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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 dipyrindamole 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 (dipyrindamole > 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 (K_m) for ADP was found to be 1525 ± 461 μ M based on the on column dose-dependent increase in ATP production.

Keywords

Mitochondrial membrane affinity chromatography; translocator protein; ATPase; ATP synthase; platelets; skeletal muscle

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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 [4]; cardiovascular diseases [5]; hyper proliferative diseases [6]; metabolic disorders [7]. The etiology and treatment of these mitochondrial diseases has 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]. F-ATPase/ATP synthase or complex V is composed of two functional domains (Figure 1), F_1 , that is positioned in the mitochondrial matrix, and F_0 , 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_0) 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 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 [18]. 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 [19]. 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 [18]. 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'-triphosphate (ATP), ethylenediaminetetraacetic acid, 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 [18] 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 seconds with a PRO200 homogenizer (PRO Scientific, Oxford, CT, USA). The resulting suspension was centrifuged for 10 min at $1000 \times g$ 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 $12,000 \times g$ 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 [18,19]. 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 10K MWCO (Thermo Fisher Scientific, 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 B.05.00, Agilent).

2.6. Binding affinity determination of DIPY

These experiments were carried out on System 2. Ammonium acetate [10 mM, pH 5.9] containing DIPY (0.125–7.5 µM) was delivered at 0.4 ml/min. DIPY was monitored in the positive ion mode at m/z 505.32, with the capillary voltage at 3500 V and the nebulizer pressure at 60 psig, fragmentor at 100 V and drying gas flow at 9 l/min set at 320 °C.

2.7. F-ATPase/ATP synthase activity study

These experiments were carried out on System 1. Ammonium acetate [10 mM, pH 7.4] was delivered at 0.2 ml/min at room temperature with an injection volume of 20 µl. ATP and ADP were monitored in the negative ion mode at m/z 551.14 and 427.20, respectively, with the capillary voltage at –3000 V and the nebulizer pressure at 35 psig. The fragmentor was at 100 V and drying gas flow at 11 L/min at a temperature of 350 °C. 100 mM stocks of ATP and ADP in H₂O were prepared and stored in aliquots of 50 µl at –20°C. Fresh aliquots of 3 mM ATP and 10 mM ADP were prepared prior to each experiment by diluting the 100 mM aqueous stock solutions using mobile phase (for ATP) or 1× PBS buffer (for ADP). Each 100 mM stock had only one freeze thaw cycle.

The MMAC-Muscle column was equilibrated for 15 min with seven concentrations of oligomycin A from 10 nM to 2 µM. Subsequently a zonal injection of 3 mM ATP was carried out for ATP hydrolysis experiments. The extent of the conversion of ATP to ADP was calculated as ATP:ADP ratio using peak areas. ATP and ADP injections on blank MMAC column was used as a control.

For the ATP synthesis experiments, 20 μl of ADP (1.56 – 8000 μM) in $1\times$ PBS, pH 7.4, were injected on MMAC-Muscle column. For the conversion of ADP to ATP, only the area of ATP was used.

2.8. Single frontal displacement for ranking TSPO ligands

Single frontal displacement chromatography was carried out as previously described [18]. Briefly, frontal analysis was carried out for 0.125 μM DIPY in ammonium acetate [10 mM, pH 5.9] in the absence or presence of 2.875 μM of rotenone, PIX, PK or DIPY. The data was normalized to the change in retention volume observed with the addition of 2.875 μM DIPY.

2.9. Data analysis

The K_d values for the displacer ligands were determined using a previously reported approach [18,19]. The displacer ligand (D), dissociation constant (K_d), and number of active binding sites of the immobilized TSPO (B_{Max}), can be calculated using Eq. (1):

$$[D](V - V_{\text{min}}) = P[D](K_d + [D])^{-1}, \quad (1)$$

where V is the retention volume of ligand, V_{min} is the retention volume of ligand when the specific interaction is completely suppressed, and P is the product of the B_{max} (number of active binding sites) and K_d/K_{dM} , where K_{dM} is the dissociation constant for the marker. The K_d for D is obtained from the plot of $[D](V - V_{\text{min}})$ versus $[D]$. The data was analyzed by nonlinear regression with a sigmoidal response curve using Prism 5 software (GraphPad Software, San Diego, CA, USA) running on a personal computer.

