Cell-free synthesis of membrane subunits of ATP synthase in phospholipid bicelles: NMR shows subunit *a* fold similar to the protein in the cell membrane

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Abstract: NMR structure determination of large membrane proteins is hampered by broad spectral lines, overlap, and ambiguity of signal assignment. Chemical shift and NOE assignment can be facilitated by amino acid selective isotope labeling in cell-free protein synthesis system. However, many biological detergents are incompatible with the cell-free synthesis, and membrane proteins often have to be synthesized in an insoluble form. We report cell-free synthesis of subunits *a* and *c* of the proton channel of *Escherichia coli* ATP synthase in a soluble form in a mixture of phosphatidylcholine derivatives. In comparison, subunit *a* was purified from the cell-free system and from the bacterial cell membranes. NMR spectra of both preparations were similar, indicating that our procedure for cell-free synthesis produces protein structurally similar to that prepared from the cell membranes.

Keywords: ATP synthase; protein NMR; cell-free synthesis; membrane protein

Introduction

The F₀F₁ ATP synthase is a multisubunit protein complex, which catalyzes ATP synthesis using the driving force of transmembrane gradient of protons in mitochondria, chloroplasts, and most microorganisms, or sodium ions in some bacteria.¹ In *Esche-*

richia coli, the membrane domain of ATP synthase (F₀) consists of three types of subunits in a ratio of *a:b:c* = 1:2:10²⁻⁴ and contains the transmembrane proton channel. The F₁-domain protruding into the cytoplasm consists of two major subunits α and β arranged in a hexamer, and three minor subunits present in a ratio of ($\alpha\beta$)₃ $\gamma\delta\epsilon$ (Fig. 1). Flow of protons through the F₀-channel induces rotation of the cylindrical oligomer built of the *c* subunits. This rotation is transmitted inside the core of the F₁ complex through the shaft built of the elongated subunit γ . Cyclical conformational changes in the three substrate binding centers, which are located on the β subunits, caused by the subunit γ rotation, constitute the structural basis of ATP formation in F₁.^{9,10}

Molecular steps of enzymatic catalysis in mitochondrial F₁ have been illuminated by a series of high-resolution X-ray structures capturing the protein at different stages of the catalytic cycle.¹¹⁻¹³ In contrast, structure of the F₀-complex is still

Abbreviations: DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DTT, dithiotreitol; LMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)].

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Figure 1. Composite picture of the *E. coli* ATP synthase. The structure of F₁-complex (3OAA⁵) includes subunits α (cyan), β (green), γ (navy), and ϵ (orange). Location of subunit δ , which is missing from the structure, is shown by the arrow. In the F₀-complex, transmembrane helices II–V of subunit a^6 are modeled as cylinders (blue). The subunit c ring⁷ is red-yellow. Only the membrane anchor of subunit b dimer⁸ (green-yellow), which forms the stalk connecting F₀ to the F₁, is shown.

largely unknown, with the exception of the X-ray structures of the rotor module built of multiple copies of the c -subunits from several bacterial species^{14–16} and the NMR structure of the membrane anchor of the subunit b .¹⁷ Model structures of the subunit c oligomer in *E. coli* calculated from the solution structure of the subunit c monomer and extensive intersubunit cross-linking data^{18,19} show overall architecture similar to the subsequent high-resolution structures of the c -oligomer from other bacteria. No high-resolution structure of subunit a is currently available. However, secondary structure prediction in combination with membrane topology studies suggests a bundle of five transmembrane α -helices^{20,21} connected by loops of different length.²²

The proton channel in ATP synthase is believed to lie at the interface of subunit a and the c oligomer.^{23–25} Protons enter the F₀-complex from outside the cell through the periplasmic half-channel and protonate the carboxyl group of a conserved glutamate or aspartate residue (Asp-61 in *E. coli*) located in the subunit c , approximately half-way across the cell membrane. The positively charged side-chain of a conserved arginine residue in subunit a (Arg-210 in *E. coli*) gates access to the exit chan-

nel, which leads to the cell interior. Movement of the subunit c oligomer, and possibly one or several helices of subunit a , results in the opening of the exit channel to the cytoplasm, deprotonation of the Asp-61, and exit of the proton.^{19,23,26} In the absence of detailed information on the structure of subunit a , this model is by necessity very general. To advance toward atomic resolution in describing the mechanism of proton translocation, a high-resolution structure of subunit a is needed.

NMR of large α -helical membrane proteins is challenging due to spectral overlap and poor chemical shift dispersion. Because of these difficulties, only few structures of polytopic membrane proteins have been solved by NMR.^{27,28} Amino acid selective isotopic labeling is essential to facilitate chemical shift assignments and resolve multiple NOE ambiguities. Several procedures for amino acid selective incorporation of isotopic labels in proteins expressed in cell cultures are available. These procedures either exploit metabolic pathways leading from isotopically labeled precursors to specific amino acids^{28–31} or require use of auxotrophic strains³² or specific inhibitors of amino acid biosynthesis³³ to allow direct incorporation of externally added isotopically labeled amino acids in the expressed protein without metabolic scrambling. Although some of these techniques, such as selective labeling of methyl groups, are widely used for NMR structure determination of large proteins, their flexibility is limited by the nature of metabolic pathways, the specificity and effectiveness of the inhibitors of amino acid synthesis, and practicality of constructing multiple auxotrophic strains.

