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Identification of the Binding Site of the Quinone-Head Group in Mitochondrial Coq10 by Photoaffinity Labeling

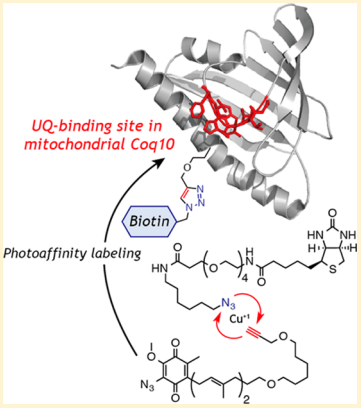
Masatoshi Murai,[†] Kohei Matsunobu,[†] Sawako Kudo,[†] Kentaro Ifuku,[‡] Makoto Kawamukai,[§] and Hideto Miyoshi*,[†]

[†]Division of Applied Life Sciences, Graduate School of Agriculture, and [‡]Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

[§]Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue 690-8504, Japan

Supporting Information

ABSTRACT: Mitochondrial Coq10 is a ubiquinone (UQ)-binding protein that is a member of the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain superfamily. Deletion of the *COQ10* gene was previously shown to cause a marked respiratory defect in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which indicated that Coq10 may support efficient electron transfer between the respiratory complexes; however, its physiological role remains elusive. To elucidate the role of Coq10, we attempted to identify the binding site of UQ in recombinant *S. pombe* Coq10 expressed in an *Escherichia coli* cell membrane through photoaffinity labeling with the photoreactive UQ probe, UQ-**1**, in combination with biotinylation of the labeled peptide by means of the so-called click chemistry. Comprehensive proteomic analyses revealed that the quinone-head ring of UQ-**1** specifically binds to the N-terminal region of Phe39−Lys45 of Coq10, which corresponds to the ligand-binding pocket of many proteins containing the START domain. The labeling was completely suppressed in the presence of an excess amount of artificial short-chain UQ analogues, such as UQ_2. In the Phe39Ala and Pro41Ala mutants, the extents of labeling were ∼40 and ∼60%, respectively, of that of wild-type Coq10. While Coq10 has been thought to bind UQ, our work first provides the direct evidence of Coq10 accommodating the quinone-head ring of UQ in its START domain. On the basis of these results, the physiological role of Coq10 has been discussed.



The biosynthesis of ubiquinone (UQ) occurs in the mitochondrial inner membrane of eukaryotes.[1,2] Extensive studies using budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* identified at least nine enzymes (proteins) Coq1−Coq9, which are required for the biosynthesis of UQ.[3−6] Similar Coq protein homologues (except for Coq4 and Coq9) have been identified in *Escherichia coli*[7] and all homologues in humans.[8] The mitochondrial binding protein Coq10 was recently reported to be critical for proper electron transport in mitochondria because deletion of the *COQ10* and *coq10* genes in *S. cerevisiae* and *S. pombe*, respectively, led to a marked respiratory defect.[9,10] In contrast to the respiratory-deficient yeast *coq1*−*coq9* mutants, Coq10 is not essential for the biosynthesis of UQ because *S. cerevisiae* and *S. pombe coq10* null mutants contain almost normal levels of UQ in their mitochondria.[9,10] Coq10 homologues have been found in various organisms, including bacteria, fungi, plants, and mammals.[10]

Using the *S. cerevisiae coq10* null mutant, Barros et al. showed that the *coq10* mutant was rescued by overexpression of the *COQ8* gene, which led to an increase in the mitochondrial content of UQ_6.[9] The addition of an artificial quinone analogue, such as UQ_2, also restored the respiration in the mitochondria isolated from the *S. cerevisiae coq10* null mutant.[9] When these hallmark phenotypes of the *coq10* mutant are taken into consideration, Coq10 may support proper electron transfer between the respiratory complexes. However, stoichiometric consideration suggested that Coq10 is unlikely to play a direct role in shuttling UQ between the respiratory complexes because the mitochondrial content of Coq10 was estimated to be 3 orders of magnitude lower than that of UQ_6 and 2 orders of magnitude lower than those of most components of the respiratory chain.[9] Thus, the specific role of Coq10 has yet to be elucidated.

The nuclear magnetic resonance (NMR) structure of the *Caulobacter crescentus* Coq10 homologue CC1736 revealed that Coq10 is a member of the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain superfamily, which is involved in the intracellular trafficking of hydrophobic molecules such as cholesterols, phospholipids, and polyketides.[11] In spite of the low level of sequence identity among their phylogenetic subgroups, the START domain has a conserved structure that consists of curved antiparallel β-sheets gripped by two α-helices and is thought to form a ligand-binding pocket called the "hydrophobic tunnel", which can

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accommodate hydrophobic molecules.[12] Mutational studies of the putative tunnel in yeast Coq10 suggested some important amino acid residues required for mitochondrial respiration.[13]

Several studies have attempted to characterize the role of Coq10 as a START protein in mitochondria.[9,10,13−15] The purified *S. pombe* Coq10, which was expressed in an *E. coli* membrane, was shown to contain an almost equimolar endogenous UQ (UQ_8), suggesting that Coq10 can accommodate UQ[10] even though the amount of bound UQ_6 in Coq10 purified from *S. cerevisiae* mitochondria was far smaller than that at the equimolar level.[9] Allan et al. recently demonstrated that expression of the *C. crescentus* Coq10 homologue CC1736 restored the respiratory growth of the *S. cerevisiae coq10* mutant.[15] Their *in vitro* binding assays indicated that CC1736 is able to bind UQ_2 and UQ_3 in an almost equimolar ratio;[15] however, it remains unclear whether the binding of these quinones was a specific event involving the hydrophobic tunnel or merely hydrophobic association under their experimental conditions. Thus, how the UQ molecule interacts with Coq10 remains elusive. Elucidating this interaction in more detail may provide insight into the role of Coq10 in mitochondrial electron transfer.

