Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supplementary Information

Self-oxidation of Cytochrome *c* at Methionine80 with Molecular Oxygen Induced by Cleavage of the Met−Heme Iron Bond

Zhonghua Wang,[ab#] Yuki Ando,[a#] Ari Dwi Nugraheni,[a] Chunguang Ren,[a] Satoshi Nagao,[a] and Shun Hirota*[a]

[a] Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan
[b] Chemical Synthesis and Pollution Control Key Laboratory, College of Chemistry and Chemical Engineering, China West Normal University, Nanchong 637002, China.

Contents

Experimental Procedure

Plasmid construction

Human cyt *c* expression was performed by co-expressing the cyt *c* and cyt *c* heme lyase (CCHL) genes.[1,2] The human cyt *c* gene was amplified by polymerase chain reaction (PCR) of the pME18SFL3 plasmid containing the human cyt *c* gene (Toyobo, Osaka), and sub-cloned into the Sma*I*−Pst*I* site of the pUC18 plasmid. The cyt *c* heme lyase (CCHL) gene was obtained from the *Saccharomyces cerevisiae* gene (strain: ATCC 18824) by PCR. The CCHL gene was sub-cloned into the Pst*I*−Hind*III* site of the human cyt *c* gene-containing plasmid, where a *lac* promoter sequence was placed upstream of the CCHL gene. After digestion of the obtained plasmid with Sma*I* and Hind*III*, the fragment containing both human cyt *c* and CCHL genes was sub-cloned into the Sma*I*−Hind*III* site of the pEMBL18+ plasmid,[3] producing the pEHC plasmid. Δ8384 human cyt *c* mutant was constructed by removing the sequence of Val83 and Gly84 from the pEHC plasmid using a KOD-Plus-Mutagenesis kit (Toyobo).

Protein expression and purification

Expression and purification of wild-type human cyt *c* were performed according to methods described elsewhere.[1,2] Protein expression was carried out in *Escherichia coli* (*E. coli*) strain Rosetta 2(DE3) pLysS (Novagen). A single transformed colony of Rosetta 2(DE3) pLysS containing the pEHC plasmid was inoculated into 200 mL LB medium in a 500 mL flask. After shaking overnight (150 rpm), 15 mL of the culture was transferred into 1.5 L modified LB media (Tryptone: 10 g/L, NaCl: 10 g/L, yeast extract: 8 g/L, $NaNO_3$: 2 g/L, glycerin: 1.5 ml/L) containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) in a 2 L flask. The flask was incubated for 20–30 h (130 rpm). The obtained cells were harvested by centrifugation (8,500 g, 5 min), and re-suspended in a minimal volume of 50 mM potassium phosphate buffer, pH 7.0. The cell mixture was treated with three cycles of flash-freezing in liquid nitrogen and thawing at room temperature. After addition of a small amount of DNase (about 0.1 mg/g cell pellet), the cell mixture was stirred on ice for 2 h before centrifugation (13,700 g, 30 min). The supernatant was dialyzed overnight in distilled water (4 °C), and centrifuged again (13,700 g, 30 min). The protein solution was loaded on a CM-52 (Whatman) column equilibrated with the same buffer. After the column was washed with the same buffer and the same buffer containing 75 mM NaCl, the protein was eluted with the buffer containing 250 mM NaCl. The protein solution was concentrated with an Amicon ultrafiltration tube (Merck Millipore, Darmstadt) to a volume of no more than 5 mL in ultrapure water. The protein was oxidized with $K_3[Fe(CN)_6]$ immediately before further purification with a CM Sepharose column (GE healthcare, Buckinghamshire) using a fast protein liquid chromatography system (BioLogic DuoFlow 10, Bio-Rad, CA) (flow rate: 0.8

mL/min, monitoring wavelength: 410 nm, solvent: 50 mM potassium phosphate buffer, pH 7.0, temperature: 4 °C) with a linear NaCl gradient. Fractions with an absorbance ratio of $A_{410}/A_{280} > 4.5$ were collected, concentrated, and dissolved in the same buffer. The protein solution was frozen in liquid nitrogen and stored at -80 °C until use.

Preparation of Δ8384 human cyt *c* mutant was performed with the same procedure as that of the wild-type protein. Unmodified Δ8384 cyt *c* was purified using N_2-bubbled buffer to prevent oxidation of Met80 by molecular oxygen.

