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Cell-free synthesis of cytochrome *c* oxidase, a multicomponent membrane protein

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Abstract Cell-free protein synthesis is a useful technique that can site-specifically incorporate isotope-labeled amino acids into proteins. This incorporation is essential for infrared analyses of the electronic state of a specific amino acid residue used to elucidate protein function. Although 17 membrane proteins have been synthesized in their active state by cell-free systems, to date no hetero-subunit protein has been synthesized with this technique, suggesting that there are serious technical limitations. Here we report the cell-free synthesis of *Paracoccus denitrificans* cytochrome *c* oxidase, a membrane protein complex composed of three distinct subunits that contain two heme A molecules and two redox-active copper centers. The synthesized protein exhibited normal Soret/vis absorption spectra and ferrocytochrome *c* oxidation activity.

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Abbreviations

CcO	cytochrome *c* oxidase
DDM	*n*-dodecyl-β,D-maltoside
P. denitrificans	*Paracoccus denitrificans*

Introduction

Crystallographic structural and infrared spectral analyses of the amino acid residues at the functional site of a protein are required for elucidating the mechanism of the physiological function of a protein. Crystallography determines the three dimensional position of each atom and prosthetic group while infrared spectral analysis reveals the chemical reactivity (or electronic state) of each atom by measuring the vibrational spectra of the chemical bonds of each atom. The chemical reactivity of the functional group of an amino acid residue is greatly influenced by its structural environment. For example, the pKa of the carboxyl group of an acidic amino acid increases by approximately 5 pH units when the solvent is changed from water to methanol (Isaacs 1995). Thus, the chemical reactivity of each amino acid residue at the functional site must be examined directly by infrared spectroscopy.

To identify the infrared bands associated with a particular amino acid in the functional site, the amino acid residue should be isotopically labeled using methods that were developed to incorporate an unnatural amino acid at a specific site within a protein using a cell-free protein expression system (Ellman et al. 1991; Kimata et al. 1995). This method is necessary since the infrared bands of the

active site amino acid residue may overlap the infrared bands of many amino acid residues with the same functional group that are located outside the active site. Site-directed mutagenesis is a powerful technique that can identify amino acid residues that are directly involved in protein function. However, because mutating the amino acid causes a loss or change of function (Imai et al. 1989; Shimokata et al. 2007; Tsukihara et al. 2003), site-directed mutagenesis does not provide direct information on the role of the amino acid residue in the physiological function of the protein.

One of the most challenging steps in performing infrared analyses on a protein is establishing a cell-free expression system. To date, 17 simple membrane proteins and one complex membrane protein (bacteriorhodopsin with one molecule of retinal as the prosthetic group), each showing full or partial physiological activity, have been synthesized using cell-free systems (Hovijitra et al. 2009; Schwarz et al. 2008). The low number of complex membrane proteins synthesized with this system suggests that there are serious technical limitations for synthesizing multicomponent membrane proteins. The process of folding and assembling large multicomponent membrane proteins is facilitated by a biological membrane scaffold in addition to various intracellular chaperones and transport systems (Greiner et al. 2008; Herrmann and Funes 2005; Xie and Dalbey 2008). Consistent with these findings, we found that it is critical to add an *Escherichia coli* cell membrane fraction that retains its periplasmic space to successfully express and synthesize cytochrome *c* oxidase (CcO) from *Paracoccus denitrificans* (*P. denitrificans*) in the current cell-free systems.

