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Novel factors essential for human mitochondrial F_{0}F_{1}-ATP synthase activity found by MASC (Mitochondrial Activity of SLO-permeabilized Cells) screening

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We and other groups have studied the regulatory mechanism of human F_{0}F_{1}-ATP synthase, especially, mechanism related to IF1 (inhibitor of F_{1}) [1]. It is thought that IF1 in mammalian mitochondria inhibits ATP hydrolysis when cells encounter conditions unfavorable for synthesis such as ischemia. Factors other than IF1 may be also involved in the regulation of human F_{0}F_{1}-ATP synthase activity as demonstrated by the recent reports on the effect of PKC delta and TMEM70. However, collective screening of the regulatory factors of mitochondrial ATP synthesis has not been done mainly because there is no high throughput assay for mammalian F_{0}F_{1}-ATP synthase activity. Conventional protocol to measure F_{0}F_{1} activity requires sub-cellular components are washed out and mitochondrial ATP synthesis activity of culture cells. We utilize streptolysin O (SLO) to make pores in plasma membrane of host cells without damaging organelle membranes. Cytosolic components are washed out and mitochondrial ATP synthesis activity is measured (Biochem. Biophys. Res. Commun., 2010, 401 (4) 538–543). The new method, MASC (Mitochondrial Activity of SLO-permeabilized Cells) assay, enables us to directly measure ATP synthesis activity of F_{0}F_{1} of culture cells on 96-well plate, suitable for high throughput screening for factors that affect ATP synthesis. First, factors assumed to be interacting with F_{0}F_{1}-ATP synthase were knocked down and screened. By this screening, DAPIT (inhibitor of F_{1}) [1]. It is thought that IF1 in mammalian mitochondria inhibits ATP hydrolysis when cells encounter conditions unfavorable for synthesis such as ischemia. Factors other than IF1 may be also involved in the regulation of human F_{0}F_{1}-ATP synthase activity as demonstrated by the recent reports on the effect of PKC delta and TMEM70. However, collective screening of the regulatory factors of mitochondrial ATP synthesis has not been done mainly because there is no high throughput assay for mammalian F_{0}F_{1}-ATP synthase activity. Conventional protocol to measure F_{0}F_{1} activity requires sub-cellular fractionation from large amounts of culture cells. Furthermore, the purified mitochondrial fractions should be used in short time due to rapid decay of the ATP synthesis activity. We invented new assay method for the mitochondrial ATP synthase activity of human culture cells. We utilize streptolysin O (SLO) to make pores in plasma membrane of host cells without damaging organelle membranes. Cytosolic components are washed out and mitochondrial ATP synthesis activity is measured (Biochem. Biophys. Res. Commun., 2010, 401 (4) 538–543). The new method, MASC (Mitochondrial Activity of SLO-permeabilized Cells) assay, enables us to directly measure ATP synthesis activity of F_{0}F_{1} of culture cells on 96-well plate, suitable for high throughput screening for factors that affect ATP synthesis. First, factors assumed to be interacting with F_{0}F_{1}-ATP synthase were knocked down and screened. By this screening, DAPIT (J. Biol. Chem., 2011, 286 (23) 20292–20296) was found to be essential for ATP synthesis of F_{0}F_{1}. Next, unknown genes coding mitochondrially localized proteins were knocked down and screened. By this screening, a critical gene for F_{0}F_{1} activity was found. To summarize, we established a new screening method for human F_{0}F_{1}-ATP synthase activity and found new factors that are essential for ATP synthesis activity.


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Characterization of CyPD - F_{0}F_{1}ATP synthase interaction: Role of the OSCP subunit

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The mitochondrial F_{0}F_{1}ATP synthase is a large multisubunit complex of 600 kDa anchored to the inner mitochondrial membrane, where it catalyzes ATP synthesis or hydrolysis at high and low proton electrochemical gradient, respectively. We have previously demonstrated that Cyclophilin D (CyPD, the only known mitochondrial immunophilin) binds the lateral stalk and inhibits the ATP synthase. The exact binding site in the lateral stalk and the mechanism(s) through which CyPD modulates ATP-synthesis/hydrolysis have not been defined yet. Here we studied the association of CyPD to ATP synthase in bovine, human and mouse heart mitochondria in order to define the site and mechanism of interaction. We found that OSCP subunit is the binding site for CyPD; consistently (i) decreasing the amount of OSCP subunit by RNA interference also reduced CyPD association to the ATP synthase; (ii) Benzodiazepine 423 (Bz423, an OSCP subunit-targeted inhibitor of F_{0}F_{1}ATP synthase) inhibited ATP hydrolysis both in wild-type and in CyPD-null mitochondria, yet higher Bz423 concentrations were required to inhibit the ATP synthase in wild-type mitochondria, where the drug displaced CyPD from the enzyme complex. Since Bz423 binds OSCP helices 3 and 4, these data suggest that CyPD binding specifically affects this site on the ATP synthase. Consistent with a site-specific effect of Bz423, no difference in inhibitory titer between wild-type and CyPD-null mitochondria was observed with oligomycin (which inhibits the ATP synthase at subunit a) and resveratrol (which binds the α/β subunits). Like binding of Bz423 to OSC, the interaction between CyPD and the lateral stalk of the ATP synthase was disrupted by increasing ionic strength, suggesting that the interaction itself is electrostatic in nature.