3. Results and Discussion

3.1. Immobilization of Human Platelet mitochondria

Frontal affinity chromatography characterization was carried out on the MMAC-Platelet columns prepared by isolating mitochondria using both, the Abcam and Miltenyi kits, by determining the binding affinity of a specific ligand that binds to TSPO with high affinity (DIPY) [18]. The calculated binding affinity (K_d) was determined to be $1.279 \pm 0.3428 \mu\text{M}$ (Abcam) vs $1.788 \pm 0.283 \mu\text{M}$ (Miltenyi), similar to the previously reported value of $1.08 \mu\text{M}$ [18], indicating that the mitochondrial membranes isolated from platelets were immobilized in a functional state. Further, the number of binding sites was similar between the two kits (44 vs 41 nmoles, respectively) (Table 1). However, MMAC columns prepared using Miltenyi kit had a shorter lifetime. Additionally, as there was a slight improvement for the number of binding sites using the Abcam kit, only this method was used for the remainder of the studies.

3.2. Immobilization of Monkey Skeletal Muscle mitochondria

The optimal amount of skeletal muscle needed for the development of a MMAC-Muscle column was determined by immobilizing 5, 20 and 50 mg of rhesus monkey skeletal muscle. The mitochondria were isolated using the Abcam kit and the resulting mitochondria were immobilized onto the surface of the IAM stationary phase. The resulting columns were

characterized by frontal affinity chromatography using DIPY, the TSPO ligand. The determined binding affinities of DIPY were 0.856 ± 0.365 , 0.847 ± 0.089 and 1.097 ± 0.084 μM for 5, 20 and 50 mg columns respectively (Table 2). This was similar to the previously reported binding affinity for DIPY of 1.08 μM [18]. While 5 mg of muscle resulted in a functional column, the number of binding sites (B_{max}) was significantly higher for the columns made with 20 mg of skeletal muscle, therefore all studies were carried out using 20 mg of muscle tissue to prepare the columns.

3.3. Characterization of F-ATPase/ATP synthase

The presence of functional mitochondrial F-ATPase/ATP synthase on the MMAC-Muscle columns was tested. It has been reported that F-ATPase/ATP synthase retains the hydrolytic and synthase activities in isolated mitochondria [21]. Further, it is known that under the right storage conditions, the mitochondrial ATPase has been shown to remain active for weeks after isolation [22]. Therefore, the immobilization of a functional mitochondrial F-ATPase/ATP synthase is not unexpected. Figure 2B demonstrates an on-column conversion of ATP to ADP on the MMAC-Muscle column, which was not observed on the control column prepared without isolated mitochondrial fragments (Figure 2A). Further, the presence of functional F-ATPase/ATP synthase was demonstrated in the presence of oligomycin A, a specific inhibitor of proton translocation in F-ATPase/ATP synthase, which can inhibit both ATP hydrolysis and ATP synthesis [9,15,16]. Figure 2C shows that oligomycin A inhibits the hydrolysis of on-column ATP. Further, a series of concentrations of oligomycin A in the mobile phase were run on the MMAC-Muscle column and an IC_{50} of 40.2 ± 13.5 nM (Figure 3A) with a maximum reduction of ~60% in ATP hydrolysis was observed, $p < 0.001$, which is consistent with previously reported IC_{50} values [20]. While it was clearly demonstrated that hydrolytic activity of F-ATPase/ATP synthase activity was present, a series of concentrations of ADP [1.56 μM to 8 mM] were run on the MMAC-Muscle column to determine whether the ATP synthase activity was also retained. Injection of 0.03 – 160 nmoles (20 μL of 1.56 μM to 8 mM) of ADP in the presence of phosphate buffered saline [10 mM, pH 7.4], resulted in a dose-dependent increase in ATP production (5-fold) on the MMAC-Muscle column (Figure 3B), with the most significant increase in ATP production from 0.8 mM ADP. No production of ATP was observed on the control column (data not shown). The Michaelis-Menten constant (K_m) for ADP calculated based on the dose-dependent increase in ATP production was 1525 ± 461 μM ($n=3$), which was found to be higher than the reported values i.e. 10–100 μM [24]. The observed high K_m value maybe the result of reduced F-ATPase/ATP synthase activity due to the absence of proton gradient under *in vitro* conditions. Overall the results demonstrate that the F-ATPase/ATP synthase has been immobilized in the functional state and in this particular *in vitro* system a proton gradient was not required for activation of the F-ATPase/ATP synthase activity, however, it may be necessary for a more robust response, considering that in this study there was a greater than 10 fold decrease in activity relative to reported values. Further, the data suggests that this may be a viable option to study the F-ATPase/ATP synthase.

