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Assembly of human mitochondrial ATP synthase through two separate intermediates, F_1-*c*-ring and *b*–*e*–*g* complex

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ABSTRACT

Mitochondrial ATP synthase is a motor enzyme in which a central shaft rotates in the stator casings fixed with the peripheral stator stalk. When expression of *d*-subunit, a stator stalk component, was knocked-down, human cells could not form ATP synthase holocomplex and instead accumulated two subcomplexes, one containing a central rotor shaft plus catalytic subunits (F_1-*c*-ring) and the other containing stator stalk components ("*b*–*e*–*g*" complex). F_1-*c*-ring was also formed when expression of mitochondrial DNA-coded *a*-subunit and A6L was suppressed. Thus, the central rotor shaft and the stator stalk are formed separately and they assemble later. Similar assembly strategy has been known for ATP synthase of yeast and *Escherichia coli* and could be common to all organisms.

1. Introduction

Mitochondrial ATP synthase catalyzes ATP synthesis driven by a transmembrane H^+ (proton) translocation via rotary motion of the central shaft [1]. Active human mitochondrial ATP synthase was purified [2] and it contains at least seventeen kinds of subunits (Table 1) [3–5]. A membrane-protruding portion, called F_1, has a subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$ and can be isolated as a water-soluble ATP hydrolyzing enzyme. F_1 by itself is an ATP hydrolysis-driven motor in which a rotor made of $\gamma\delta\varepsilon$-subunits rotates in a stator casing $\alpha_3\beta_3$-hexamer [6,7]. Membrane-integrated portion, called F_o, contains a ring of oligomeric *c*-subunits (*c*-ring) that rotates relative to a stator casing *a*-subunit when protons flow through *c*-ring. Then the whole central rotor shaft, made of *c*-ring and $\gamma\delta\varepsilon$-subunits, rotates and induces cyclic conformational changes in the $\alpha_3\beta_3$-hexamer necessary for synthesis reaction of ATP. To prevent the dragged rotation, the peripheral stator stalk, made of OSCP, F6, *b*- and *d*-subunit, connects the stator casing in F_1 ($\alpha_3\beta_3$) and that in F_o (*a*-subunit). Other minor subunits, namely, *e*, *f*, *g*, A6L, DAPIT and MLQ are assumed to be located in F_o portion. We previously showed that, when expression of DAPIT or MLQ was suppressed by knockdown, human cells failed in assembly (and/or stabilization) of ATP synthase [8,9]. Here, we report that, in the *d*-subunit knockdown human cells, ATP synthase holocomplex is not formed and instead two subcomplexes, one containing F_1 plus *c*-ring and the other containing *b*-, *e*- and *g*-subunits, are accumulated. This finding indicates that the portion containing the central rotor shaft and the portion containing the stator stalk are formed separately and they assemble after recruiting *d*-subunit to the stator stalk.

2. Materials and methods

2.1. Cells and plasmids

HeLa cells were purchased from the Health Science Research Resources Bank (JCRB9004). They were cultured at 37 °C under

Abbreviations: MASC assay, mitochondrial activity of SLO-permeabilized cells assay; VDAC, voltage-dependent anion channel of mitochondria

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Table 1
Subunits of human mitochondrial ATP synthase.

Subunit	Gene	Ms (kDa) (mature protein)	Sector	Role in motor	Antibody used for detection
α	ATP5A1	55.2	F_1	Stator	Anti-α
β	ATP5B	51.8	F_1	Stator	Anti-β
γ	ATP5C1	30.2	F_1	Rotor	Anti-γ
δ	ATP5D	15	F_1	Rotor	Anti-δ
ε	ATP5E	5.6	F_1	Rotor	Anti-ε
b	ATP5F1	24.6	F_o	Stator stalk	Anti-*b*
c	ATP5G1/2/3	7.6	F_o	Rotor	Anti-myc tag
d	ATP5H	18.4		Stator stalk	Anti-*d*
e	ATP5I	7.8	F_o	Stator	Anti-HA tag
F6	ATP5 J	9		Stator stalk	Anti-HA tag
f	ATP5J2	10.8	F_o	Stator	Not tested
g	ATP5L	11.4	F_o	Stator	Anti-HA tag
OSCP	ATP5O	20.9		Stator stalk	Anti-OSCP
a	MT-ATP6	24.8	F_o	Stator	Anti-*a*
A6L	MT-ATP8	8	F_o	Stator	Anti-myc tag
DAPIT	USMG5	6.5	F_o	Stator	Anti-DAPIT
MLQ	C14orf2	6.7	F_o	Stator	Anti-MLQ

5% CO_2 in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). An shRNA-expressing retroviral vector of pSuper.retro.puro (Oligoengine) was used for gene knockdown of *d*-subunit. The target sequence for shRNA was 5′-ACCATTGACTGGG TAGCTT-3′ which was predicted by an application of iRNAi (freeware). The control cells of the gene knockdown was prepared by retroviral infection of the empty vector (pSuper.retro.puro). When indicated, subunits of ATP synthase were exogenously expressed in HeLa cells with pcDNA3.1/mycHis vector (Invitrogen). GP cells for retrovirus package were cultivated and co-transfected with pSuper.retro.puro and pCI-VSVG (AddGene) plasmid vectors. The transfectants were incubated for 48 h and the culture supernatants were subjected to filtration of with a syringe filter (Millipore 0.45 μm PVDF). The filtrated solution was used as a retroviral solution for target HeLa cells. The infected cells were selected by 1.0 μg/ml of puromycin (Sigma–Aldrich) for 4 days.

