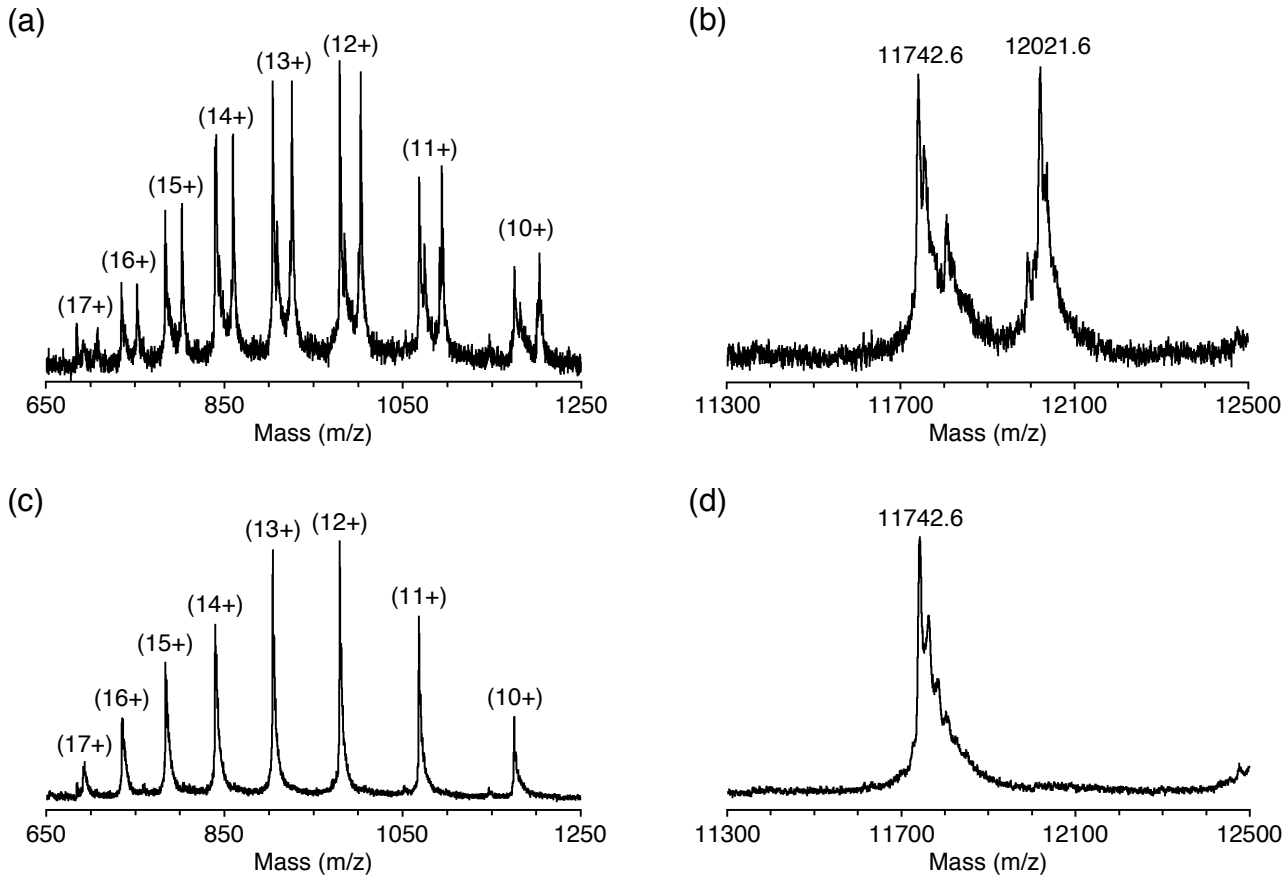

Fig. S1 ESI-TOF MS spectra of H-apocyt *c* and apocyt *c*; (a) the raw spectrum of H-apocyt *c*, (b) deconvoluted spectrum of H-apocyt *c*, the calculated mass is 12021.0, (c) the raw spectrum of apocyt *c*, and (d) deconvoluted spectrum of apocyt *c*. The calculated mass number of H-apocyt *c* is 11743.2. The sample was prepared in 10% MeOH–NH_4OAc buffer solution (50 mM, pH 8).