Experimental procedures

Preparations Recombinant *P. denitrificans* CcO containing a C-terminal His-tagged subunit I was purified from *n*-dodecyl-β,D-maltoside (DDM)-solubilized membrane fractions of *P. denitrificans* using Ni^{2+}-NTA (Ni-nitrilotriacetic acid) column chromatography with Ni^{2+}-NTA superflow (QIAGEN) (Mitchell and Gennis 1995), followed by Mono Q column chromatography on a SMART system (GE Healthcare Bioscience). Heme A was extracted from a crystalline bovine heart CcO sample using the acid-acetone method (Morrison and Horie 1965) and then neutralized with KOH. The heme A solution was diluted to 3 μM with 10 mM Tris-HCl, pH 7.2 and then stored at −80 °C until use. The *E. coli* cell membrane fractions that were added to the cell-free protein synthesis systems were prepared as follows at 4 °C unless stated otherwise. BL21 *E. coli* were grown overnight in LB medium at 37 °C. The overnight culture was diluted 1:20 with terrific broth (TB) medium and incubated at 37 °C for 3 h with vigorous shaking. The cells were harvested by centrifugation and then washed with buffer A (10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA). The cell pellet was suspended in five volumes of buffer B (buffer A containing 40 mM KCl) and mildly disrupted with a French Press (Ohtake Works) at a low pressure (500 kg/cm^2). The disrupted cells were centrifuged at 8,000×*g* for 20 min. The supernatant fraction was re-centrifuged at 100,000×*g* for 1 h. The pellets were suspended in ten volumes of sterile buffer A and centrifuged at 15,000×*g* for 15 min to precipitate the membrane fraction. The above resuspension-precipitation procedure was repeated four times with a final suspension in an appropriate volume of sterile buffer A. After adjusting the concentration of the membrane fractions to a turbidity of 0.2 at 550 nm, they were quickly frozen in liquid N_2 and stored at −80 °C until use. The above procedures were performed without digesting the outer membrane with enzymes such as lysozyme in order to obtain membrane fractions with intact membrane structures that retain the periplasmic space. Negative staining electron microscopy of these fractions revealed U-shaped structures, indicating that the membrane portions lacked whole cells but retained intact membrane structures.

Expression vectors Three genes (*cta*DII, *cta*C and *cta*E) encoding subunits I, II and III, respectively (Raitio et al. 1987, 1990), of *P. denitrificans* CcO were PCR amplified from chromosomal DNA that was extracted from cell strain #19367. Each gene had a 5′-flanking DNA sequence of approximately 20 bp that contained a Shine-Dalgarno sequence. The primer used to amplify the subunit I gene encoded six histidine residues in order to insert a His-tag at the C-terminus of subunit I. The genes for subunits I, II and III were cloned in tandem into pUC18 downstream of the *lac* promoter to generate the pSI-II-III plasmid. Plasmids pSI, pSII and pSIII harbor only the genes for subunits I, II or III in pUC18, pUC19 and pUC18, respectively. The 155-bp 5′-flanking DNA sequence of the CYP101 gene from the soil bacteria *Pseudomonas putida* (Unger et al. 1986) was PCR amplified with a 5′-*Eco*RI site and 3′-*Nde*I site. This fragment was substituted for the short 5′-flanking sequence of the subunit I gene in pSI-II-III to create pSI*-II-III. Closed circular DNA was prepared (Sambrook and Russel 2001) from the plasmid fractions that were extracted from *E. coli* using alkaline methods and used as templates for cell-free synthesis.

Cell-free protein synthesis The *E. coli* cell-free system was obtained from Promega and protein synthesis was performed as follows. Typically, 1 ml of the cell-free synthesis reaction mixture (amino acid mixture, S30 extract and S30 premixture containing an ATP generating system, rNTPs

and tRNAs provided in the kit were mixed as recommended by the manufacturer) was supplemented with 50 μg template DNA, 50 μl of 3 μM heme A solution, 30 μl of 5 mM $CuSO_4$ (pH 7.4), and 50 μl of the *E. coli* cell membrane fraction prepared as described above. The reaction mixture was incubated for 4 h at 32 °C with shaking at 2,500 rpm, and the reaction was terminated by cooling to 4 °C. The synthesized proteins were pelleted by centrifuging at 15,000×*g* for 15 min. The proteins were isotope-labeled by adding 20 μl/ml of 15 mCi [^{35}S]-methionine (GE Healthcare Bioscience) to the reaction mixture in the absence of external nonlabeled methionine.

Purification of cell-free synthesized proteins Solubilization of the membrane fraction and subsequent column chromatography of the synthesized protein were performed at 4 °C. The reaction mixture was pelleted at 15,000×*g* for 15 min, homogenized with five volumes of buffer B containing 2% DDM, and then mixed for 30 min by shaking at 2,500 rpm. Undissolved materials were removed by centrifuging at 100,000×*g* for 20 min. The supernatant fraction was loaded onto a SMART system equipped with a Mono Q column (PC 1.6/5), which was pre-equilibrated with buffer C (buffer B plus 0.1% DDM). After washing with 1 ml buffer C, the proteins were eluted with a linear 200–400 mM NaCl gradient in buffer C. The fractions that corresponded to the expected elution volume of authentic CcO were pooled and concentrated using a centrifugal filter device (Millipore).