Preparation of liposome and interaction of cyt *c* with liposome

Liposomes were prepared from 1,2-dioloeyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Inc., Alabaster) with and without bovine heart CL (Avanti Polar Lipids, Inc., Alabaster). DOPC (final concentration, 3 mM) was dissolved in 1 mL $CHCl_2$ with and without CL (final concentration, 3 mM) at room temperature. After evaporation of the DOPC solution with an evaporator, 1 mL of 25 mM HEPES buffer, pH 7.4, was added to the precipitate at room temperature. The mixture was shaken with a vortex for 1−2 min at room temperature, and successively ultrasonicated for 1−2 min at 25 °C. The DOPC solution with and without CL was frozen with liquid nitrogen and incubated at 50 °C until the sample was melted. The shaking, ultrasonication, freezing, and melting were repeated three times.

Ferric wild-type human cyt *c* (12 μM) was incubated with DOPC liposome (120 μM DOPC) with and without 120 μM CL in 25 mM HEPES buffer, pH 7.4, in the presence of 500 μM dithiothreitol (DTT) under air at room temperature for 1 h. Unmodified Δ8384 cyt *c* in 50 mM potassium phosphate buffer, pH 7.0, was incubated in the presence of DTT or tris(2-carboxyethyl)phosphine hydrochloride under air at 25 °C for 3 h.

Mass measurements

MALDI-TOF mass spectroscopy of wild-type human cyt *c* and its Δ8384 mutant were performed with an Autoflex II mass spectrometer (Bruker Daltonics) using sinapinic acid as a matrix in linear mode. The buffer of the sample was exchanged to ultrapure water using an Amicon ultrafiltration tube (Merck Millipore) or dialysis.

ESI mass spectroscopy (ESI-MS) was performed with an AccuTOF mass spectrometer (JEOL, Tokyo). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses were performed with an LCMS-8030 triple quadrupole mass spectrometer using a Nexera UHPLC system (Shimadzu, Kyoto). Chromatographic separations were carried out using an ODS column (Shim-pack XR-ODS II, Shimadzu, 150 mm × 2.0 mm, 2.2 μm) at 40 °C. Protein samples were digested by incubation of 300 μM cyt *c* with 0.1 μM trypsin or 0.05 μg/μL lysyl endopeptidase in 50 mM ammonium carbonate buffer, pH 7.8, under N_2

atmosphere at 37 °C for 12 h. The digested samples were diluted 20 times with 0.05% formic acid and 50% acetonitrile aqueous solution before measurements.

Optical absorption and CD measurements

Optical absorption spectra of cyt *c* were measured with a UV-2450 spectrophotometer (Shimadzu) using a 1-cm path-length quartz cell at 25 °C. The extinction coefficient of ferric Δ8384 cyt *c* was determined to be 1.15×10^5 $M^{-1}cm^{-1}$ at 406 nm with the prydine hemochrome method.[4] The concentration of ferric Δ8384 cyt *c* in the solution was calculated using the extinction coefficient. Ferric Δ8384 cyt *c* (12 μM) was incubated under air and N_2 atmosphere in the presence of 500 μM DTT at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, and the changes in the optical absorption spectra were monitored with the UV-2450 spectrophotometer (Shimadzu).

CD spectra were measured with a J-725 circular dichroism spectropolarimeter (Jasco, Japan) using a 0.1-cm path-length quartz cell at room temperature.

Repair of modified Δ8384 cyt *c*

Modified Δ8384 cyt *c* was purified from *E. coli* under air. Repair of modified Δ8384 cyt *c* was performed by incubation of the protein sample (70 μM) with 0.2 mg/ml MsrA (Abcam, Cambridge) in the presence of 20 mM DTT at 37 °C for 2 h in 50 mM potassium phosphate buffer, pH 7.0. Sample solutions were desalted and concentrated with an Amicon ultrafiltration tube (Merck Millipore) before mass measurements.

References

1 W. B. Pollock, F. I. Rosell, M. B. Twitchett, M. E. Dumont and A. G. Mauk, *Biochemistry*, 1998, **37**, 6124-6131.

2 Z. H. Wang, Y. W. Lin, F. I. Rosell, F. Y. Ni, H. J. Lu, P. Y. Yang, X. S. Tan, X. Y. Li, Z. X. Huang and A. G. Mauk, *ChemBioChem*, 2007, **8**, 607-609.