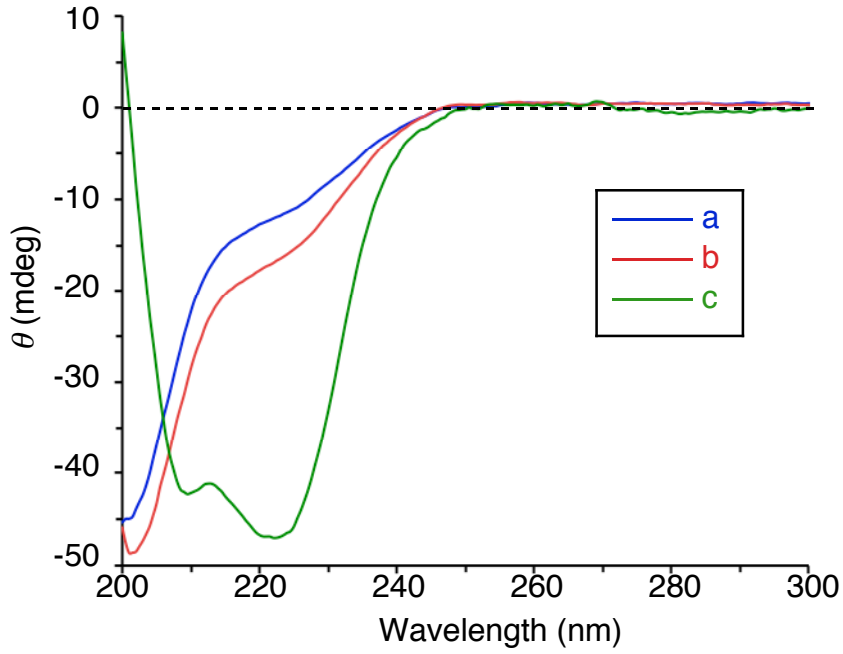

Fig. S2 Overlaid CD spectra of (a) apocyt *c* (30 μM), (b) H-apocyt *c* (30 μM) and (c) cyt *c* (30 μM) in Tris/HCl buffer (50 mM, pH 8) at 25 ºC.