Blue native two-dimensional electrophoresis Blue native PAGE (13%) as the first dimension and subsequent SDS-PAGE (15% gel) as the second dimension were performed as previously described (Nijtmans et al. 2002) with some modifications. Pellets from a 50 μl cell-free synthesis mixture containing ^{35}S-labeled proteins were solubilized with two volumes of extraction buffer (50 mM Bis-Tris-HCl, pH 7.0, containing 2% DDM and 750 mM 6-aminocaproic acid) and stirred for 30 min at 4 °C. The solubilized protein fractions were mixed in a 20:1 vol/vol ratio with loading buffer (50 mM Bis-Tris-HCl, pH 7.0, containing 5% Coomassie brilliant blue G-250 (Serva blue G) and 500 mM 6-aminocaproic acid) and subjected to two-dimensional electrophoresis. Size markers for blue native PAGE were obtained from GE Healthcare Bioscience (a high molecular weight native marker kit).

Analyses Synthesized ^{35}S-labeled proteins were separated by SDS-PAGE and visualized by autoradiography. The gels were vacuum-dried and autoradiographic images were acquired and quantified using a Molecular Imager system (Bio-Rad) and Quantity One Software (Bio-Rad). Immunoblot analyses of the synthesized proteins were performed following standard procedures using a PVDF (polyvinylidene difluoride) membrane (Millipore). The membranes were probed with an antibody (prepared in house) raised against *P. denitrificans* CcO subunits I and II, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody as the secondary antibody. The immunoreactive proteins were visualized with 3,3′-diaminobenzidine (Sigma) as the chromogen and then quantified as described above with the Molecular Imager system.

Measurements Ferrocytochrome *c* oxidation by the enzyme was monitored spectrophotometrically by repetitively recording the absorption spectra from 600 nm to 500 nm at 25 °C on a Perkin Elmer spectrophotometer model λ18. The reaction was initiated by adding 15 μl of the enzyme fractions to a 280 μl reaction mixture (50 mM sodium phosphate buffer, pH 6.5, 10 μM ferrocytochrome *c*, 0.1% DDM). A USB2000 CCD spectrometer (Ocean Optics) equipped with a xenon lamp and a lamp power supply from Hamamatsu Photonics was used to measure the absorption spectra of CcO in the 0.05–70 nM range using a capillary microcuvette with a 100-mm light path (Model MicroVette™, World Precision Instruments). Approximately 45 μl of the protein solution was reduced with a trace amount of dithionite and then introduced into the capillary though a loading well at one end with the aid of gentle suction from a drain port at the other end. Each spectrum was the

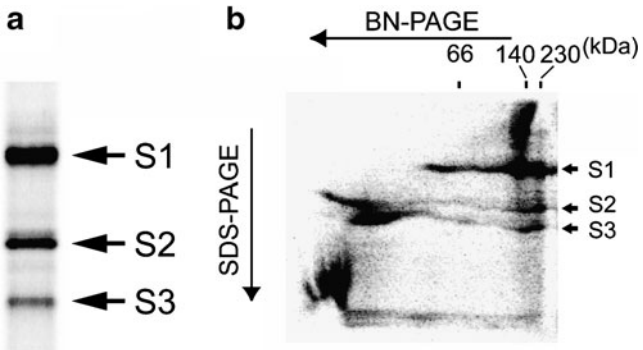

Fig. 1 Autoradiography of ^{35}S-labeled *P. denitrificans* CcO subunits I to III synthesized in the cell-free system. **a** SDS-PAGE of subunits I to III synthesized from template DNA consisting of plasmids pSI*-II-III, pSII and pSIII (3: 4: 3) in the cell-free system supplemented with *E. coli* membrane fractions, heme A and $CuSO_4$. The synthesis of the subunits was not noticeably influenced by each of these additives. The faint band adjacent to the clear band for subunit II is caused by β-lactamase, a selection marker for the pUC plasmid. **b** Two-dimensional blue native/SDS PAGE of cell-free synthesized proteins. The ^{35}S-labeled protein samples from the pellets of a 50-μl reaction mixture were solubilized and subjected to electrophoresis. The numbers above the upper side of the gel indicate the molecular mass of the marker proteins (bovine liver catalase 230 kDa, bovine heart lactate dehydrogenase 140 kDa, and bovine serum albumin 66 kDa) for the first blue native page. The locations of subunits I, II and III are indicated in the right side of each figure as S1, S2 and S3, respectively

accumulation of 200 scans recorded with a single scan of 150 ms. The spectra in the visible region were collected after manual adjustment of the light intensity level with a neutral density filter (Kenkou filter) and a variable neutral filter (Edmund Optics) inserted between the light source and the capillary cuvette, while the spectra at the Soret region were collected without the filters.