3.4. Ranking of TSPO ligands

Further characterization of the TSPO was carried out using single frontal displacement chromatography. We had previously shown, that a single displacement experiment could be used to correctly rank known TSPO ligands on a mitochondrial column. In this study, a similar experiment was conducted with 0.125 μ M DIPY as the marker ligand and 2.875 μ M of known TSPO ligands (DIPY, PK, PIX, and Rot) both for platelet (Figure 4A) and monkey muscle derived mitochondria (4B). The ranking order of displacement observed in case of DIPY > PK > PIX > Rot was in agreement with the previously published ranking orders [18].

4. Conclusions

In this study, it was shown for the first time that functional mitochondrial membrane affinity chromatography (MMAC) columns could be generated by immobilizing mitochondrial fragments isolated from human platelets and monkey's skeletal muscles onto IAM stationary phase. Single displacement studies confirmed that the generated MMAC-Platelet and MMAC-Muscle columns could be used for rapid screening of TSPO ligands and to study the dual functions of the F-ATPase/ATP synthase.

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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

References

1. Olszewska A, Szewczyk A. Mitochondria as a pharmacological target: magnum overview. *IUBMB Life*. 2013; 65:273–281. [PubMed: 23441041]
2. Szewczyk A, Wojtczak L. Mitochondria as a pharmacological target. *Pharmacol Rev*. 2002; 54:101–127. [PubMed: 11870261]