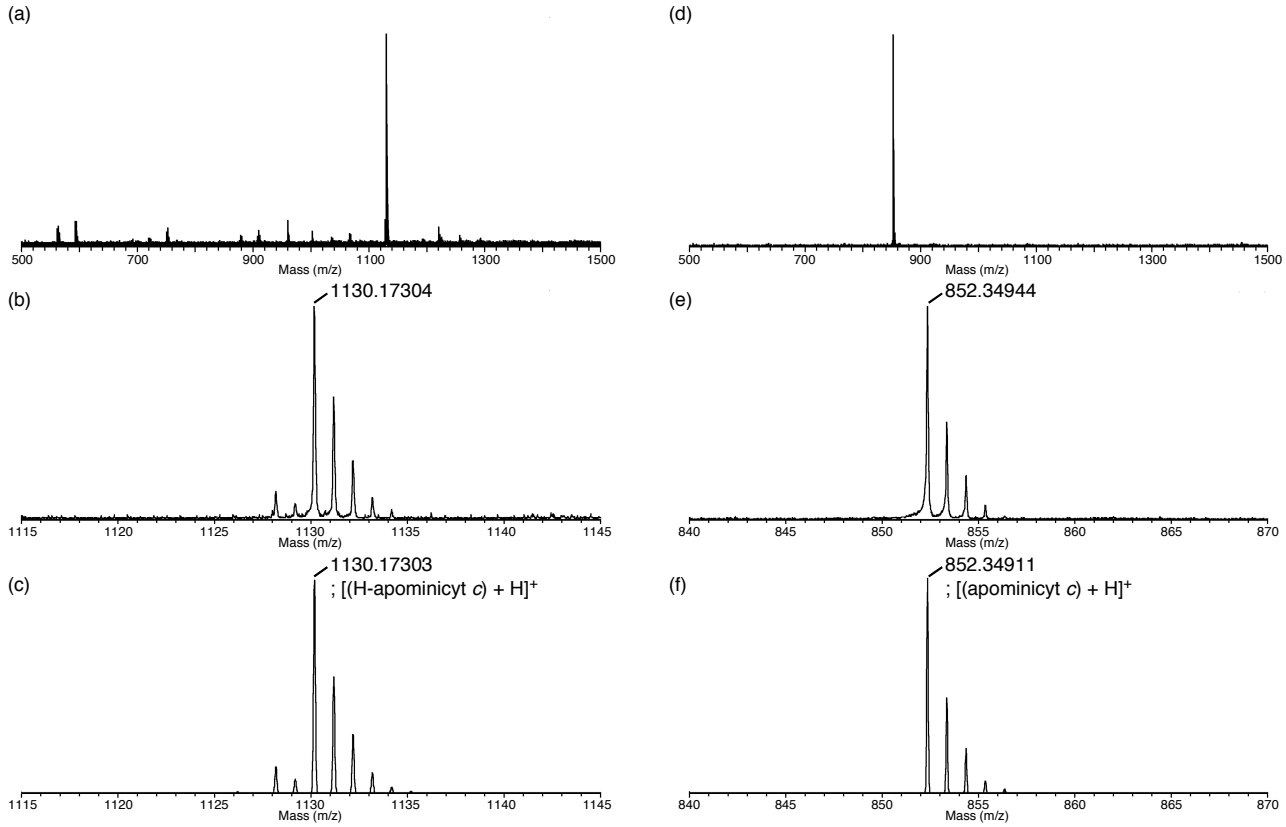

Fig. S3 ESI-TOF MS spectra of H-apominicyt *c* and apominicyt *c*; (a) the raw spectrum, (b) the expanded spectrum and (c) the theoretical spectrum of H-apominicyt *c* (HRMS (ESI+) m/z calcd for $C_{41}H_{52}Fe_2N_{11}O_{16}S_2$ $[M + H]^+$ 1130.17303, found 1130.17304); (d) the raw spectrum, (e) the expanded spectrum and (f) the theoretical spectrum of apominicyt *c* (HRMS (ESI+) m/z calcd for $C_{35}H_{54}N_{11}O_{10}S_2$ $[M + H]^+$ 852.34911, found 852.34944). The sample was prepared in 10% MeOH–NH_4OAc buffer solution (50 mM, pH 8).

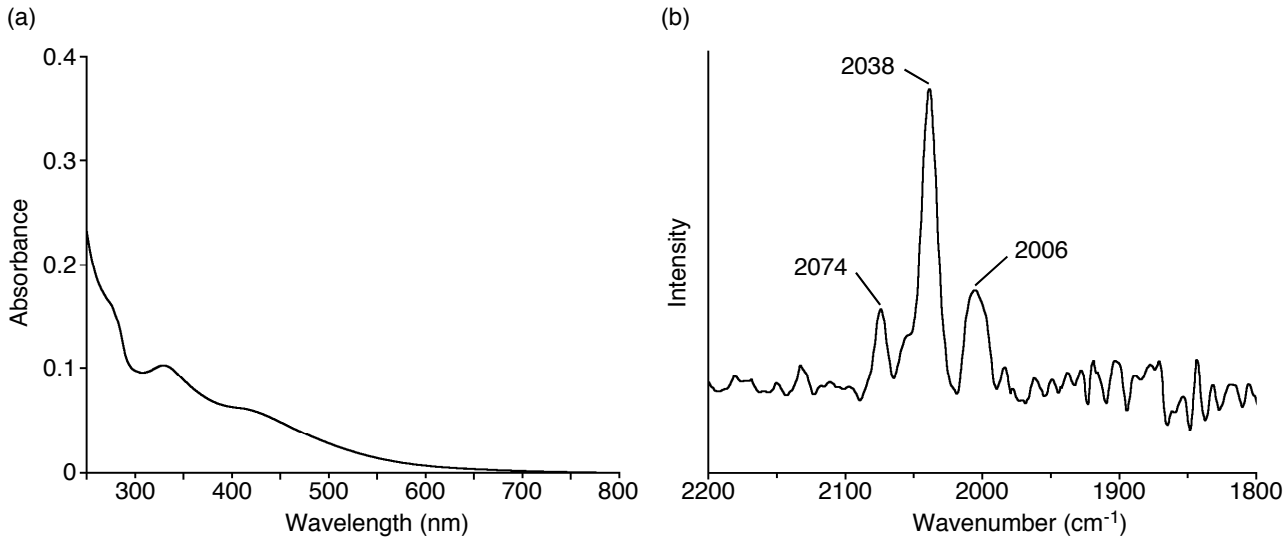

Fig. S4 (a) UV-vis spectrum of H-apominicyt *c* (ε_{330} = 8250 M^{-1} cm^{-1}; 12.4 μM) and (b) FT/IR spectrum of H-apominicyt *c* in Tris/HCl buffer (50 mM, pH 8) at 25 ºC.