A much more flexible approach to amino acid selective labeling is protein synthesis in a cell-free system or *in vitro* translation. In this approach, crude cell extract provides the protein synthesis machinery such as ribosomes and protein cofactors, while other components, including amino acids and DNA template for the protein of interest are added externally. In contrast to protein production in cell culture, *in vitro* translation offers essentially unlimited flexibility of amino acid selective labeling patterns, because each amino acid can be added to the synthesis mixture separately in either labeled or unlabeled form.

Efficient cell-free synthesis protocols have been developed both for prokaryotic³⁴ and eukaryotic systems.^{35,36} Synthesis of membrane proteins in cell-free system presents special challenges. Most integral membrane proteins do not insert into the lipid membrane spontaneously and require participation of the protein translocase complex and other chaperone proteins.³⁷ Although the chaperones can be added to the cell-free system to facilitate protein incorporation into the membrane,³⁸ complexity of reconstituting membrane insertion *in vitro* makes

this approach impractical for preparative purposes. Alternatively, membrane proteins can be synthesized in a soluble form in the presence of a detergent or as precipitate that can be separated from the reaction mixture and solubilized in detergent after completion of the synthesis reaction.^{39–43} Both approaches have obvious drawbacks. Many detergents favored for structural studies inhibit protein synthesis, whereas proper refolding of denatured protein upon solubilization in detergent is uncertain.

We have demonstrated effective incorporation of newly synthesized integral membrane subunits of ATP synthase into biologically compatible lipid-detergent bicelles in a cell-free system and developed new protocols for purification of subunit *a* from the cell membranes and cell-free protein synthesis mixture. NMR spectra show similar folding of subunit *a* isolated from the cell membrane and prepared by cell-free synthesis. Both approaches will facilitate high-resolution structural studies of subunit *a* and will likely be useful for other integral membrane proteins.

Results and Discussion

The main goal of this work was to design a method for the synthesis of integral membrane subunits of ATP synthase in biologically compatible detergents. Subunit *a* is 271 amino acids long and consists of five predicted transmembrane helices. Although a large number of detergents has been tested for use in cell-free synthesis of membrane proteins in soluble form,⁴⁰ consistent success with polytopic α -helical membrane proteins, such as major facilitator superfamily transporters and G-protein coupled receptors, was achieved mostly with nonbiological detergents, such as Brij and Tween derivatives.^{39,42,44,45} In contrast, detergents composed of the building blocks of natural structural lipids, such as dodecyl phosphocholine,²⁸ short chain phosphatidylcholine analogs (DHPC),²⁷ and lysolipids derived from phosphatidyl glycerol (LMPG and LPPG)⁴⁶ were found to be more useful for high-resolution NMR of α -helical integral membrane proteins. We focused on the last two types of detergents, which are structurally most similar to lipids of the cell membranes. In the initial experiments, subunit *a* synthesized as precipitate in a cell-free protein system in the absence of detergents could be resolubilized in either LMPG or DHPC (data not shown). However, both detergents severely inhibited *in vitro* translation system at the concentrations needed to solubilize subunit *a*. As a next step, we tested mixtures of short-chain (DHPC) and long-chain (DMPC) synthetic phosphatidylcholines with the rationale that mixed micelles, or bicelles, formed in such mixtures will be better tolerated by the components of cell-free synthesis system, while allowing synthesis

of subunit *a* in a soluble form. Indeed, cell-free synthesis of subunit *a* was observed in DHPC/DMPC mixtures, with a maximum yield of soluble protein found at 2% DHPC and 1% DMPC (Fig. 2). Under these conditions, most of subunit *a* was found in the soluble fraction.

DHPC taken without DMPC completely inhibited protein synthesis above 0.5%, whereas lower DHPC concentrations were insufficient to solubilize newly synthesized subunits *a* or *c*. Addition of DMPC apparently prevented binding of DHPC to the components of protein synthesis machinery and allowed protein synthesis at detergent concentrations sufficient for protein solubilization. Complex phase behavior of DHPC/DMPC mixtures as function of DMPC to DHPC molar ratio (*q*), temperature, salt concentration, and other factors has been studied extensively due to the growing interest in the use of bicelles for structural studies of proteins by NMR and X-ray crystallography.⁴⁷ In a mixture of 2% DHPC and 1% DMPC (*q* = 0.5), which proved to be optimal in our experiments, the most likely detergent-lipid phase is isotropic bicelle, a disc-shaped aggregate of detergent and lipid molecules of about 10 nm diameter,⁴⁸ although lipid from the *E. coli* cell membrane present in the extract used for protein synthesis can change the composition and geometry of the bicelles reported for pure DHPC/DMPC mixtures.