3. Pourahmad, J., Salimi, A., Seydi, E. Mitochondrial Targeting for Drug Development. In: Andreazza, AC., Scola, G., editors. Toxicology Studies - Cells, Drugs and Environment. InTech; 2015. p. 61-82.
4. Martin LJ. Mitochondrial and Cell Death Mechanisms in Neurodegenerative Diseases. Pharmaceuticals (Basel). 2010; 3:839–915. [PubMed: 21258649]
5. Ballinger SW. Mitochondrial dysfunction in cardiovascular disease. Free Radic Biol Med. 2005; 38:1278–1295. [PubMed: 15855047]
6. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. Nature Reviews Drug Discovery. 2010; 9:447–464. [PubMed: 20467424]
7. Alam MA, Rahman MM. Mitochondrial dysfunction in obesity: potential benefit and mechanism of Co-enzyme Q10 supplementation in metabolic syndrome. J Diabetes Metab Disord. 2014; 13:60. [PubMed: 24932457]
8. Nakanishi-Matsui M, Sekiya M, Nakamoto RK, Futai M. The mechanism of rotating proton pumping ATPases. Biochim Biophys Acta. 2010; 1797:1343–1352. [PubMed: 20170625]
9. Jonckheere AI, Smeitink JA, Rodenburg RJ. Mitochondrial ATP synthase: architecture, function and pathology. J Inherit Metab Dis. 2012; 35:211–225. [PubMed: 21874297]
10. Capaldi RA, Aggeler R, Turina P, Wilkens S. Coupling between catalytic sites and the proton channel in F1F0-type ATPases. Trends Biochem Sci. 1994; 19:284–289. [PubMed: 8048168]
11. de Vries DD, van Engelen BG, Gabreëls FJ, Ruitenbeek W, van Oost BA. A second missense mutation in the mitochondrial ATPase 6 gene in Leigh's syndrome. Ann Neurol. 1993; 34:410–412. [PubMed: 8395787]
12. Sergeant N, Watzel A, Galván-valencia M, Ghestem A, David JP, Lemoine J, Sautière PE, Dachary J, Mazat JP, Michalski JC, Velours J, Mena-López R, Delacourte A. Association of ATP synthase alpha-chain with neurofibrillary degeneration in Alzheimer's disease. Neuroscience. 2003; 117:293–303. [PubMed: 12614671]
13. Arakaki N, Kita T, Shibata H, Higuti T. Cell-surface H⁺-ATP synthase as a potential molecular target for anti-obesity drugs. FEBS Lett. 2007; 581:3405–3409. [PubMed: 17612527]
14. Fu Y, Zhu Y. Ectopic ATP synthase in endothelial cells: a novel cardiovascular therapeutic target. Curr Pharm Des. 2010; 16:4074–4079. [PubMed: 21247400]
15. Hong S, Pedersen PL. ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. Microbiol Mol Biol Rev. 2008; 72:590–641. [PubMed: 19052322]
16. Devenish RJ, Prescott M, Boyle GM, Nagley P. The oligomycin axis of mitochondrial ATP synthase: OSCP and the proton channel. J Bioenerg Biomembr. 2000; 32:507–515. [PubMed: 15254386]
17. Symersky J, Osowski D, Walters DE, Mueller DM. Oligomycin frames a common drug-binding site in the ATP synthase. Proc Natl Acad Sci U S A. 2012; 109:13961–13965. [PubMed: 22869738]
18. Habicht KL, Singh NS, Indig FE, Wainer IW, Moaddel R, Shimmo R. The development of mitochondrial membrane affinity chromatography columns for the study of mitochondrial transmembrane proteins. Anal Biochem. 2015; 484:154–161. [PubMed: 26049098]
19. Habicht KL, Singh NS, Khadeer MA, Shimmo R, Wainer IW, Moaddel R. Characterization of a multiple endogenously expressed adenosine triphosphate-binding cassette transporters using nuclear and cellular membrane affinity chromatography columns. J Chromatogr A. 2014; 1339:80–85. [PubMed: 24642394]
20. Stock U, Matter H, Diekert K, Dörner W, Dröse S, Licher T. Measuring interference of drug-like molecules with the respiratory chain: toward the early identification of mitochondrial uncouplers in lead finding. Assay Drug Dev Technol. 2013; 11:408–422. [PubMed: 23992120]
21. De Rasmio D, Micelli L, Santeramo A, Signorile A, Lattanzio P, Papa S. cAMP regulates the functional activity, coupling efficiency and structural organization of mammalian FOF1 ATP synthase. Biochim Biophys Acta. 2016; 1857:350–358. [PubMed: 26775111]
22. Li Z, Neufeld GJ. Isolation and characterization of mitochondrial F(1)-ATPase from crayfish (*Orconectes virilis*) gills. Comp Biochem Physiol B Biochem Mol Biol. 2001; 128:325–338. [PubMed: 11207445]

23. Martinez-Cruz O, Calderon de la Barca AM, Uribe-Carvajal S, Muhlia-Almazan A. The function of mitochondrial F₀F₁ ATP-synthase from the whiteleg shrimp *Litopenaeus vannamei* muscle during hypoxia. *Comp Biochem Physiol B Biochem Mol Biol*. 2012; 162:107–112. [PubMed: 22507548]
24. Matsuno-Yagi A, Hatefi Y. Kinetic modalities of ATP synthesis. Regulation by the mitochondrial respiratory chain. *J Biol Chem*. 1986; 261:14031–8. [PubMed: 2945814]

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Highlights

- Immobilization of mitochondrial membranes from platelets and skeletal muscle.
- Characterization of MMAC columns by ranking TSPO ligands.
- Functional immobilization of the F-ATPase/ATP synthase was demonstrated.