In this study, we performed photoaffinity labeling with *S. pombe* Coq10, which was expressed in an *E. coli* cell membrane, using a photoreactive UQ probe (UQ-**1**) in combination with biotinylation of the labeled peptide by means of the so-called click chemistry to examine the interaction between Coq10 and UQ. Careful proteomic analyses indicated that UQ-**1** specifically labels the region Phe39−Lys45, which forms a part of the hydrophobic tunnel in recombinant Coq10. Our results provide the first direct evidence that Coq10 specifically accommodates the quinone-head ring of UQ in a manner similar to that of other START domain proteins with their ligands.

EXPERIMENTAL PROCEDURES

Materials. Ubiquinone-1 (UQ_1) and ubiquinone-2 (UQ_2) were generously provided by Eisai Co., Ltd. (Tokyo Japan). Ubiquinone-0 (UQ_0) and *trans,trans*-farnesol were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. Protein standards for SDS−PAGE were from Bio-Rad (Hercules, CA). Other reagents were of analytical grade.

Synthesis of UQ-1. The procedure used to synthesize UQ-**1** (Figure 1) is described in the Supporting Information. Biotin tags **1** and **2** were synthesized according to the previously reported method.[16]

Fission Yeast and *E. coli* Strains. The *S. pombe* strains used in this study (wild-type and Δ*coq10*) were described previously.[10] *E. coli* BL21(DE3)pLysS was used as a host strain to express *coq10* in the pET28c vector.[10] For the construction of pET28c-*coq10*(F39A) to express the F39A *coq10* mutant in *E. coli*, four oligonucleotides [Coq10-NdeI, F39A-F, F39A-R, and Coq10-BamHI (Table S1 of the Supporting Information)] were used to amplify a DNA fragment containing the F39A mutation in *coq10*. The fragment was then cloned into the pET28c vector at the NdeI and BamHI sites. pET28c-*coq10*(P41A) was also created by cloning a DNA fragment that had been amplified by COQ10-NdeI, P41A-F, P41A-R, and Coq10-BamHI primer. The proper construction of plasmids was verified by DNA sequencing.

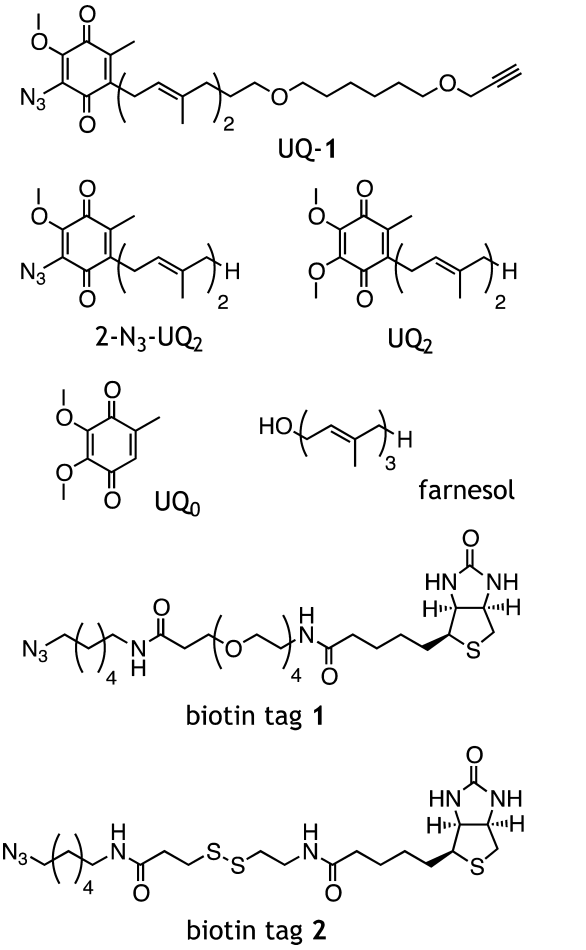

Figure 1. Structures of UQ-**1**, biotin tags **1** and **2**, and ubiquinone analogues used in this study.

Preparation of *E. coli* Membrane Vesicles Expressing *S. pombe* Coq10. *E. coli* cells were grown in 10 mL (5 mL × 2 test tubes) of Terrific Broth containing 50 μg/mL kanamycin and 25 μg/mL chloramphenicol at 28 °C for 16 h with 250 rpm rotation.[10] The culture was used to inoculate 500 mL (250 mL × 2 flasks) of the same medium, and cells were grown at 250 rpm and 37 °C until A_{600} reached 0.6. Then, 0.5 mM isopropyl D-thiogalactopyranoside was added, and the culture was continued for an additional 3 h at 28 °C. The cells were harvested by centrifugation at 6000*g* for 10 min. They were washed and resuspended in a buffer containing 10 mM Tris-HCl (pH 7.0) 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 15% (w/v) glycerol at 10% (cell weight/buffer volume). They were then sonicated four times for 15 s each (TOMY, Tokyo, Japan) and passed three times through a French pressure cell (Thermo, Waltham, MA) at 15000 psi.[17] Unbroken cells and inclusion bodies were removed by centrifugation (10000*g*, 20 min, twice), and inside-out membrane vesicles were collected by ultracentrifugation (200000*g*, 1 h). The pellet was resuspended in the same buffer, frozen in liquid nitrogen, and stored at −80 °C until use.

Photoaffinity Labeling. The *E. coli* membrane preparation (0.5−1.0 mg of protein/mL, 250−500 μL) was incubated with UQ-**1** (2−10 μM) in 50 mM KP_i buffer (pH 7.0). The samples were photoirradiated with a long wavelength UV lamp (Black Ray model B-100A, UVP, Upland, CA) for 10 min on ice, positioned 10 cm from the light source. The labeled membrane

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preparation was collected by ultracentrifugation and solubilized in a buffer containing 50 mM Tris-HCl (pH 8.0) and 1% (w/v) SDS. The proteins labeled with UQ-**1** were covalently conjugated with biotin tags (Figure 1) by means of click chemistry using the Click-iT reaction buffer kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The biotinylated and nonbiotinylated proteins were recovered by precipitation with a methanol and chloroform and subjected to further analyses. Photoaffinity labeling, click chemistry, and consequent analysis are schematically illustrated in Figure S1 of the Supporting Information.