Reaction mixtures after the cell-free synthesis contained some incompletely elongated polypeptides, along with the full-length subunit *a* (Fig. 2). At the optimum DHPC and DMPC concentrations, fraction of incompletely elongated subunit *a* was low, and subsequent optimization further improved yield of fully elongated product (Fig. 3). Although polyhistidine tag at the C-terminus would allow efficient removal of the truncated products by Ni-NTA chromatography, C-terminal His-tag was shown to significantly reduce amount of subunit *a* in the cell membrane,⁴⁹ probably due to its effect on assembly of the F_0 -complex and therefore is not ideal for structural studies. Optimal concentrations of DHPC and DMPC for the synthesis of soluble subunit *c* were the same as for subunit *a*, although even under these conditions, significant amount of subunit *c* was found in the insoluble fraction (Fig. 3). Only soluble fraction of subunit *c* was used for preparative protein purification.

Subunits *a* and *c* prepared by cell-free synthesis were purified by Ni-NTA chromatography followed by ion-exchange chromatography. For NMR studies, both proteins were purified in LMPG, a lysophosphatidyl glycerol derivative previously shown to produce spectra of superior quality in a survey of detergents for NMR studies of membrane proteins.⁴⁶ Final preparations of both subunits were essentially pure and yielded 0.06 mg subunit *a* and 0.007 mg

Figure 2. Cell-free synthesis of ATP synthase subunit *a* in DHPC/DMPC mixtures. DHPC and DMPC were present in the reaction mixture at the concentrations shown above. Reaction mixture was separated by centrifugation, and the supernatant (S) and pellet (P) were analyzed by SDS-PAGE. Subunit *a* was detected using antibodies against polyhistidine tag at the N-terminus of subunit *a*. Positions of molecular weight markers are shown by the arrows. Subunit *a* has a molecular weight of 30 kDa, but migrates on SDS gels at an apparent molecular weight of about 23 kDa.⁴ Lower molecular weight fragments visible on the blot are incompletely elongated subunit *a* polypeptides.

subunit *c* per milliliter of cell-free protein synthesis mixture [Fig. 4(A,C)]. The same yield of 0.06 mg protein per milliliter reaction mixture was found when subunit *a* was synthesized as precipitate in the absence of any detergent. Thus, use of DHPC/DMPC bicelles allows production of subunit *a* in soluble form without affecting the yield of the protein.

In parallel, we have developed a new procedure for purification of subunit *a* from the cell membrane. First, F_0 -complex was purified from the cell membranes using an established protocol.⁴ At the next step, in the original procedure, F_0 was dissociated in 3M sodium trichloroacetate, and the F_0 -subunits were separated by gel filtration. Because of incomplete separation of subunits *a* and *c*, we found it difficult to achieve both high purity and high yield of subunit *a* using this method (data not shown). At the same time, Ni-NTA chromatography yielded poor results in the presence of high concentrations of trichloroacetate. Therefore, we substituted another chaotropic agent, lithium bromide, for trichloroacetate. Dissociation of F_0 -complex in lithium bromide followed by subunit separation by Ni-NTA chromatography yielded subunit *a* of exceptionally high purity essentially free of contamination with subunit *c* [Fig. 4(B)]. The yield of subunit *a* was 0.006 mg per milligram total membrane protein or 0.35 mg per liter cell culture. We are currently using this purification method for structural studies of subunit *a*.

Figure 3. Solubility of subunits *a* and *c* synthesized in DHPC/DMPC bicelles. Cell-free synthesis of subunits *a* and *c* was performed in 2% DHPC and 1% DMPC. Whole reaction mixture (W) was separated by centrifugation, and the supernatant (S) and pellet (P) were analyzed by SDS-PAGE. Subunits *a* and *c* were detected using antibodies against polyhistidine tag at the N-terminus of the protein. Positions of molecular weight markers are shown by the arrows.

Figure 4. Purification of subunits *a* (A, B) and *c* (C) prepared by cell-free synthesis (A, C) or isolated from the cell membranes (B). Purified proteins were analyzed by SDS-PAGE with silver staining (*a*) and Western blotting with antibodies directed against polyhistidine tag (*b*) or against subunit *c* (*c*). Positions of molecular weight markers are shown by the arrows.

Comparative NMR analysis of subunit *a* prepared by cell-free synthesis [Fig. 5(A)] and isolated from cell membranes [Fig. 5(B)] showed rather similar spectra. Both preparations of subunit *a* were found to be monodisperse by dynamic light scattering (data not shown). Although further optimization of sample conditions is clearly required for high-resolution NMR studies, this type of spectra is consistent with the long correlation time expected for the 30 kDa subunit *a* in detergent micelles and poor chemical shift dispersion typical for largely α -helical membrane proteins. TROSY-based experiments (Supporting Information Fig. 1) will likely be useful for high-resolution studies of subunit *a*. As a test of amino acid selective labeling in the cell-free system, we prepared subunit *a* labeled with ^{15}N -methionine [Fig. 5(C)]. Subunit *a* contains 10 potentially observable methionines. The spectrum of ^{15}N -Met subunit *a* contained six well-resolved peaks. Strong intensity and irregular shape of some of the peaks suggest that they result from overlapping signals of two or three individual residues, consistent with chemical shift degeneracy of amide groups observed in the assigned spectra of subunit *a* in organic solvent-water mixture,⁵⁰ where such peak overlap was resolved by three-dimensional experiments with ^{13}C , ^{15}N -labeled protein.