Figure 1.

A schematic figure of human mitochondrial ATP synthase (adapted from [18]). It contains two functional domains, F_1 and F_0 . 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_0 is composed of c, a, b, d, F6, oligomycin sensitivity-conferring protein (OSCP), e, f, 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_0 unit of human mitochondrial ATP synthase.

Figure 2.

Representative chromatogram of ATP and ADP following 20 μl of 3mM ATP zonal injection on (A) blank IAM column; (B) MMAC-Muscle column (0.531 cm $\times$ 2 cm); and (C) MMAC-Muscle column equilibrated with 1 μM oligomycin; running at a flow rate of 0.2 ml/min with ammonium acetate [10 mM, pH 7.4] as the mobile phase.

Figure 3.

Figure 3A. The effect of increasing concentration of oligomycin A [10–2000 nM] on the conversion ATP to ADP in the MMAC-Muscle column running at a flow rate of 0.2 ml/min with ammonium acetate [10 mM, pH 7.4] as the mobile phase.

Figure 3B. Dose-response of increasing concentrations of ADP [1.56–200 μM] (20 ul injection) in the presence of phosphate buffered saline [10 mM, pH 7.4] on the conversion ADP to ATP in the MMAC-Muscle, running at a flow rate of 0.4 ml/min with ammonium acetate [10 mM, pH 7.4] as mobile phase.

Figure 4.

Single frontal displacement studies of 4 compounds (2.875 μM), DIPY (I), PK (II), PIX (III), Rot (IV), carried out on the MMAC columns (0.531 cm $\times$ 2 cm) using ammonium acetate [10 mM, pH 5.9] in the presence 0.125 μM DIPY as the mobile phase. (A) MMAC-Platelet; (B) MMAC-Muscle.

Table 1

Binding affinities (μM) of DIPY determined by frontal affinity chromatography on the human platelet ($\sim 30 \times 10^6$) mitochondria immobilized onto the surface of an IAM stationary phase resulting in the MMAC-Platelet columns. Mitochondria used for preparation of the column were either extracted using Abcam or Miltenyi mitochondria extraction kit.

Source	Mitochondrial Isolation Kit	K_d of DIPY
Human Platelets	Abcam	$1.279 \pm 0.3428 \mu\text{M}$, $r^2 = 0.9614$, $B_{\text{max}} = 44.28 \pm 3.940$
Human Platelets	Miltenyi	$1.788 \pm 0.283 \mu\text{M}$, $r^2 = 0.9911$, $B_{\text{max}} = 40.51 \pm 3.098$

Table 2

Binding affinities (μM) of DIPY determined by frontal affinity chromatography on the monkey skeletal muscle immobilized onto the surface of an IAM stationary phase resulting in the MMAC-Muscle columns.

Source	K_d of DIPY
Monkey skeletal muscle (50mg)	$1.097 \pm 0.084 \mu\text{M}$, $r^2 = 0.9971$, $B_{\text{max}} = 53.30 \pm 1.569$
Monkey skeletal muscle (20mg)	$0.847 \pm 0.089 \mu\text{M}$, $r^2 = 0.9937$, $B_{\text{max}} = 29.97 \pm 1.128$
Monkey skeletal muscle (5mg)	$0.856 \pm 0.365 \mu\text{M}$, $r^2 = 0.8456$, $B_{\text{max}} = 13.78 \pm 5.343$