Small bubbles often form when the sample solution is introduced into the capillary cell and can cause a significant noise level due to interference of the measuring light beam. This noise was effectively separated by Fourier transformation of the raw spectra using the Igor Pro program (WaveMetrics). The applicability of the data analysis was confirmed by processing the raw spectra of the authentic CcO with the known concentration obtained under the same conditions.

Determinations The concentration of the authentic CcO samples was determined from absorption spectra of the dithionite-reduced form using an extinction coefficient of 15.6 cm^{-1} mM^{-1} (605 nm–630 nm) (Ludwig and Schatz 1980). The heme A concentration was determined from the absorption spectra of the pyridine hemochrome using an extinction coefficient of 25 mM^{-1} cm^{-1} of the absorbance difference between 587 nm and 620 nm of the reduced-minus-oxidized difference spectra (Berry and Trumpower 1987).

Results and discussion

Cell-free synthesis

The catalytic core of CcO is composed of the three largest subunits (subunits I, II and III) that contain two heme A molecules (each with a long isoprenoid tail), three copper ions with unique coordination structures and 22 transmembrane helices that are stabilized by several phospholipid molecules (Iwata et al. 1995; Shinzawa-Itoh et al. 2007; Tsukihara et al. 1996). The genes encoding subunits I, II, and III of *P. denitrificans* CcO were expressed in the *E. coli* cell-free system under the control of the *lac* promoter in pSI-II-III. Autoradiographic analyses of [^{35}S]-methionine-labeled proteins that were separated by SDS-PAGE revealed that subunits I and III were synthesized at low levels, which did not noticeably increase when the subunits were placed under the stronger T7 promoter in pET21. However, replacing the 5′-flanking DNA sequence of the subunit I gene in pSI-II-III with that of the CYP101 gene (the 155-bp DNA sequence) to generate plasmid pSI*-II-III increased the production of subunit I by over 1,000-fold and the synthesis of other subunits, although to a lesser extent (Fig. 1a). Therefore, we used plasmids pSI*-II-III, pSII and pSIII as the DNA template. The latter two plasmids encoding the genes for subunits II and III were added in order to produce comparable amounts of the three proteins.

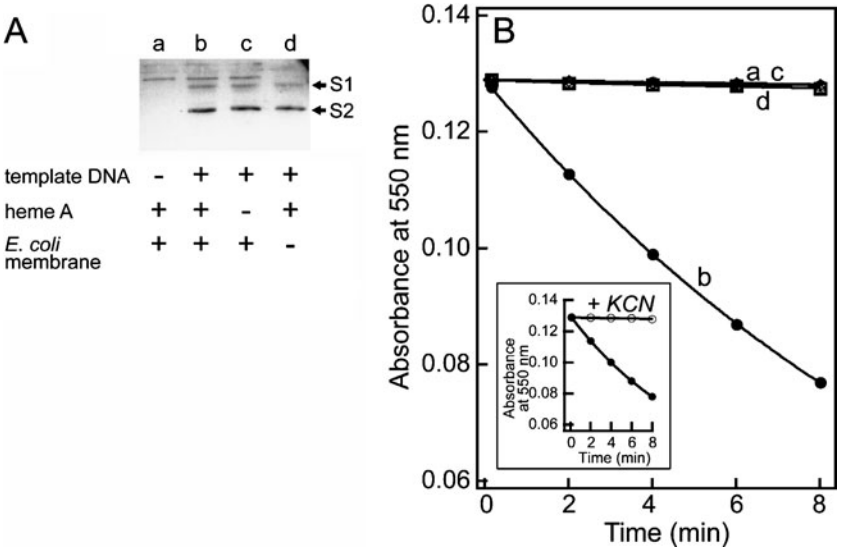

Fig. 2 Characterization of *P. denitrificans* CcO synthesized in the cell-free system. **a** Immunoblot analyses with antibodies against subunits I and II were used to examine the proteins that were synthesized under the four different conditions (*a–d*) indicated at the *bottom* of the panel. The pellets from the reaction mixture were mixed with the loading buffer and then separated by SDS-PAGE. **b** Ferrocytochrome *c* oxidation assay. The proteins synthesized under the above conditions were solubilized and chromatographed on a Mono Q column. The fractions that eluted at a volume that corresponded to the authentic enzyme were subjected to the assays. *Inset*: Ferrocytochrome *c* oxidation by the enzymatically active fraction in the absence (*filled circle*) and presence (*open circle*) of 1 mM KCN