2.2. Analysis of mitochondrial proteins

Cultured cells on a 15 cm-dish were harvested and the cell pellets were suspended into 1× mitochondria homogenization solution (6.6 mM imidazole/HCl [pH7.0] and 83 mM sucrose). They were homogenized by Potter homogenizer and the homogenates were centrifuged at 500×g for 5 min at 4 °C. The supernatants were centrifuged at 15 000×g for 10 min at 4 °C. The precipitated mitochondria were suspended in 3× mitochondrial homogenization solution (20 mM imidazole/HCl [pH 7.0] and 250 mM sucrose). For polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS–PAGE), the mitochondrial suspensions (5 μg protein per lane throughout this study) were mixed with 10× reducing reagents (Invitrogen) and 4× LSD sample buffer (Invitrogen) and the mixtures were incubated at 70 °C for 10 min. They were applied to 12% SDS–PAGE and the gel was electroblotted onto PVDF membrane (BioRad) at 12 V for 30 min. For Clear Native (CN-) PAGE, digitonin was used to solubilize mitochondria because subcomplexes tend to be destroyed in dodecylmaltoside and TritonX-100. The mitochondrial suspensions (5 μg protein per lane) were mixed with digitonin (15 μg per lane) and CNE solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl [pH7.0], 2 mM 6-aminohexanoic acid and 1 mM EDTA) and incubated on ice for 10 min. They were centrifuged at 100 000×g for 15 min at 4 °C and the supernatants were mixed with loading buffer (0.5% Ponceau S and 5% glycerol). The samples were applied to 3.5–18% CN-PAGE [10]. After the run, the gel was immersed in Bjerrum buffer [11] supplemented with 20% ethanol and 1% SDS for 30 min at room temperature. Then the gel was washed by Bjerrum buffer and electroblotted onto PVDF membrane at 12 V for 30 min with Bjerrum buffer supplemented with 20% ethanol and 0.037% SDS. The blotted membranes were blocked with 2% advanced blocking reagent (GE healthcare) for 1 h at room temperature and further incubated at 4 °C overnight in 2% advanced blocking reagent including antibodies against α- (Molecular Probes, A21350), β- (Molecular Probes, A21351), γ- (Protein Tech, PTG10910–1-AP), δ- (Sigma–Aldrich, R04042), ε- (Abnova, H00000514-M01), *a*- (Abcam, ab102573), *b*- (Protein Tech, 15999-1-AP), *d*-subunits (Invitrogen, 459000) and OSCP (MitoScience, C0655), DAPIT [8], MLQ [9] and VDAC (Abcam, ab16816)). The membranes were washed by TBS buffer supplemented with 0.005% Tween-20 and incubated with anti-mouse or anti-rabbit IgG antibody in 2% advanced blocking reagent. The membrane was immersed with ECL advance reagent (GE healthcare) and the chemiluminescence was detected by LAS-4000 (Fujifilm). For analysis of whole proteins, the gels were incubated in the protein staining solution (5 g/l $Al_2(SO_4)_3{\cdot}(H_2O)_{14-18}$, 10% ethanol, 0.2 g/l Coomassie brilliant blue-G250 and 8% phosphoric acid) at room temperature for 3–16 h. The stained gels were washed with de-staining solution (10% ethanol and 2% phosphoric acid) at room temperature for 30 min and were rinsed by water. Endogenous *e*-, *g*- and *c*-subunits were not detected with our experimental procedures. These subunits tagged with HA- and Myc-epitope at C-termini were expressed in *d*-KD and the control cells, and were detected with the antibodies against HA (Invivogen, ab-hatag) and Myc (SantaCruz, sc-40) epitopes. To suppress expression of mitochondria-coded *a*- and A6L subunits, *d*-KD and the control cells were cultured in the medium containing 40 μg/ml chloramphenicol for 48 h.

2.3. ATP synthesis activity assay (MASC assay)

Cells were passaged at 5000 cells per well of 96-well plate and incubated in CO_2-incubator overnight. The cells were applied to MASC assay as described [12]. Briefly, cells were treated with activated streptolysin O on ice for 10 min and were washed with PBS (−). The treated cells were incubated at 37 °C for 10 min to allow cytosolic components leak out through pores of streptolysin O. The buffer was replaced with the potassium phosphate buffer containing luciferase, luciferin, Ap5A (an adenylate kinase inhibitor), ADP, and succinic acid, and chemiluminescence was measured by a luminometer (Berthold, LB96V). When indicated, oligomycin (final concentration, 10 μg/ml) was included in the buffer.