Electrophoresis. Proteins were separated on a Laemmli-type SDS gel,[18] and the biotinylated proteins were detected with the streptavidin−alkaline phosphatase conjugate (Streptavidin-AP, Dako Cytomation, Glostrup, Denmark) according to the procedures described previously.[19] The position of recombinant Coq10 was detected using an anti-His tag antibody (mouse monoclonal, GE healthcare, Little Chalfont, U.K.). Biotinylated Coq10 was enriched with immobilized streptavidin on agarose.[19] The Coq10 captured on the avidin resin was eluted in Laemmli's sample buffer containing 2.5% mercaptoethanol.

To analyze the binding site of the quinone-head group in Coq10, Coq10, which had been reacted with UQ-**1** and consequently conjugated with biotin tag **1**, was partially purified by SDS−PAGE and electroelution using a model 422 Electro-Eluter (Bio-Rad). The purified protein was digested with lysylendopeptidase (Lys-C, Wako Pure Chemicals, Osaka, Japan), endoprotease Asp-N (Roche, Penzburg, Germany), endoprotease Arg-C (Roche), or cyanogen bromide (CNBr) in 50 mM Tris-HCl buffer (containing 0.1% SDS), 50 mM NaP_i buffer (containing 0.01% SDS), 50 mM Tris-HCl buffer (containing 4.0 M urea), or 70% formic acid, respectively.[19] The digests were separated on a Schägger-type SDS gel (16.5% T, 6% C containing 6.0 M urea[20]).

Isolation of Mitochondria from *S. pombe*. Mitochondria were prepared according to the procedure of Khalimonchuk et al.[21] with some modifications. *S. pombe* wild-type or Δ*coq10* cells were grown in 10 mL (5 mL × 2 test tubes) of YES medium at 28 °C for 36 h with 250 rpm rotation. The preculture was used to inoculate 500 mL (250 mL × 2 flasks) of the same medium, and they were grown at 120 rpm and 28 °C until A_{600} reached 2−4. *S. pombe* cells were harvested by centrifugation at 2000*g* for 10 min, washed with water, and reduced in buffer containing 0.1 M Tris-HCl (pH 9.3) and 0.3% mercaptoethanol for 10 min at 30 °C. The cells were centrifuged at 2000*g* for 10 min and washed in a buffer containing 0.5 M KCl and 10 mM Tris-HCl (pH 7.0). They were resuspended in digestion buffer containing 1.2 M sorbitol and 20 mM KP_i buffer (pH 7.4) at 3 mL/g of the wet weight of the cells. Zymolyase (Nacalai-tesque, Kyoto, Japan) was added at a concentration of 1 mg/mL, and the suspension was incubated at 30 °C for 15 min with gentle agitation. Lysing enzymes (from *Trychoderma harzianum*, Sigma) were then added at a concentration of 2 mg/mL, and the incubation was continued for a further 15 min.

All subsequent steps were performed at 4 °C. The obtained spheroplasts were pelleted at 400*g* for 10 min, washed in buffer containing 1.2 M sorbitol, 10 mM MOPS (pH 6.8), and 0.1% BSA, and resuspended in lysis buffer containing 0.65 M mannitol, 10 mM MOPS-KOH (pH 6.8), 2 mM EDTA, 0.5% BSA, and protease inhibitor cocktail (Sigma) at 6.7 mL/g of the wet cells. After careful homogenization with five strokes of a loose-fit Teflon homogenizer, homogenates were centrifuged twice at 1000*g* for 10 min. The supernatant was centrifuged at 17000*g* for 10 min. The crude mitochondrial pellet was resuspended in a buffer containing 0.7 M sorbitol, 20 mM HEPES-KOH (pH 7.4), and 2 mM EDTA and centrifuged at 1000*g* for 10 min. The supernatant was centrifuged at 12000*g* for 10 min. The final mitochondrial pellet was resuspended in a small volume of the same buffer containing 0.5% BSA. A total of 3−5 mg of mitochondria was typically obtained from a 500 mL culture.

Enzyme Assays. The NADH oxidase activity in *S. pombe* mitochondria was measured spectrometrically with a Shimadzu UV-3000 instrument (340 nm; $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) at 30 °C.[22] The reaction medium (2.5 mL) contained 0.45 M sorbitol, 20 mM Tris-HCl (pH 6.8), 100 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, and 10 mM K_2HPO_4. The final protein concentration was set to 20 μg/mL. The reaction was started by adding 100 μM NADH, followed by the addition of uncoupler SF6847 (0.2 μM) and UQ_2 (2 μM). Antimycin A was used at a final concentration of 0.2 μM to confirm that the NADH oxidase activity is fully inhibited.

Proteomic Analyses. Protein identification was conducted by mass spectrometric analysis. The proteins were in gel digested with trypsin in a buffer containing 25 mM NH_4HCO_3 at 37 °C overnight. The tryptic digests were desalted with ZipTip (Millipore, Billerica, MA) and spotted onto the target plate using CHCA as a matrix.[23] Mass spectrometric analysis was then conducted using a Bruker Autoflex III Smartbeam instrument (MALDI-TOF/TOF, Bruker Daltonics, Billerica, MA). The obtained MS spectra were analyzed according to the previously described procedures.[23]

Some protease digests of Coq10 were identified by Edman degradation. The digests were transferred onto the PVDF membrane (Immobilon-PSQ, Millipore) according to the previously described procedure.[19] Their N-terminal amino acid residues were determined with a Procise 494 HT protein sequencing system (Applied Life Sciences, Foster City, CA) at the APRO Life Science Institute, Inc. (Tokushima, Japan).

RESULTS

Design and Synthesis of Photoreactive UQ-1. The photoaffinity labeling technique is a powerful means of studying interactions between biologically active compounds and their target proteins. We previously demonstrated that photoreactive azido-quinones work as effective photoaffinity probes with cytochrome *bd* in *E. coli*,[24] alternative NADH-quinone oxidoreductase (Ndi1) in *S. cerevisiae*,[19] and Na^+-pumping NADH-quinone oxidoreductase in *Vibrio cholerae*.[25] In these studies, we directly attached biotin to the terminal end of the isoprenyl tail as the "tag" to detect and/or enrich the labeled proteins. However, we cannot exclude the possibility that the direct attachment of bulky biotin to the isoprenyl tail of UQ probes may interfere with the interaction between the probes and their target proteins.

