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Short communication

Development and characterization of mitochondrial membrane affinity chromatography columns derived from skeletal muscle and platelets for the study of mitochondrial transmembrane proteins

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ABSTRACT

Mitochondrial membrane fragments from human platelets and monkey skeletal muscles were successfully immobilized onto immobilized artificial membrane chromatographic support for the first time, resulting in mitochondrial membrane affinity chromatography (MMAC) columns. These columns were validated by characterization of translocator protein (TSPO), where multiple concentrations of dipyridamole were run and the binding affinities (K_d) determined. Further, the relative ranking data of TSPO ligands was consistent with previously reported rankings for both, the platelet (MMAC-Platelet) and the skeletal muscle (MMAC-Muscle) column (dipyridamole > PK11195 > protoporphyrin IX > rotenone). The functional immobilization of the F-ATPase/ATP synthase was demonstrated on MMAC-Muscle column. Online hydrolysis of ATP to ADP and synthesis of ATP from ADP were both demonstrated on the MMAC-Muscle column. Hydrolysis of ATP to ADP was inhibited by oligomycin A with an IC_{50} of 40.2 ± 13.5 nM (~60% reduction in ATP hydrolysis, $p < 0.001$), similar to previously reported values. Additionally, the Michaelis-Menten constant (Km) for ADP was found to be 1525 ± 461 μM based on the on column dose-dependent increase in ATP production.

1. Introduction

Mitochondria are highly dynamic organelles with their own DNA and translational machinery providing cellular energy and are essential for calcium and reactive oxygen species homeostasis, amino acid and lipid metabolism, cell growth and differentiation and apoptosis [1–3]. Their morphology, distribution, and activity are regulated by the fusion and fission machinery. Several components of these machineries have been identified in the mitochondria's outer and inner membrane (OMM and IMM respectively). Mitochondrial membrane proteins are potential targets for xenobiotics to induce either harmful or therapeutic effects on cellular function and survival associated with mitochondrial dysfunction which has been associated with number of acute and chronic diseases, including neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease [2–4]; cardiovascular diseases [5]; hyper proliferative diseases [6]; metabolic disorders [7]. The etiology and treatment of these mitochondrial diseases have been associated with the changes in expression and function of specific receptors and transporters expressed in the OMM and IMM. Thus these mitochondrial membrane proteins are important clinical targets for pharmacotherapy and drug discovery [1–3].

One of the key IMM receptors is the human mitochondrial F-ATPase/ATP synthase. This is a double-motor enzyme that is responsible for ATP synthesis and/or hydrolysis [8,9]. F-ATPase/ATP synthase or complex V is composed of two functional domains (Fig. 1), F_1, that is positioned in the mitochondrial matrix, and F_o, that is located in the IMM. The F-ATPase/ATP synthase is the fifth multi-subunit oxidative phosphorylation complex and produces ATP from ADP using the energy provided by the proton electrochemical gradient. This phenomenon causes rotation of two rotary motors, the ring of c subunits (F_o) and the subunits γ, δ and ε (F_1), to which c subunits are attached. This phenomenon is called "rotary catalysis". For ATP synthesis, each of the subunits switch co-operatively through conformations by which ADP and P_i bind resulting in ATP formation. In case of ATP hydrolysis the same pathway is utilized but in reverse order [9,10]. Changes in function, expression and structure of F-ATPase/ATP synthase have been

Abbreviations: OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; TSPO, translocator protein; MMAC, mitochondrial membrane affinity chromatography; DIPY, dipyridamole; PIX, protoporphyrin IX; PK, PK-11195; Rot, rotenone; IAM, immobilized artificial membrane

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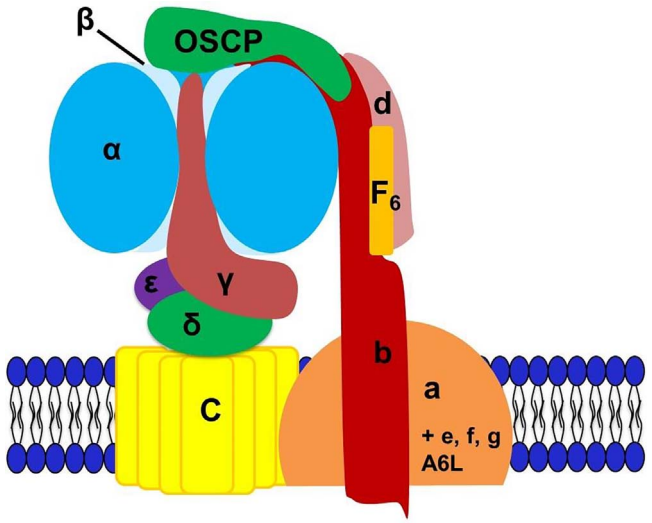

Fig. 1. A schematic figure of human mitochondrial ATP synthase (adapted from [9]). It contains two functional domains, F_1 and F_o. F_1 is composed of five different subunits (three units of α, three units of β, and one unit of γ, δ and ε). F_o is composed of c, a, b, d, F6, oligomycin sensitivity-conferring protein (OSCP), e–g and A6L subunits. γ, δ and ε subunits of F_1 are the part of the central stalk, whereas b, d, F6 and OSCP subunits constitute the peripheral stalk of the human mitochondrial ATP synthase. ATP conversion to ADP or vice-versa is derived via proton gradient which cross the IMM from the intermembrane space into the matrix through the F_o unit of human mitochondrial ATP synthase.

associated with a number of diseases [11–15] including but not limited to neurodegenerative diseases [11,12], metabolic disorders [13] and cardiovascular diseases [14].