The synthesis of the subunits in the reaction mixture (typically 1 ml) that contained 50 μg template DNA (pSI*-II-III: pSII: pSIII≈3: 4: 3) was not noticeably influenced by the addition of 50 μl of 3 μM heme A, 30 μl of 5 mM $CuSO_4$, and 50 μl of *E. coli* cell membrane fractions (turbidity of 0.2 at 550 nm), which were prepared as described in the "Experimental procedures" and had large U-shaped structures in electron microscopic images (data not shown). These images suggest that these cell membrane structures had retained the periplasmic space between the outer and inner membranes. The synthesized proteins were incorporated within the membrane fractions and could be collected by pelleting the reaction mixture with low speed sedimentation (15,000×*g*, 15 min). The ^{35}S-labeled proteins in the membrane fractions were solubilized with 2% DDM and subjected to two-dimensional blue native and SDS PAGE. Blue native-PAGE showed the monomeric and dimeric forms of the normal enzyme complex at 120 kDa and 240 kDa, respectively (Fig. 1b). Each protein fraction was separated by SDS-PAGE in the second dimension to reveal the individuals subunits I, II, and III (Fig. 1b). The intensities of the autoradiograms of the three subunits were roughly proportional to the number of methionine residues in the subunit proteins (3:1:1 in the order of subunits I to III). These results indicate that the complex had equimolar subunit assembly.

Subunit assembly and insertion into the membrane were achieved with the addition of the membrane fractions, while noticeable insertion was not detectable with other membrane fractions such as artificial phospholipids vesicles, canine pancreas microsomal membrane fractions and *E. coli* cell membrane vesicles that were prepared at high pressure (1,500 kg/cm^2) or by sonicating lysozyme-treated cells. The U-shaped structures described above were not detected in these *E. coli* cell membrane fractions.

Large scale protein synthesis (5-ml reaction mixture) was carried out using four different medium compositions: 1) complete medium (containing the coupled translation/transcription system, template DNA, *E. coli* cell membrane fractions, heme A and $CuSO_4$), 2) a control mixture without template DNA, 3) a control mixture without heme A and 4) a control mixture without the membrane fractions. The efficiency of protein synthesis is essentially constant regardless of the medium composition as long as the template DNA is included. This was demonstrated by immunoblot analyses of the synthesized proteins using antibodies specific to subunits I and II (Fig. 2a). Typically, approximately 1.2 mg of protein was synthesized in a 5-ml reaction mixture.

DDM solubilized samples were chromatographed on a Mono Q column. The fractions that eluted at a volume that corresponded to the authentic enzyme were subjected to the ferrocytochrome *c* oxidation assay (Fig. 2b). The fraction obtained from the complete medium showed cyanide-sensitive ferrocytochrome *c* oxidation activity (Fig. 2b, inset), while the other three did not. Based on six independent preparations, the molecular activities ((electron equivalent/sec)/mole of the enzyme molecule) were determined to be 142±15 (n=6) compared to 236±21 (n=6) for the authentic enzyme samples. The synthesized enzyme was spectrophotometrically quantified as described below. The specific enzyme activity of the active enzyme fraction was comparable to that of the authentic enzyme.

Spectral analysis using a capillary microcuvette with a 100-mm light path revealed that the enzymatically active protein samples in the fully reduced state had absorption peaks at 605 nm and 445 nm, which are identical to those of the authentic enzyme (Fig. 3). This indicates that the synthesized proteins had a normal enzyme structure. The amount of the enzymatically active form, which was