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Conformational changes of the ε-subunit of the H^{+}-ATP synthase by binding of nucleotides

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Movements of the ε-subunit of the H^{+}-ATP synthase from Escherichia coli (EFOF1) are investigated by single molecule spectroscopy. Two mutants of EFOF1 that differ in the binding site of the fluorescent dye on the ε-subunit (εA93C and εY114C) have been used. Both mutants have the same binding site on the γ-subunit (γT106C). The ε- and the γ-subunits of EFOF1 have been covalently labeled with fluorescent dyes. Depending on the investigated distances two fluorescent pairs, ATTO 532/ATTO 610 or ATTO 532/ATTO 647N, have been used. The labeled E_{d}F_{1} is integrated into liposomes. Single-pair fluorescence resonance energy transfer is measured in freely diffusing proteoliposomes with a confocal two channel microscope. It has been shown with both mutants that addition of the nucleotide ADP changes the conformation of the ε-subunit from the “up” conformation to the “up extended” conformation. Addition of the nucleotides ATP or AMPPNP changes the “up” conformation to the “down” conformation.

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The interaction of the diarylquinoline TMC207, a new tuberculosis antibiotic, with its target mycobacterial ATP synthase

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The interaction of the diarylquinoline TMC207, a new tuberculosis antibiotic, with its target mycobacterial ATP synthase

Movements of the ε-subunit of the H^{+}-ATP synthase from Escherichia coli (EFOF1) are investigated by single molecule spectroscopy. Two mutants of EFOF1 that differ in the binding site of the fluorescent dye on the ε-subunit (εA93C and εY114C) have been used. Both mutants have the same binding site on the γ-subunit (γT106C). The ε- and the γ-subunits of EFOF1 have been covalently labeled with fluorescent dyes. Depending on the investigated distances two fluorescent pairs, ATTO 532/ATTO 610 or ATTO 532/ATTO 647N, have been used. The labeled E_{d}F_{1} is integrated into liposomes. Single-pair fluorescence resonance energy transfer is measured in freely diffusing proteoliposomes with a confocal two channel microscope. It has been shown with both mutants that addition of the nucleotide ADP changes the conformation of the ε-subunit from the “up” conformation to the “up extended” conformation. Addition of the nucleotides ATP or AMPPNP changes the “up” conformation to the “down” conformation.
Energy metabolism has emerged as a new target-pathway for development of new anti-tubercular drugs [1], which are urgently needed to combat concomitantly emerging drug-resistant bacterial strains. A new ATP synthase inhibitor [2], the diarylquinoline TMC207, acts in a highly selective manner with strong affinity for ATP synthase from Mycobacterium tuberculosis and only minimal effect on the human homologue [3]. These features are prerequisite for clinical application of ATP synthase inhibitors and make TMC207 a promising drug candidate for shortening and simplifying tuberculosis chemotherapy. We used biochemical assays and binding studies to characterize the interaction between TMC207 and ATP synthase [3]. Using inverted membrane vesicles from the non-pathogenic strain M. bovis BCG [4] we show that TMC207 acts independent of the proton motive force and does not compete with protons for a common binding site. The drug is active on mycobacterial ATP synthase at neutral and acidic pH with no significant change in affinity between pH 5.25 and pH 7.5 (IC50 = 5–10 nM), indicating that the protonated form of TMC207 is the active drug entity. The interaction of TMC207 with ATP synthase can be explained by a one-site binding mechanism, the drug molecule thus binds to a defined binding site on ATP synthase. TMC207 affinity for its target decreases with increasing ionic strength, suggesting that electrostatic forces play a significant role in drug binding. These results will be discussed in view of existing docking studies and may provide input for structure-based design of new ATP synthase inhibitors and make TMC207 a promising drug candidate for shortening and simplifying tuberculosis chemotherapy.

References

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Localization and orientation of TMEM70 protein in the inner mitochondrial membrane
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Isolated ATP synthase deficiency of nuclear origin leads to a rather frequent fatal neonatal encephalo-cardiomyopathy (OMIM604273). The most common cause of the disease is c.317-2A>G mutation in TMEM70 gene, coding for specific factor of mitochondrial ATP synthase biogenesis with unknown function [1]. Characterization of TMEM70 protein properties is thus necessary for elucidation of its biological role and better understanding of the pathogenic mechanism of the disease. In this study we analyzed in detail the biosynthesis and presumed mitochondrial localization of the protein using cells expressing GFP- or FLAG-tagged forms of TMEM70 protein as the experimental model. We have found that TMEM70 gene encoded precursor of 29 kDa is processed into 21 kDa mature protein. The import study revealed that the TMEM70 protein is processed after transport into mitochondrial matrix, but the level of transported protein and its cellular content are very low. Immuno-cytchemical analysis and fractionation of mitochondria confirmed the localization of the TMEM70 protein in the inner mitochondrial membrane [2]. Accessibility of the tagged forms of TMEM70 protein to trypsin followed by electrophoretic or microscopic analysis demonstrated the orientation of C- and N- protein termini into mitochondrial matrix. 2D BN/SDS electrophoretic analysis of digitonin-solubilized proteins further showed, that TMEM70 protein is able either form an oligomeric structures or associate with other, yet unknown proteins. Supported by GACR 303/11/0970, GA UK 370411, 37710 and RVO:67985823.

References

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Technical design of a module for the continuous production of biochemical energy for cell-free protein synthesis
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Introduction and aim
In order to develop a flow reaction chamber for the continuous cell free protein synthesis, one key challenge is to provide energy in form of Adenosine 5′-triphosphate (ATP) for the process. To feed the system with ATP molecules from an external source is one strategy to solve this problem. However in this case, the major drawback is that the reaction products Adenosine 5′-diphosphate (ADP) and phosphate accumulate in the reaction solution and have to be removed frequently. Another approach is the direct regeneration of ATP from ADP and the phosphate molecule within the reaction compartment. Therefore, our aim is to isolate the ATP-Synthases from Escherichia coli and to immobilize these proteins onto custom made carrier membranes. In the presented work, an ATP regeneration module is designed employing computational modeling to transfer a batch reaction into a continuous protein synthesis process.