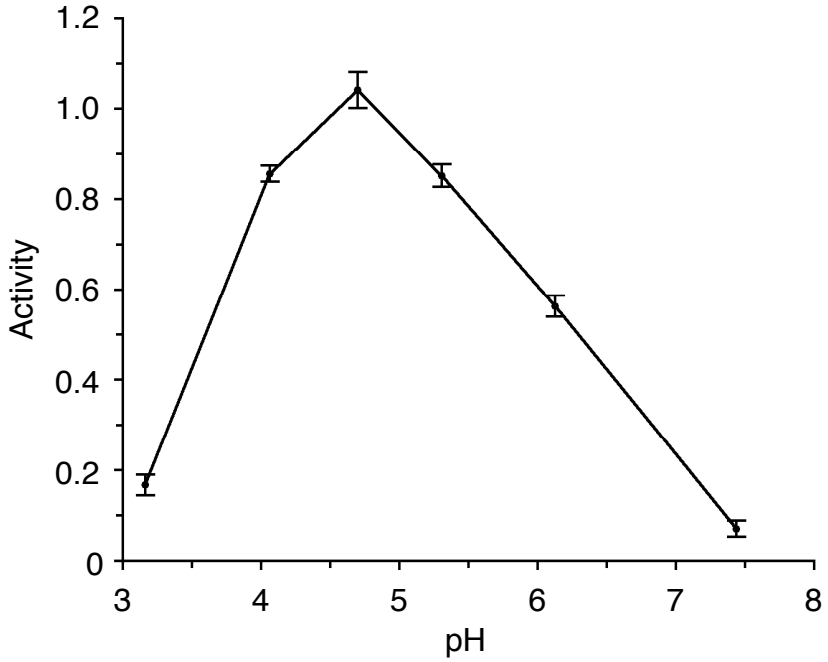

Fig. S5 pH dependence of photocatalytic H_2 evolution activity in the presence of H-apocyt *c* (14 μM, 1.4 nmol) and $[Ru(bpy)_3]^{2+}$ (140 μM) in Tris/HCl buffer (50 mM, pH 3–8) containing ascorbate (100 mM) as a sacrificial reagent at 25 ºC relative to its activity at pH 4.7.

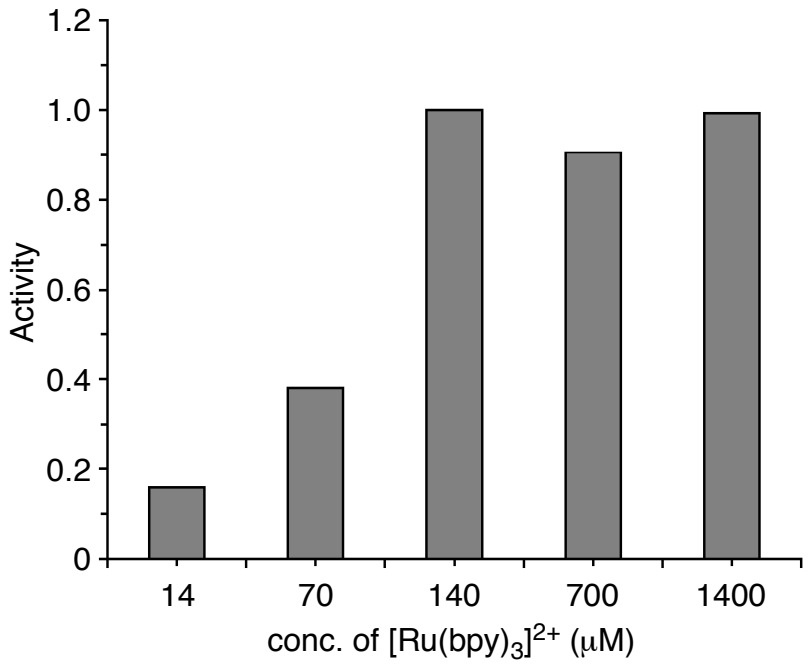

Fig. S6 $[Ru(bpy)_3]^{2+}$ dependence of photocatalytic H_2 evolution activity in the presence of H-apocyt *c* (14 μM, 1.4 nmol) and $[Ru(bpy)_3]^{2+}$ (14–1400 μM) in Tris/HCl buffer (50 mM, pH 4.7) containing ascorbate (100 mM) at 25 ºC relative to its activity at 140 μM.