To overcome this problem and appropriately characterize the binding of UQ to Coq10 in membrane preparations, we newly synthesized UQ probe UQ-**1** (Figure 1). We attached an alkyne to the terminal end of the hydrophobic tail of UQ-**1** to allow covalent attachment of a biotin tag via click chemistry (i.e., azide−alkyne [3+2] cycloaddition in water).[26,27] The experimental procedures are schematically outlined in Figure S1 of the Supporting Information. Once UQ-**1** labels Coq10 by UV irradiation, biotin tag **1** or **2** can be covalently conjugated with the terminal alkyne via click chemistry, allowing subsequent

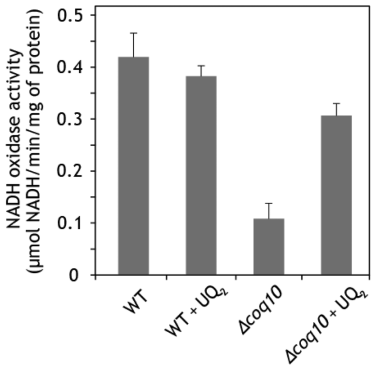

Figure 2. Characterization of electron transfer in mitochondria from wild-type and Δ*coq10* mutant *S. pombe* cells. NADH oxidase activity in mitochondria (20 μg of protein/mL) from wild-type and Δ*coq10* mutant cells at 30 °C. The activity was fully sensitive to antimycin A (0.2 μM).

biochemical analyses such as Western blotting and avidin precipitation.

Characterization of the Electron Transfer Activity in Mitochondria from Δ*coq10 S. pombe*. In the previous study,[10] we did not examine the biochemical phenotype of isolated mitochondria from Δ*coq10 S. pombe* cells. Therefore, we here characterized the electron transfer activity in mitochondria isolated from wild-type and Δ*coq10* cells. The concentration of UQ_{10} in Δ*coq10* mitochondria was similar to that in wild-type mitochondria, as described previously.[10] Wild-type *S. pombe* mitochondria oxidized exogenous NADH by external NADH dehydrogenase (NDH-2-type enzyme), the rate of which could be stimulated and fully inhibited by the addition of uncoupler SF6847 and cytochrome bc_1 complex (complex III) inhibitor antimycin A (data not shown). However, NADH oxidase activity in Δ*coq10* mitochondria was ∼25% of that of wild-type mitochondria and was restored to ∼70% by adding the short-chain UQ, UQ_2 (Figure 2). The restored activity was completely inhibited by antimycin A. This response to the exogenous quinone was only observed with mitochondria from Δ*coq10* cells (Figure 2), as observed with mitochondria from Δ*coq10 S. cerevisiae*.[9] Addition of UQ_2 did not restore NADH oxidase activity in Δ*coq10* mitochondria to that in wild-type mitochondria (Figure 2). Because the UV−visible spectra of Δ*coq10* mitochondria showed lower concentrations of type *a* and *b* cytochromes (60−70% of wild-type mitochondria), this phenomenon may be attributed to the lower expression level (or instability) of the proteins in complex III and cytochrome *c* oxidase (complex IV) in Δ*coq10* mitochondria, as had been reported for Δ*coq10 S. cerevisiae*.[9,14]

Photoaffinity Labeling of Recombinant *S. pombe* Coq10. N-Terminally His_6-tagged *S. pombe* Coq10 was expressed on the *E. coli* membrane. The membrane preparations were used for photoaffinity labeling to ensure the intactness of recombinant Coq10. As expected, an ∼20 kDa protein, which reacted with the anti-His_6 antibody, was present in the membrane preparations and not in the corresponding vector control (Figure 3). The membrane was incubated with UQ-**1**, irradiated with a UV lamp, and covalently conjugated with biotin tag **1** via click chemistry. Western analysis using streptavidin-AP (Figure 3) revealed that the ∼20 kDa protein was biotinylated, which strongly suggested that this protein is Coq10. The labeled (biotinylated) Coq10 shifted to a slightly higher position probably because of an increase in its molecular mass (anti-His_6 vs streptavidin-AP).

To verify the ∼20 kDa protein was Coq10, we purified the biotinylated protein using immobilized streptavidin. The protein biotinylated by biotin tag **1** was captured by streptavidin-agarose and released by incubating the resin in Laemmli's SDS−PAGE sample buffer at 90 °C. When the once captured protein was released from the avidin resin, two bands were detected on the SDS gel (Figure 4, elute). Because both bands were identified as *S. pombe* Coq10 by peptide mass fingerprinting (Table S2 of the Supporting Information), the upper and lower bands are thought to be UQ-**1**-labeled and UQ-**1**-dropped Coq10, respectively. This result indicated that as UQ-**1** once covalently attached to Coq10 is easily dissociated from the protein in the releasing step, the releasing treatment must be conducted under milder conditions. To address this point, we synthesized another biotinylation reagent