Notably, overall appearance of the NMR spectra and chemical shifts of well-separated signals, such as those of glycine backbone amides and arginine side chains, are similar for both preparations suggesting similar global fold. In fact, spectrum of subunit *a* prepared by cell-free synthesis shows better resolution than the spectrum of the protein isolated from the cell membrane. Purification of subunit *a* presents an unusual challenge, because subunit *a* is toxic for the cells and requires other subunits of the

F_0 -complex for stable expression and incorporation into the cell membrane. This in turn requires treatment with chaotropic agents to dissociate F_0 in the course of subunit *a* purification, which may lead to partial protein denaturation and consequent degradation of spectral quality. Cell-free synthesis procedure described here may offer an additional advantage over purification from the cell membrane, because subunit *a* is produced without other F_0 -subunits and is purified without the harsh dissociation step.

Signals of arginine side chains are useful indicators of sample purity as they are located in an uncrowded region of the spectrum and commonly appear in ^1H , ^{15}N -HSQC spectra with a negative sign as a result of ^{15}N frequency aliasing. In addition, arginine side chains generally have high-local mobility producing fairly narrow spectral lines even in large proteins. Four arginine side-chain signals were observed for subunit *a* [Fig. 5(A,B)], in agreement with the number of arginines in the protein. Arginine-210 is a uniformly conserved residue, which is believed to play gatekeeper's role between the two proton half-channels in the F_0 -complex of ATP synthase, possibly through interaction with the Asp-61 of subunit *c*, which is believed to be the key relay point for protons on their way across the membrane. To facilitate studies of functional interaction between subunits *a* and *c*, we have assigned the Arg-210 side chain by side-directed mutagenesis. Comparison of the NMR spectra of the wild-type subunit *a* and the R210Q mutant allowed easy identification of the R210 signal [Fig. 5(D)].

Although there is no direct proof that isolated subunit *a* preserves its native fold, subunit *a* purified from the cell membranes was successfully

Figure 5. ^1H , ^{15}N -HSQC spectra of subunits *a* (A–D) and *c* (E, F) prepared by cell-free synthesis (A, C, E, F), or isolated from the cell membranes (B, D). Proteins were uniformly ^{15}N -labeled (A, B, D, E), or selectively labeled with ^{15}N -methionine (subunit *a*, C), or ^{15}N -valine (subunit *c*, F). Signals of arginine side chains (A, B, D) are boxed and shown in red. For R210 assignment (D), spectra of the wild-type subunit *a* (WT) and of the R210Q mutant (R210Q) were compared. Only the part of the spectrum corresponding to the boxed region in panel B is shown in panel D. Position of the R210 side chain signal peak is marked by a dot. All spectra were recorded on a 600 MHz spectrometer in 0.5% (subunit *a*, A–D) or 0.1% (subunit *c*, E, F) LMPG at pH 6.0 and 45°C (A–F), or 37°C (D), with 0.2 mM (A, B, D) or 0.1 mM (C) subunit *a*, or with 0.05 mM (E) or 0.1 mM (F) subunit *c*.

reconstituted with the other F_0 -subunits to produce a functional proton channel.^{51,52} Taken together, these results suggest that cell-free synthesis of subunit *a* will be useful for amino acid selective labeling to facilitate structural studies of this protein by high-resolution NMR. In comparison, subunit *c* prepared by cell-free synthesis showed NMR spectra of very good quality. As a preliminary test of spectrum

completeness, all nine glycine residues and the side chains of the two arginines can be identified in the subunit *c* spectrum [Fig. 5(E)]. In the spectrum of subunit *c* selectively labeled with ^{15}N -valine, five of six valine residues in subunit *c* produce well-dispersed peaks of similar intensity. An additional weak peak, which may correspond to the sixth valine residue, was also observed [Fig. 5(F)].

In summary, we demonstrated effective incorporation of integral membrane subunits of ATP synthase into the biologically compatible lipid-detergent bicelles and developed new methods for preparation of subunit *a* for structural studies. Subunit *a* was purified from the cell membranes at high yield and exceptional purity. This preparation is suitable for structural studies by NMR and X-ray crystallography. Cell-free synthesis of subunit *a* will be useful for amino acid selective labeling to facilitate, functional studies, chemical shift assignment, and NOE analysis. Subunit *c* prepared by cell-free synthesis produced NMR spectra of very good quality and will be used for functional studies of this protein, which plays critical role in proton transport through the membrane complex of ATP synthase. Successful cell-free synthesis of these two structurally rather different membrane proteins in DHPC/DMPC bicelles demonstrates applicability of this method for various membrane proteins and should be useful for structural as well as mechanistic studies of membrane proteins by high-resolution NMR.