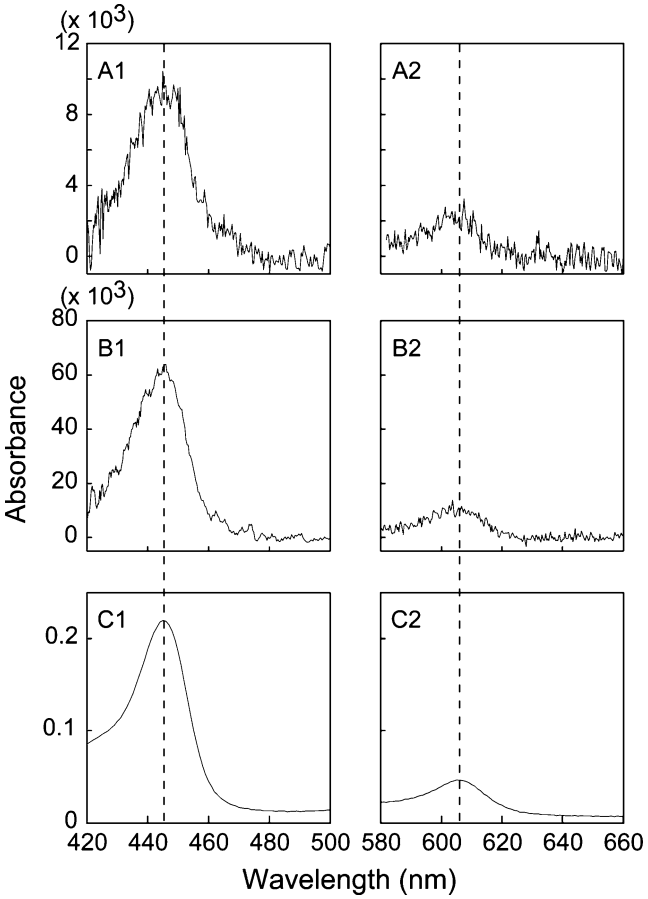

Fig. 3 The absorption spectra of the dithionite-reduced enzymatically active samples (**a**) and authentic CcO purified from *P. denitrificans* (**b** (5 nM) and **c** (1.15 μM)). The visible (A2 and B2) and Soret (A1 and B1) spectra were obtained with a USB2000 CCD spectrometer using a capillary microcuvette with a 100-mm light path with and without neutral density filters to adjust the light intensity and processed using the methods described in the "Experimental procedures". The spectra C were recorded with a Perkin Elmer spectrophotometer λ18 using a 1-cm light path cuvette

estimated based on the peak height at 605 nm in the fully reduced state, accounted for approximately 0.01% of the total synthesized proteins. When the authentic enzyme was subjected to Mono Q column chromatography under the same conditions as those for the synthesized proteins, the yield was approximately 10%, suggesting that approximately 0.1% of the synthesized protein is assembled as a native enzyme in the present cell-free system. Ni^{2+}-NTA column chromatography was much less efficient in isolating the active enzyme from the present cell-free synthesis system because of unsuccessful removal of imidazole from the eluted sample.

The subunit proteins were produced in this cell-free synthesis system with a fairly high efficiency (1.2 mg/5 ml). However, compared to cell-free systems for water-soluble proteins, the recovery of native CcO from the synthesized subunit proteins was much less efficient. For example, a recently constructed system for soluble bacterial cytochrome P450cam, a heme containing monooxygenase of *d*-camphor, has approximately a 10% yield (unpublished observation). Although the recovery efficiency is quite low, it is remarkable that a multisubunit complex membrane protein like CcO is formed in a cell-free system. The commercially available S30 extracts aside from the one used in this study did not produce a detectable amount of the active enzyme. Therefore, the present S30 extracts seem to contain the protein factors that are necessary to form the native enzyme.

The cell-free synthesis system containing an *E. coli* inner membrane fraction that did not show U-shaped structures as described above did not yield the active enzyme. This finding suggests that it is necessary to retain some integrity of the *E. coli* cell membrane structure, as indicated by the U-shaped appearance, for proper folding and assembly of the subunits. Furthermore, the critical function of the *E. coli* membrane fraction for folding and assembling *P. denitrificans* CcO indicates that this process does not require membrane preparations that are specific to the source of the membrane protein. This indicates that the present system is likely to be applicable to cell-free synthesis of various multicomponent oligomeric membrane proteins.

It should be noted that this cell-free synthesis system is expected to provide efficient methods not only for site-specific isotope labeling of amino acid residues but also for exchanging non-protein constituents (heme A, Cu ion and phospholipids) with isotope-labeled compounds or artificially synthesized derivatives. Therefore, this system will be particularly useful for various spectroscopic analyses of enzymes, such as Mössbauer, EPR, NMR, and electronic and vibrational spectroscopies.

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