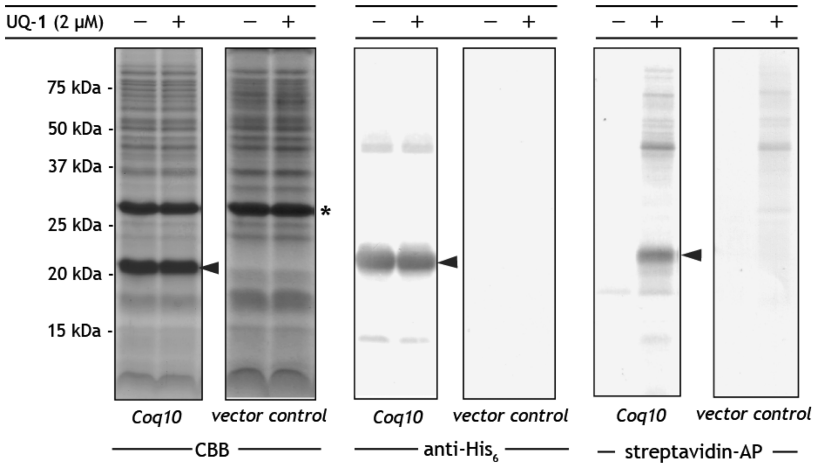

Figure 3. Photoaffinity labeling of recombinant Coq10. *E. coli* membrane preparations expressing *S. pombe* Coq10 (0.5 mg of protein/mL, 500 μL) were incubated with UQ-**1** (2 μM), irradiated with a UV lamp, and denatured with 1% (w/v) SDS, followed by the attachment of biotin tag **1** via click chemistry. Proteins were separated on a 15% Laemmli-type SDS gel and subjected to CBB staining and Western analysis using an anti-His_6 antibody and streptavidin-AP. Signals were developed by NBT/BCIP colorimetric reagents in Western analysis. *E. coli* membranes expressing the empty vector were used as a negative control (vector control). Approximately 20 and 10 μg of proteins were loaded on each well for CBB staining and Western analysis, respectively. Arrowheads indicate the Coq10 protein. The ∼30 kDa protein indicated by an asterisk was identified as *E. coli* outer membrane protein A by MADLI-TOF MS (45% coverage).

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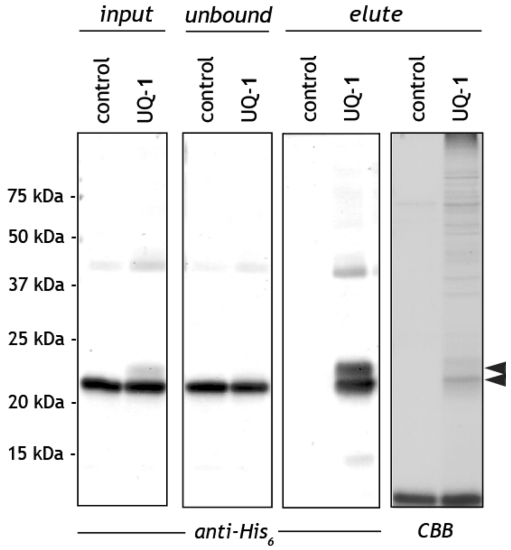

Figure 4. Enrichment of biotinylated Coq10 using immobilized avidin. *E. coli* membrane preparations (0.5 mg of protein/mL, 1 mL) were labeled with UQ-**1** (5 μM) and conjugated with biotin tag **1** via click chemistry. The biotinylated proteins were enriched using streptavidin-agarose, according to the procedure described in Experimental Procedures. The *E. coli* membrane without UQ-**1** was used as a negative control (control vs UQ-**1**). The two enriched proteins were released from streptavidin in Laemmli's sample buffer at 90 °C for 10 min (elute). The two major proteins (arrowheads) were identified as *S. pombe* Coq10 by MALDI-TOF MS (Table S2 of the Supporting Information).

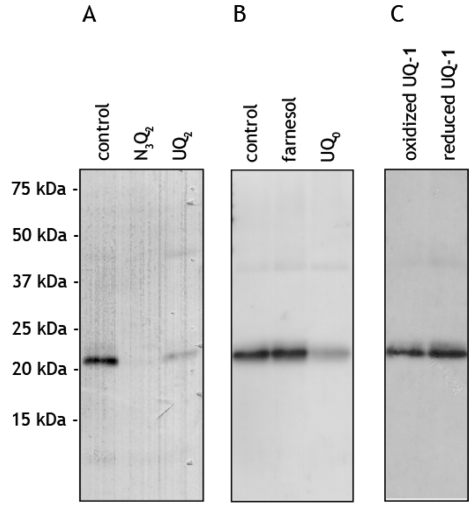

Figure 5. Specificity of labeling of Coq10 by UQ-**1**. (A) Competition test with 2-N_3-UQ_2 and UQ_2 (Figure 1). *E. coli* membrane preparations (0.5 mg of protein/mL, 500 μL) were labeled with UQ-**1** (2 μM) in the presence of 2-N_3-UQ_2 (40 μM) or UQ_2(100 μM). The proteins were denatured and biotinylated with cleavable biotin tag **2** (Figure 1) via click chemistry. The biotinylated proteins were enriched using streptavidin-agarose and released from avidin in Laemmli's sample buffer containing 2% mercaptoethanol at 40 °C for 1 h. The eluted proteins were separated on a 15% Laemmli-type SDS gel, followed by the specific detection of Coq10 using an anti-His_6 antibody. (B) Competition test with UQ_0 and farnesyl alcohol. The *E. coli* membrane (0.5 mg of protein/mL) was labeled with UQ-**1** (2 μM) in the presence of UQ_0 (100 μM) and farnesol (40 μM), followed by biotinylation, enrichment with streptavidin-agarose, and detection by the anti-His_6 antibody. (C) Comparison of binding affinities between the oxidized and reduced forms of UQ-**1**. The *E. coli* membrane (0.5 mg of protein/mL) was labeled by the oxidized or reduced form of UQ-**1** (2 μM), followed by biotinylation and enrichment with streptavidin-agarose. Coq10 was detected by Western analysis using streptavidin-AP.

containing a disulfide bond [biotin tag **2** (Figure 1)] and used this reagent when necessary. Using biotin tag **2**, the captured protein can be released under milder conditions (40 °C, 1 h, in Laemmli's buffer) to minimize the dissociation of covalently bound UQ-**1**.

Characterization of the Binding Manner of UQ-1. To characterize the binding specificity of UQ-**1**, Coq10 was labeled in the presence of short-chain UQ analogues, followed by biotinylation with biotin tag **2**, avidin precipitation, and Western analysis using the anti-His tag antibody. The labeling of Coq10 was strongly suppressed in the presence of a 20- or 50-fold molar excess of 2-N_3-UQ_2 or UQ_2, respectively (Figure 5A). We note that the UQ-**1**-dropped Coq10 band mentioned above was not detected using biotin tag **2**. In addition, UQ_0, which has no isoprenyl chain, effectively suppressed the binding of UQ-**1**, whereas farnesol, which mimics three isoprene units, did not (Figure 5B). These results strongly suggested that Coq10 accommodates the quinone-head ring of UQ, but not the polyisoprene tail moiety.