Materials and Methods

Plasmid construction and expression of subunits *a* and *c* in cell free protein synthesis system

The *atpB* gene encoding subunit *a* was amplified from the plasmid pBWU13-aN-His₆ containing complete *E. coli atp* operon with *atpB* modified to encode a hexahistidine tag following the N-terminal methionine^{49,53} using a forward primer designed to introduce a *NdeI* restriction site immediately before the start codon and a reverse primer designed to introduce *NheI* restriction site after the *atpB* stop codon. For subunit *c* synthesis, the *atpE* gene was amplified from the plasmid pCP35⁵⁴ using a forward primer to introduce an *NdeI* restriction site before initiation ATG of *atpE* and a hexahistidine tag following the start codon and a reverse primer introducing *NdeI* restriction site after the second stop codon of *atpE*. Purified PCR fragments were ligated into vector pEXP1-DEST (Invitrogen, Carlsbad, CA) using *NheI* and *NdeI* restriction sites producing plasmids pEU2 (subunit *a*) and pEOE-N-His (subunit *c*). The plasmids were transformed into *ccdB* resistant One Shot competent cells (Invitrogen, Carlsbad, CA). Plasmids were purified for cell-free protein synthesis using Maxi, Mega, or Giga kits (Qiagen, Germantown, MD), or NucleoBond PC kit (Macherey-Nagel, Bethlehem, PA).

Cell-free protein synthesis

Cell-free protein synthesis protocol was developed on the basis of previously described method³⁴ with the following modifications. The ¹⁵N-ammonium acetate was used to prevent loss of asparagine and glutamine side-chain signals due to transamination. The

Mg²⁺ and K⁺ concentrations were optimized for protein yield, and the synthesis reactions were routinely performed at 11 mM Mg²⁺ and 330 mM K⁺. Plasmid DNA was added to a concentration of 30–50 µg/mL. For natural isotopic abundance samples, the 20 individual amino acids (Roche, San Francisco, CA or 5 Prime, Gaithersburg, MD) were added at 1 mM each. For selectively labeled samples, one amino acid was provided in ¹⁵N-labeled form at 1 mM, and the others as described earlier. For U-¹⁵N-labeled samples, “cell-free” U-¹⁵N-amino acid mix (Cambridge Isotope Laboratories, Andover, MA) was used at a final concentration of 2 mg/mL. RiboLock™ RNase Inhibitor and T7 RNA Polymerase were from Fermentas (Burlington, ON). A stock solution of 20% DHPC with 10% DMPC (Avanti Polar Lipids, Alabaster, AL) was prepared in 10 mM Tris-Cl, pH 8.2, and added to the synthesis mixture to a final concentration of 2% DHPC and 1% DMPC under the standard conditions. Protein synthesis was carried out in batch mode for 5 h for subunit *a* and for 3 h for subunit *c* at 30°C under gentle tumbling.

Purification of subunits *a* and *c* prepared by cell-free synthesis

After the completion of protein synthesis, insoluble material was removed by centrifugation at 14,000 × *g* for 10 min at 4°C. LMPG was added to the supernatant to a final concentration of 0.2%, and the EDTA-free protease inhibitor cocktail (Roche, San Francisco, CA) was added as recommended by the manufacturer. The mixture was incubated on a shaking platform for 10 min at room temperature. Then, 500 mM NaCl and 4 mM imidazole were added, and the mixture was incubated with ~50 mg/mL Ni-NTA agarose (Qiagen, Germantown, MD), pre-equilibrated with 25 mM potassium phosphate, pH 8.0 containing 0.1% LMPG for subunit *a*, or 0.02% LMPG for subunit *c* with gentle shaking for 25 min at room temperature. The mixture was transferred into a chromatography column, and the protein solution was drained under gravity flow. The column was washed with five bed volumes of 25 mM potassium phosphate, pH 8.0, containing 0.1% (subunit *a*) or 0.02% (subunit *c*) LMPG, and 500 mM NaCl, and then with five bed volumes of 25 mM potassium phosphate, pH 8.0, containing 0.1% (subunit *a*) or 0.02% (subunit *c*) LMPG. The protein was eluted with the same buffer containing 50 mM imidazole. Chromatography fractions were analyzed by SDS-PAGE.

Fractions containing subunit *a* or *c* were combined and pH was adjusted to 8.0. The protein solution was then applied to 1 mL anion exchange HiTrap Q column, connected to A KTA Prime protein chromatography system (GE Healthcare, Baie d'Urfe QB), and pre-equilibrated with 25 mM potassium phosphate, pH 8.0, containing 0.1% (subunit *a*) or

0.02% (subunit *c*) LMPG. Protein was eluted with a 0–1.0M linear gradient of NaCl concentration in the same buffer in a total volume of 15 mL at a flow rate of 1 mL/min. Fractions containing purified subunit *a* or subunit *c* were combined and dialyzed against 50 volumes of 25 mM potassium phosphate, pH 6.0 containing 0.1% (subunit *a*) or 0.02% (subunit *c*) LMPG using 12–14,000 (subunit *a*) or 6–8,000 (subunit *c*) molecular weight cutoff dialysis membrane at 4°C. For NMR, protein was concentrated to 0.1–0.2 mM (subunit *a*) or 0.05–0.1 mM (subunit *c*) by membrane filtration using Vivaspin 6 (GE Healthcare, Baie d'Urfe, QB) for subunit *a* or Amicon Ultra 0.5 (Millipore, Billerica, MA) for subunit *c*.