Previously, we had demonstrated that mitochondrial membrane fragments from cultured cells can be immobilized onto a stationary phase resulting in a functional mitochondrial membrane affinity chromatography (MMAC) column [16]. The co-immobilization of both OMM and IMM in MMAC column was confirmed by characterization of translocator protein (TSPO), mitochondrial permeability transition pore and sulfonylurea receptor. It was also previously demonstrated that the expression of ABC transporters on the cellular versus nuclear surface resulted in a difference in the binding characteristics [17]. As a result, the characterization of mitochondrial proteins from different matrices could provide insight into changes in binding characteristics.

The mitochondrial membranes derived from monkey skeletal muscle and human platelets were isolated and immobilized onto an immobilized artificial membrane (IAM) stationary phase generating functional MMAC-Muscle and MMAC-Platelet columns. The functionality of these columns was validated by characterization of TSPO involving determination of the binding affinity of dipyridamole (DIPY) and establishing the screening for TSPO ligands as previously reported [16]. In addition, F-ATPase/ATP synthase activity was demonstrated on the MMAC-Muscle column.

2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminoethane (Tris), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, sodium chloride (NaCl), 2-mercaptoethanol, benzamidine, protease inhibitor cocktail, N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), adenosine 5′-triphoshphate (ATP), ammonium acetate, DIPY, PK-11195 (PK), protoporphyrin IX (PIX), rotenone (Rot), adenosine 5′-diphosphate (ADP) and oligomycin A were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1 × PBS was obtained from Quality Biologicals (Gaithersburg, MD, USA). Deionized water was obtained from a Milli-Q system (Millipore, Billerica, MA). IAM particles were purchased from Regis Technologies (Morton grove, IL).

2.2. Animals

A male rhesus monkey (*Macaca mulatta*) was initially anesthetized with Telazol (6 mg/kg, IM) and subsequently euthanized with B-Euthanasia-D (80 mg/kg, IV). All procedures were approved by the Animal Care and Use Committee of the NIA Intramural Program, USA.

2.3. Preparation of mitochondria

2.3.1. Human platelets

Human platelets were obtained from Bioreclamation (Westbury, NY, USA) from 500 ml of whole blood divided equally in 10 vials. The mitochondria was isolated as previously described [16] using a Mitochondria Isolation Kit for Cultured Cells from Abcam and Miltenyi Biotec.

2.3.2. Skeletal muscle

Mitochondria from rhesus monkey skeletal muscle (5, 20 or 50 mg) was isolated using the Mitochondria Isolation Kit from Abcam (Cambridge, UK), with slight modifications. Briefly, the skeletal muscle was suspended in 2.5 ml of Reagent A. After incubation on ice for 10 min, the skeletal muscle was homogenized for 30 s with a PRO200 homogenizer (PRO Scientific, Oxford, CT, USA). The resulting suspension was centrifuged for 10 min at 1000 × *g* and 4 °C. The supernatant was saved (no. 1) and the pellet was resuspended in 2.5 ml of Reagent B. Homogenization and centrifugation steps were repeated and the resulting supernatant was saved (no. 2). Supernatants 1 and 2 were mixed thoroughly and centrifuged for 15 min at 12,000 × *g* and 4 °C to pellet the mitochondria.

2.4. Preparation of MMAC columns

Mitochondria obtained from monkey muscle tissue or from human platelets were solubilized using a previously described protocol [16,17]. Briefly, the mitochondria pellets were suspended in 10 ml of Tris buffer [10 mM, pH 7.4] containing 2% (w/v) CHAPS, 10% glycerol, 500 mM NaCl, 5 mM 2-mercaptoethanol, 100 μM benzamidine, 1:100 dilution of protease inhibitor cocktail, 50 μg/ml TPCK, 100 μM PMSF and 100 μM ATP for 18 h at 4 °C. The solution was centrifuged and the supernatant incubated with 75 mg of IAMs for 1 h. The suspension was then dialyzed using dialysis tubing with 10 K MWCO (Thermo Fisher Scietific, Waltham, USA) against 2 l of dialysis buffer for 2 days. The resulting stationary phase was collected and washed twice with ammonium acetate buffer [10 mM, pH 7.4] and packed into a Tricorn 5/20 column (GE Healthcare Life Sciences, Uppsala, Sweden) to yield a 15 × 5 mm (i.d.) chromatographic bed.

The columns were tested periodically by running 0.125 μM DIPY. If a change in retention volume > 10% was observed, the column was no longer used for any studies.

2.5. Chromatographic studies

The MMAC columns were attached to the LC/MS chromatographic system. Two different system setups were used – System 1) Series 1100 Liquid Chromatography/Mass Selective Detector (Agilent Technologies, CA, USA) equipped with an autosampler (G1313A), a mass selective detector (G1946B) and running ChemStation software (Rev B.10.00, Hewlett-Packard); System 2) Series 6200 Accurate-Mass TOF-LC/MS chromatographic system (Agilent Technologies) equipped with a series 1200 Infinity binary pump (G1312B) and a mass selective detector (G6230A). The chromatographic system was run with MassHunter Workstation Software – LC/MS Data Acquisition software (revision