We also examined a difference in binding affinities between the oxidized and reduced forms of UQ-**1**. UQ-**1** was quantitatively reduced by sodium dithionite,[28] and the reduced UQ-**1** was subjected to photoaffinity labeling under anaerobic conditions. The reduced UQ-**1** retained comparable binding affinity against Coq10 (Figure 5C), which indicated that Coq10 can accommodate both forms of UQ with similar affinity.

Localization of the Binding Site of the Quinone-Head Group. To localize the binding site of the quinone-head group, Coq10 photoreacted with UQ-**1** was conjugated with biotin tag **1** and partially purified by preparative SDS−PAGE, followed by electroelution. When Coq10 was digested by Lys-C, Asp-N, or CNBr, a single biotinylated fragment with an apparent molecular mass of ~8, ~9, or 14 kDa, respectively, was observed (Figure 6A).

The Lys-C and Asp-N fragments were subjected to Edman degradation, and their N-terminal sequences were determined to be H_2N-GSSHH in common, which corresponds to the N-terminus of the His tag. Considering this sequence information and the theoretical digest patterns of Coq10, the Lys-C and Asp-N digests were assigned to the N-terminal region of Met1−Lys45 (5.5 kDa and the His tag) and Met1−Tyr51 (6.2 kDa and the His tag), respectively (Figure 6C). From the CNBr fragment corresponding to the biotinylated band, we identified the N-terminus containing a tryptic digest H_2N-P^{20}YKP-SLFFSLISNVNEEYER^{38}-COOH [MALDI-TOF MS, m/z 2303.5 (Figure S2 of the Supporting Information)], which indicated that the CNBr fragment can be assigned to Pro20−Met131 (12.7 kDa). These results strongly suggested that UQ-**1** binds to the region of Pro20−Lys45 (26 amino acids) in Coq10 (Figure 6C).

Furthermore, we digested the Coq10, which had been conjugated with biotin tag **1**, by endoprotease Arg-C. Although the digestion was incomplete, which may have been caused by its hydrophobicity, the smallest fragment was observed at ~6 kDa (Figure 6B). Because this Arg-C fragment contained the tryptic digest H_2N-V^{78}VCDPVALTVLADASHHR^{95}-COOH [MALDI-TOF and TOF/TOF, m/z 1960.1 (Figure S3 of the Supporting Information)], the fragment was assigned to Phe39−Arg95 (6.3 kDa). Taken together, we conclude that the binding site of the quinone-head ring of UQ-**1** is

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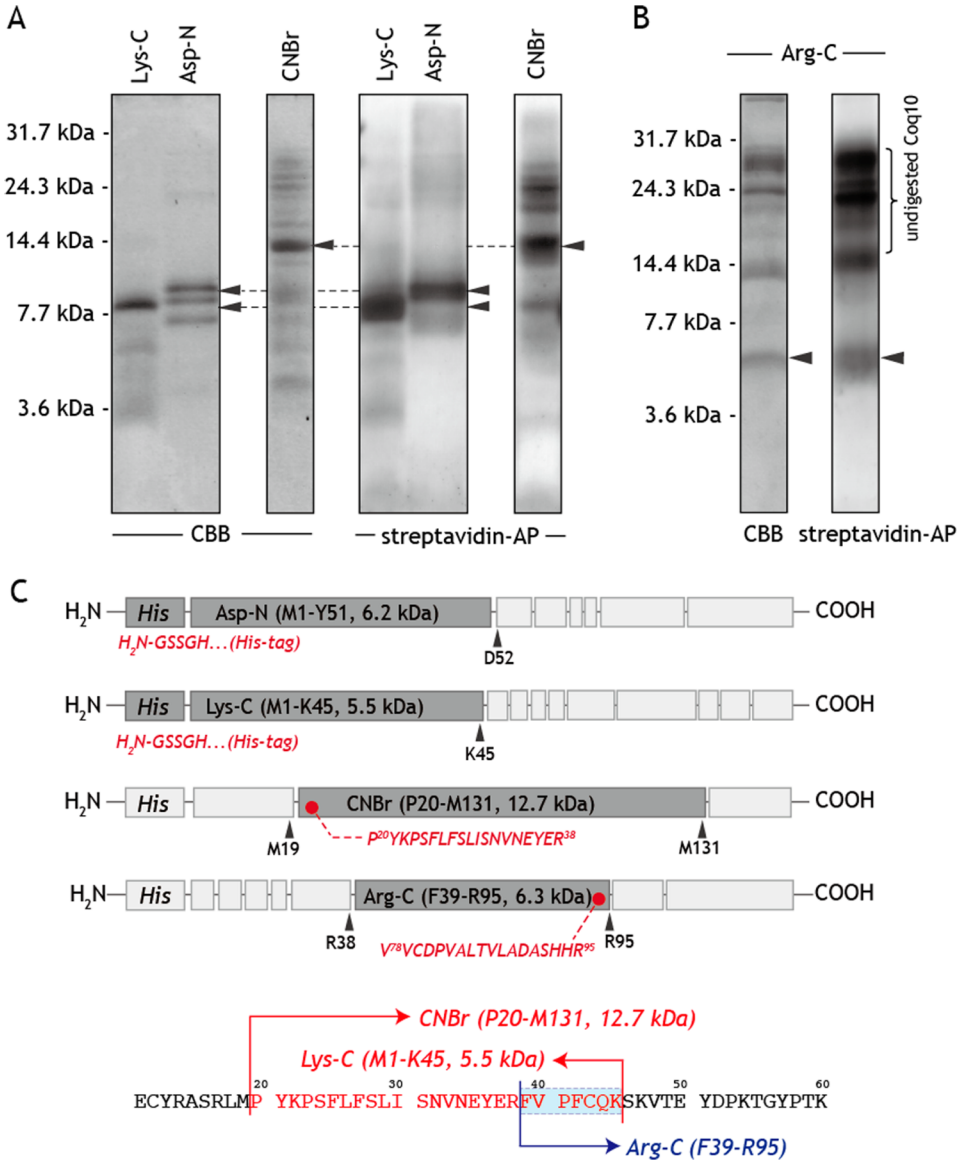