Purification of subunit *a* from the *E. coli* cell membranes

The C43(DE3) *E. coli*⁵⁵ transformed with pBWU13- α NHis₆ was grown as described previously.⁵³ For ¹⁵N-labeling, ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Andover, MA) was substituted for natural isotopic abundance NH₄Cl in the growth medium. The F₀ complex was isolated from the cells essentially as described previously.⁴ Isolated F₀ was diluted to a final concentration of 1 mg/mL in the dissociation buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 3M lithium bromide, 1% (w/v) sodium cholate, and 5% (w/v) Zwittergent 3–14 (Sigma-Aldrich, Oakville, ON) and incubated for 20 h at room temperature. This solution containing dissociated F₀ subunits was loaded onto the Ni-NTA column pre-equilibrated with the dissociation buffer at a ratio of 30 mg protein per milliliter bed volume. After loading, the column was washed first with five bed volumes of the dissociation buffer, then with five bed volumes of dissociation buffer supplemented with 20 mM imidazole, and finally with five bed volumes of 10 mM potassium phosphate, pH 8.0, 1 mM DTT, and 0.01% LMPG-containing 20 mM imidazole. Subunit *a* was eluted from the column with 250 mM imidazole in 10 mM potassium phosphate, pH 8.0 containing 1 mM DTT, and 0.01% LMPG. Fractions containing subunit *a* were concentrated to 5–8 mg/mL in a Vivaspin-20 concentrating device (GE Healthcare, Baie d'Urfe, QB) pretreated with bovine serum albumin to minimize protein adsorption to the polymer membrane. To exchange buffer for NMR, the protein sample was subjected to several cycles of dilution with 50 mM KH₂PO₄, pH 6.0, containing 0.01% LMPG and concentrating to the original volume in Vivaspin-20.

Analytical procedures

Protein samples were analyzed by SDS-PAGE.⁵⁶ Protein was visualized by silver staining.⁵⁷ For Western blotting, following SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) using Towbin

buffer.⁵⁸ Immunodetection was carried out using reaction of the hexahistidine tag on subunits *a* and *c* with anti-His HRP Conjugate Kit (Qiagen, Germantown, MD) visualized by chemiluminescence. Protein was determined by modified Lowry assay.⁵⁹

NMR spectroscopy

The NMR samples contained 0.2–0.35 mM subunit *a* or 0.05–0.1 mM subunit *c* prepared as described earlier with 5% (v/v) deuterium oxide and 0.3 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid added for frequency lock and chemical shift referencing, respectively, in a total volume of 0.35–0.4 mL. The ¹H, ¹⁵N-HSQC spectra were recorded at 45°C on a 600 or 900 MHz Bruker (Billerica, MA) NMR spectrometer equipped with a Cryoprobe and *z*-axis pulse field gradients. Water suppression was achieved using double-pulsed field gradient spin echo.⁶⁰

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References

1. Senior AE (1988) ATP synthesis by oxidative phosphorylation. *Physiol Rev* 68:177–231.
2. Fillingame RH, Jiang W, Dmitriev OY (2000) Coupling H⁺ transport to rotary catalysis in F-type ATP synthases: structure and organization of the transmembrane rotary motor. *J Exp Biol* 203:9–17.
3. Jiang W, Hermolin J, Fillingame RH (2001) The preferred stoichiometry of *c* subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10. *Proc Natl Acad Sci USA* 98:4966–4971.
4. Schneider E, Altendorf K (1986) Proton-conducting portion (F₀) from *Escherichia coli* ATP synthase: preparation, dissociation into subunits, and reconstitution of an active complex. *Methods Enzymol* 126:569–578.
5. Cingolani G, Duncan TM (2011) Structure of the ATP synthase catalytic complex (F₁) from *Escherichia coli* in an autoinhibited conformation. *Nat Struct Mol Biol* 18:701–707.
6. Pierson HE, Uhlemann EM, Dmitriev OY (2011) Interaction with monomeric subunit *c* drives insertion of ATP synthase subunit *a* into the membrane and primes a-c complex formation. *J Biol Chem* 286:38583–38591.
7. Fillingame RH, Dmitriev OY (2002) Structural model of the transmembrane F₀ rotary sector of H⁺-transporting ATP synthase derived by solution NMR and intersubunit cross-linking in situ. *Biochim Biophys Acta* 1565:232–245.
8. Dmitriev O, Jones PC, Jiang W, Fillingame RH (1999) Structure of the membrane domain of subunit *b* of the *Escherichia coli* F₀F₁ ATP synthase. *J Biol Chem* 274:15598–15604.
9. Cross RL (2000) The rotary binding change mechanism of ATP synthases. *Biochim Biophys Acta* 1458:270–275.