3 L. Dente and R. Cortese, *Methods Enzymol.*, 1987, **155**, 111-119.

4 E. A. Berry and B. L. Trumpower, *Anal. Biochem.*, 1987, **161**, 1-15.

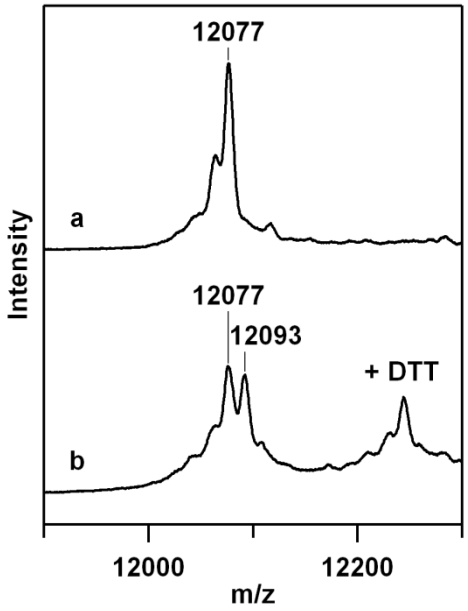

Figure S1. MALDI-TOF mass spectra of Met80-unmodified ferric Δ8384 human cyt *c* (a) before and (b) after incubation in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 500 μM DTT under air at 25 °C for 3 h. Unmodified ferric Δ8384 human cyt *c* was purified with N_2-bubbled buffer.

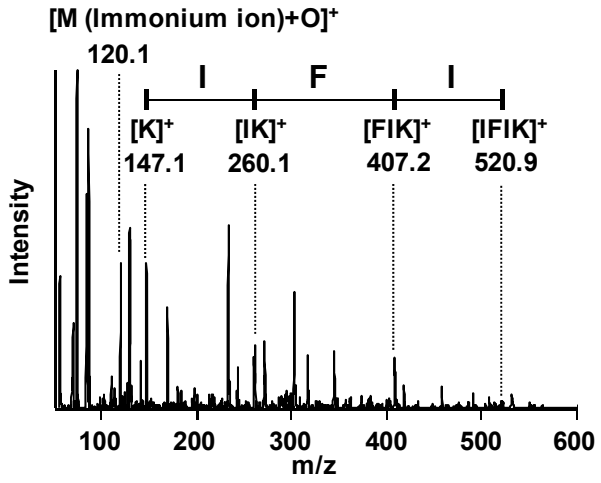

Figure S2. Tandem mass spectrum of the peptide fragment with the mass of m/z = 667.4 obtained by lysyl endopeptidase digestion of Δ8384 human cyt *c* after incubation under air in the presence of DTT. Met80-unmodified Δ8384 cyt *c* was incubated under air in the presence of DTT before digestion. Reaction conditions: ferric Δ8384 cyt *c*, 12 μM; DTT, 500 μM; buffer, 50 mM potassium phosphate buffer; pH, 7.0; incubation time, 3 h; temperature, room temperature.

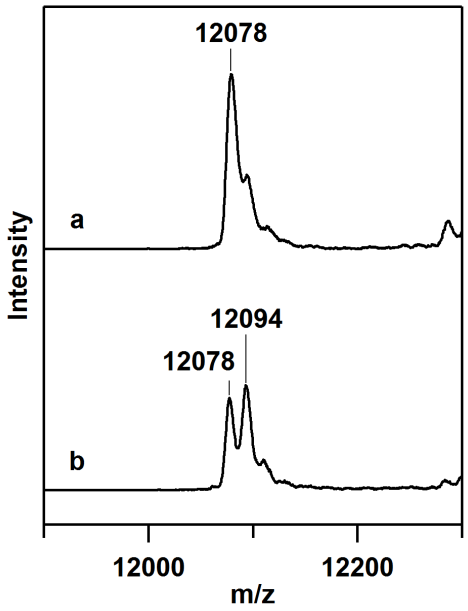

Figure S3. MALDI-TOF mass spectra of Met80-unmodified ferric Δ8384 human cyt *c* (a) before and (b) after incubation in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 500 μM tris(2-carboxyethyl)phosphine under air at 25 °C for 3 h. Unmodified ferric Δ8384 human cyt *c* was purified with N_2-bubbled buffer.