Figure 6. Localization of the binding site of UQ-**1** in Coq10. (A) *E. coli* membrane preparations (0.5 mg of protein/mL, 1.0 mL) were labeled with UQ-**1** (5 μM) and conjugated with biotin tag **1** via click chemistry. The biotinylated Coq10 was partially purified with a preparative 12.5% Laemmli-type gel and electroelution. The eluted proteins were digested with CNBr, Lys-C, and Asp-N (A) or Arg-C (B) as described in Experimental Procedures. The digests were separated on a 16% Schägger-type tricine gel (16.5% T, 6% C, 6.0 M urea), and the biotinylated proteins were then detected with streptavidin-AP. (C) Schematic presentation of the digestion of recombinant Coq10 by Asp-N, Lys-C, CNBr, or Arg-C. The residue numbers of the predicted cleavage sites refer to the sequence of *S. pombe* Coq10 (SwissProt entry Q9USM9).

located in the region of Phe39−Lys45 [seven amino acids (Figure 6C)].

To pinpoint the amino acid labeled by UQ-**1**, we performed manual Edman degradation[29] of the Arg-C fragment (Phe39−Arg65), which was conjugated with biotin tag **2**, followed by biotin detection on the PVDF membrane. Unfortunately, we could not unequivocally assign the amino acid residue containing the biotin within the first seven cycles, which corresponds to the putative labeled site, because of a low signal-to-noise ratio.

As an alternative, we compared the MALDI-TOF MS patterns of the tryptic digests between the control and labeled Coq10, which was enriched by the procedure described in the legend of Figure 4. While the peptide Phe39−Lys45 (m/z 925.5 with Cys-calbamidomethylation) was detected in common, no significant differences were observed in the spectra between the two samples (Figure S4 of the Supporting Information). As discussed in previous photoaffinity labeling studies, including our work,[19,30] labeled peptides are often determined in their free form, not in their adducted form, because of the cleavage of unstable nitrene-mediated cross-linked sites under the analytical conditions used. This may explain why the tryptic digests of the labeled Coq10 showed almost the same MALDI-TOF MS pattern as that of control Coq10. This instability may also be the cause for the failure in assigning the biotinylated residue by Edman degradation because of the large background arising from the dissociated biotin tag.

Effects of the Mutation in the Phe39−Lys45 Region on the Binding of UQ-1. To confirm the functional importance of the region labeled by UQ-**1** (Phe39−Lys45), we created two single mutants of highly conserved Phe39 and Pro41 by site-directed mutagenesis (Figure 7A). They were

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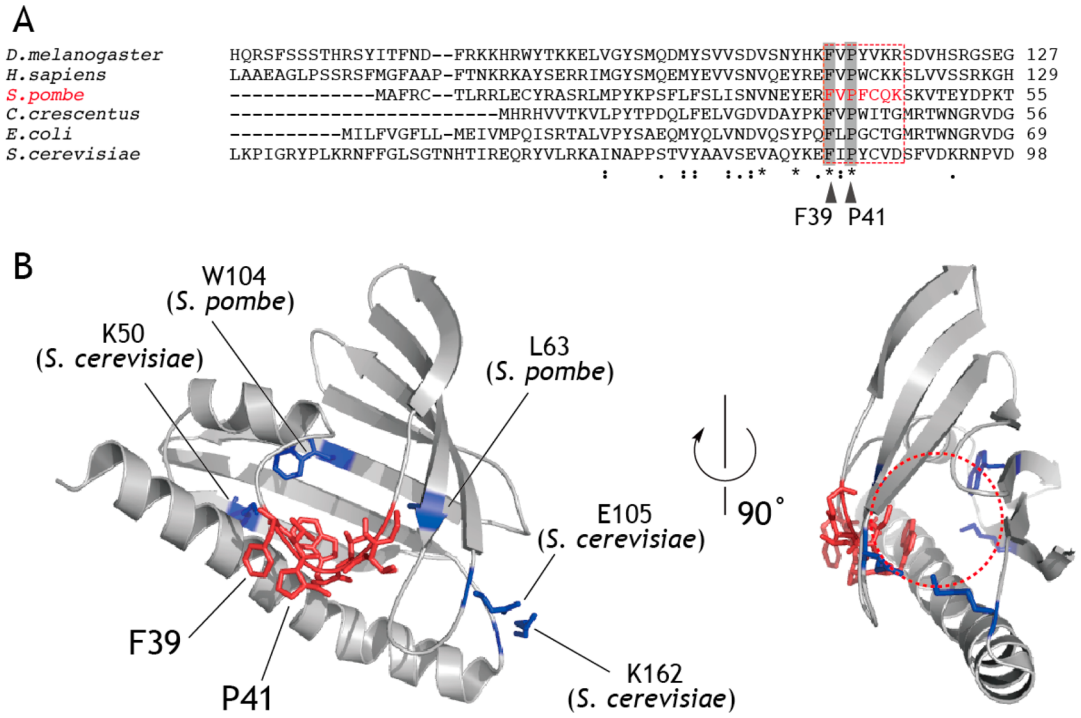

Figure 7. UQ-binding site in *S. pombe* Coq10. (A) Sequence alignment of homologues of Coq10 from various organisms. Alignment was conducted with Clastal W, using the following amino acid sequences: *Drosophila melanogaster* (Q8MLL3), *Homo sapiens* (Q96MF6), *S. pombe* (Q9USM9), *C. crescentus* (Q9A7I7), *E. coli* (P0AGL5), and *S. cerevisiae* (Q08058). The sequences corresponding to the region labeled by UQ-**1** (Phe39−Lys45) are surrounded by a red rectangle. (B) Structure of the *C. crescentus* Coq10 homologue CC1736 (Proteien Data Bank entry 1T17). Amino acids corresponding to the labeled region of Phe39−Lys45 and previously mutated ones[10,13] are shown as red and blue stick models, respectively. The red dotted circle shows the hydrophobic tunnel.