10. Yoshida M, Muneyuki E, Hisabori T (2001) ATP synthase—a marvellous rotary engine of the cell. *Nat Rev Mol Cell Biol* 2:669–677.
11. Abrahams JP, Leslie AG, Lutter R, Walker JE (1994) Structure at 2.8 Å resolution of F₁-ATPase from bovine heart mitochondria. *Nature* 370:621–628.
12. Braig K, Menz RI, Montgomery MG, Leslie AG, Walker JE (2000) Structure of bovine mitochondrial F₁-ATPase inhibited by Mg²⁺ ADP and aluminium fluoride. *Structure* 8:567–573.
13. Menz RI, Walker JE, Leslie AG (2001) Structure of bovine mitochondrial F₁-ATPase with nucleotide bound to all three catalytic sites: implications for the mechanism of rotary catalysis. *Cell* 106:331–341.
14. Meier T, Polzer P, Diederichs K, Welte W, Dimroth P (2005) Structure of the rotor ring of F-type Na⁺-ATPase from *Ilyobacter tartaricus*. *Science* 308:659–662.
15. Pogoryelov D, Yildiz O, Faraldo-Gomez JD, Meier T (2009) High-resolution structure of the rotor ring of a proton-dependent ATP synthase. *Nat Struct Mol Biol* 16:1068–1073.
16. Preiss L, Yildiz O, Hicks DB, Krulwich TA, Meier T (2010) A new type of proton coordination in an F₁F₀-ATP synthase rotor ring. *PLoS Biol* 8:e1000443.
17. Dmitriev O, Jones PC, Jiang W, Fillingame RH (1999) Structure of the membrane domain of subunit b of the *Escherichia coli* F₀F₁ ATP synthase. *J Biol Chem* 274:15598–15604.
18. Dmitriev OY, Jones PC, Fillingame RH (1999) Structure of the subunit c oligomer in the F₁F₀ ATP synthase: model derived from solution structure of the monomer and cross-linking in the native enzyme. *Proc Natl Acad Sci USA* 96:7785–7790.
19. Rastogi VK, Girvin ME (1999) Structural changes linked to proton translocation by subunit c of the ATP synthase. *Nature* 402:263–268.
20. Schwem BE, Fillingame RH (2006) Cross-linking between helices within subunit a of *Escherichia coli* ATP synthase defines the transmembrane packing of a four-helix bundle. *J Biol Chem* 281:37861–37867.
21. Valiyaveetil FI, Fillingame RH (1998) Transmembrane topography of subunit a in the *Escherichia coli* F₁F₀ ATP synthase. *J Biol Chem* 273:16241–16247.
22. Vik SB, Ishmukhametov RR (2005) Structure and function of subunit a of the ATP synthase of *Escherichia coli*. *J Bioenerg Biomembr* 37:445–449.
23. Fillingame RH, Angevine CM, Dmitriev OY (2003) Mechanics of coupling proton movements to c-ring rotation in ATP synthase. *FEBS Lett* 555:29–34.
24. Moore KJ, Fillingame RH (2008) Structural interactions between transmembrane helices 4 and 5 of subunit a and the subunit c ring of *Escherichia coli* ATP synthase. *J Biol Chem* 283:31726–31735.
25. Steed PR, Fillingame RH (2008) Subunit a facilitates aqueous access to a membrane-embedded region of subunit c in *Escherichia coli* F₁F₀ ATP synthase. *J Biol Chem* 283:12365–12372.
26. von Ballmoos C, Wiedenmann A, Dimroth P (2009) Essentials for ATP synthesis by F₁F₀ ATP synthases. *Annu Rev Biochem* 78:649–672.
27. Gautier A, Mott HR, Bostock MJ, Kirkpatrick JP, Nietlispach D (2010) Structure determination of the seven-helix transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. *Nat Struct Mol Biol* 17:768–774.
28. Van Horn WD, Kim HJ, Ellis CD, Hadziselimovic A, Sulistijo ES, Karra MD, Tian C, Sonnichsen FD, Sanders CR (2009) Solution nuclear magnetic resonance structure of membrane-integral diacylglycerol kinase. *Science* 324:1726–1729.
29. Gardner KH, Kay LE (1997) Production and incorporation of N-15, C-13, H-2 (H-1-delta 1 methyl) isoleucine into proteins for multidimensional NMR studies. *J Am Chem Soc* 119:7599–7600.
30. Goto NK, Gardner KH, Mueller GA, Willis RC, Kay LE (1999) A robust and cost-effective method for the production of Val, Leu, Ile (delta 1) methyl-protonated 15N-, 13C-, 2H-labeled proteins. *J Biomol NMR* 13:369–374.
31. Rajesh S, Nietlispach D, Nakayama H, Takio K, Laue ED, Shibata T, Ito Y (2003) A novel method for the bio-synthesis of deuterated proteins with selective protonation at the aromatic rings of Phe, Tyr and Trp. *J Biomol NMR* 27:81–86.
32. Whittaker JW (2007) Selective isotopic labeling of recombinant proteins using amino acid auxotroph strains. *Methods Mol Biol* 389:175–188.
33. Kim HW, Perez JA, Ferguson SJ, Campbell ID (1990) The specific incorporation of labelled aromatic amino acids into proteins through growth of bacteria in the presence of glyphosate. Application to fluorotryptophan labelling to the H⁺-ATPase of *Escherichia coli* and NMR studies. *FEBS Lett* 272:34–36.
34. Torizawa T, Shimizu M, Taoka M, Miyano H, Kainosho M (2004) Efficient production of isotopically labeled proteins by cell-free synthesis: a practical protocol. *J Biomol NMR* 30:311–325.
35. Madin K, Sawasaki T, Ogasawara T, Endo Y (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc Natl Acad Sci USA* 97:559–564.
36. Vinarov DA, Newman CL, Tyler EM, Markley JL, Shahan MN (2006) Wheat germ cell-free expression system for protein production. *Curr Protoc Protein Sci* 2006; Chapter 5;Unit 5.18.
37. Saier MH Jr (2006) Protein secretion and membrane insertion systems in Gram-negative bacteria. *J Membr Biol* 214:75–90.
38. Kol S, Majczak W, Heerlien R, van der Berg JP, Nouwen N, Driessen AJ (2009) Subunit a of the F₁F₀ ATP synthase requires YidC and SecYEG for membrane insertion. *J Mol Biol* 390:893–901.
39. Junge F, Haberstock S, Roos C, Stefer S, Proverbio D, Dotsch V, Bernhard F (2011) Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins. *Nat Biotechnol* 28:262–271.
40. Klammt C, Schwarz D, Fendler K, Haase W, Dotsch V, Bernhard F (2005) Evaluation of detergents for the soluble expression of alpha-helical and beta-barrel-type integral membrane proteins by a preparative scale individual cell-free expression system. *FEBS J* 272:6024–6038.
41. Klammt C, Schwarz D, Lohr F, Schneider B, Dotsch V, Bernhard F (2006) Cell-free expression as an emerging technique for the large scale production of integral membrane protein. *FEBS J* 273:4141–4153.
42. Klammt C, Schwarz D, Dotsch V, Bernhard F (2007) Cell-free production of integral membrane proteins on a preparative scale. *Methods Mol Biol* 375:57–78.
43. Reckel S, Sobhanifar S, Schneider B, Junge F, Schwarz D, Durst F, Lohr F, Guntert P, Bernhard F, Dotsch V (2008) Transmembrane segment enhanced labeling as a tool for the backbone assignment of alpha-helical membrane proteins. *Proc Natl Acad Sci USA* 105:8262–8267.