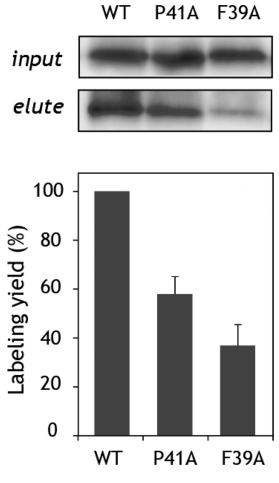

Figure 8. Photoaffinity labeling with *S. pombe* Coq10 mutants. *E. coli* membranes expressing wild-type and mutant Coq10 (0.5 mg of protein/mL, 500 μL) were labeled with UQ-**1** (2.0 μM) and conjugated with biotin tag **2**. The biotinylated proteins were enriched with streptavidin-agarose, eluted with 40 μL of Laemmli's sample buffer, and separated on a 15% Laemmli-type SDS gel. The labeled Coq10 was detected by an anti-His_6 antibody. The biotinylated Coq10 (elute; 10 μL/well) was quantified with ImageJ and normalized by the total content of Coq10 in the membrane preparations (input; 10 μg of protein was loaded onto each well).

mutated into alanine and successfully expressed in the *E. coli* membrane. These mutants were subjected to the photoaffinity labeling using a combination of UQ-**1** and biotin tag **2**, followed by avidin precipitation and Western analysis using the anti-His tag antibody, as shown in Figure 5. To correct for differences in the expression level of Coq10 between the wild type and mutants, the amount of labeled Coq10 was normalized by the total content of Coq10 in the membrane preparations. As shown in Figure 8, the replacement of Phe39 and Pro41 with alanine resulted in ∼60 and ∼40% reductions in the labeling yield, respectively, corroborating the important contribution of this region to the binding of UQ.

DISCUSSION

We used *E. coli* membrane preparations expressing *S. pombe* Coq10 instead of isolated Coq10 in the photoaffinity labeling experiments to ensure the intactness of the protein and because it is not easy to discriminate between the specific and non-specific (i.e., merely due to hydrophobic interaction) binding of hydrophobic UQ to isolated Coq10 in water. This study revealed that the quinone-head ring of UQ specifically binds to the region of Phe39−Lys45 in Coq10. *C. crescentus* CC1736 is a bacterial Coq10 homologue whose structure was determined by NMR spectroscopy,[11] and its functional identity was verified by the complementation of the *S. cerevisiae coq10* deletion mutant.[15] On the basis of the sequence alignment of various Coq10 homologues (Figure 7A), we superimposed the binding region of the quinone head in *S. pombe* Coq10 (Phe39−Lys45) onto the *C. crescentus* CC1736 structure (Phe30−Gly36). As shown in Figure 7B, this region (red) is clearly a part of the hydrophobic tunnel formed by the START domain; it was located on the loop, which may serve as the "lid" of the tunnel. The hydrophobic tunnel in Coq10 may be spacious enough to accommodate the quinone-head ring of UQ in a similar manner with other START domain proteins for their ligands.[12,31]

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Several studies characterized the role of conserved amino acid residues in Coq10, which are thought to be located at the hydrophobic tunnel in the START domain. For example, Leu63 and Trp104 in *S. pombe* Coq10[10] and Lys50, Glu105, and Lys162 in *S. cerevisiae* Coq10[13] were identified as being important amino acid residues for UQ binding and supporting mitochondrial electron transfer, respectively. The positions of these residues were also superimposed onto the *C. crescentus* CC1736 structure (Figure 7B, blue). In the Phe39−Lys45 region identified in this study, Phe39 and Pro41 are highly conserved among prokaryotes and eukaryotes, including those of humans (Figure 7A). To the best of our knowledge, these and the corresponding residues in other organisms have never been functionally characterized in previous mutagenesis studies.[10,13,15] Our photoaffinity labeling experiments using the single mutants F39A and P41A revealed the important contribution of these conserved residues recognizing the quinone-head group of UQ.

All previous studies investigated binding of the oxidized form of UQ (both endogenous and artificial UQs) to Coq10[9,10,15] and have yet to determine which form of UQ, the oxidized or reduced form, binds to Coq10 dominantly or by how much. Answering to this question may provide insight into the putative role of Coq10 in supporting efficient electron transfer between the respiratory complexes because the binding and/or transport of a minor form may be negligible in consideration of its role. Using both the oxidized and reduced forms of UQ-**1**, we revealed that Coq10 accommodates both forms of UQ-**1** with similar affinity. Therefore, Coq10 may be responsible for the binding and/or transport of both forms of UQ. This bidirectional nature of Coq10 may allow it to support the proper electron transfer between NADH dehydrogenase (NDH-II) or complex II and complex III. The result that additional UQ_2 restored the NADH oxidase activity to some extent in mitochondria from Δ*coq10* cells suggests that Coq10 may play a chaperone-like function to facilitate electron transfer[9] but is not absolutely required for the respiration. Our results also suggest that the binding domain for the quinone-head ring in the hydrophobic tunnel does not strictly recognize the ring structure in the manner of the so-called "lock and key" analogy. This may explain why the single mutations (Phe39Ala and Pro41Ala) did not result in the complete loss of UQ-**1** binding.

In conclusion, we performed photoaffinity labeling using the newly synthesized UQ-**1** to identify the binding site of the quinone-head ring in recombinant *S. pombe* Coq10. Detailed proteomic analyses revealed that the quinone-head ring specifically binds to the region of Phe39−Lys45, which is a part of the hydrophobic tunnel formed by the START domain. It was strongly suggested that Coq10 is responsible for the binding and/or transport of the oxidized and reduced forms of UQ. This bidirectional nature of Coq10 may be critical for supporting efficient electron transfer between the respiratory complexes.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedure for UQ-**1**, Figures S1−S4, and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: miyoshi@kais.kyoto-u.ac.jp. Telephone: +81-75-753-6119. Fax: +81-75-753-6408.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CBB, Coomassie brilliant blue R250; complex II, succinate-quinone oxidoreductase; complex III, cytochrome bc_1 complex; complex IV, cytochrome *c* oxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PVDF, polyvinylidene fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; START, steroidogenic acute regulatory protein-related lipid transfer; UQ, ubiquinone; UQ_1, ubiquinone-1; UQ_2, ubiquinone-2.

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