44. Schwarz D, Klammt C, Koglin A, Lohr F, Schneider B, Dotsch V, Bernhard F (2007) Preparative scale cell-free expression systems: new tools for the large scale preparation of integral membrane proteins for functional and structural studies. *Methods* 41:355–369.
45. Schwarz D, Daley D, Beckhaus T, Dotsch V, Bernhard F (2010) Cell-free expression profiling of *E. coli* inner membrane proteins. *Proteomics* 10:1762–1779.
46. Krueger-Koplin RD, Sorgen PL, Krueger-Koplin ST, Rivera-Torres IO, Cahill SM, Hicks DB, Grinius L, Krulwich TA, Girvin ME (2004) An evaluation of detergents for NMR structural studies of membrane proteins. *J Biomol NMR* 28:43–57.
47. Katsaras J, Harroun TA, Pencer J, Nieh MP (2005) “Bicellar” lipid mixtures as used in biochemical and biophysical studies. *Naturwissenschaften* 92:355–366.
48. Andersson A, Maler L (2006) Size and shape of fast-tumbling bicelles as determined by translational diffusion. *Langmuir* 22:2447–2449.
49. Stalz WD, Greie JC, Deckers-Hebestreit G, Altendorf K (2003) Direct interaction of subunits a and b of the F₀ complex of *Escherichia coli* ATP synthase by forming an ab₂ subcomplex. *J Biol Chem* 278:27068–27071.
50. Dmitriev OY, Abildgaard F, Markley JL, Fillingame RH (2004) Backbone ¹H, ¹⁵N and ¹³C assignments for the subunit a of the *E. coli* ATP synthase. *J Biomol NMR* 29:439–440.
51. Dmitriev OY, Altendorf K, Fillingame RH (1995) Reconstitution of the F₀ complex of *Escherichia coli* ATP synthase from isolated subunits. Varying the number of essential carboxylates by co-incorporation of wild-type and mutant subunit c after purification in organic solvent. *Eur J Biochem* 233:478–483.
52. Schneider E, Altendorf K (1985) All three subunits are required for the reconstitution of an active proton channel (F₀) of *Escherichia coli* ATP synthase (F₁F₀). *EMBO J* 4:515–518.
53. Dmitriev OY, Altendorf K, Fillingame RH (2004) Subunit a of the *E. coli* ATP synthase: reconstitution and high resolution NMR with protein purified in a mixed polarity solvent. *FEBS Lett* 556:35–38.
54. Fillingame RH, Peters LK, White LK, Mosher ME, Paule CR (1984) Mutations altering aspartyl-61 of the omega subunit (uncE protein) of *Escherichia coli* H⁺-ATPase differ in effect on coupled ATP hydrolysis. *J Bacteriol* 158:1078–1083.
55. Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260:289–298.
56. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379.
57. Heukeshoven J, Dernick R (1988) Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* 9:28–32.
58. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354.
59. Dulley JR, Grieve PA (1975) A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal Biochem* 64:136–141.
60. Hwang TL, Shaka AJ (1995) Water suppression that works—excitation sculpting using arbitrary wave-forms and pulsed-field gradients. *J Magn Reson A* 112:275–279.