Published in final edited form as:

Anal Biochem. 2010 December 1; 407(1): 52–57. doi:10.1016/j.ab.2010.07.031.

A Kinetic Assay of Mitochondrial ATP-ADP Exchange Rate in Permeabilized Cells

Hibiki Kawamata¹, Anatoly A Starkov¹, Giovanni Manfredi¹, and Christos Chinopoulos^{1,2}

¹Weill Medical College, Cornell University, New York, NY, 10065, USA

²Department of Medical Biochemistry, Semmelweis University, Budapest, 1094, Hungary

Abstract

We have previously described a method to measure ADP-ATP exchange rates in isolated mitochondria by recording the changes in free extramitochondrial $[Mg^{2+}]$ reported by a Mg^{2+} -sensitive fluorescent indicator, exploiting the differential affinity of ADP and ATP to Mg^{2+} . In this manuscript we describe a modification of this method suited for following ADP-ATP exchange rates in environments with competing reactions that interconvert adenine nucleotides, such as in permeabilized cells that harbor phosphorylases and kinases, ion pumps exhibiting substantial ATPase activity and myosin ATPase activity. Here we report that addition of BeF_3^- and Na_3VO_4 to media containing digitonin-permeabilized cells inhibit all ATP-ADP utilizing reactions, except the ANT-mediated mitochondrial ATP-ADP exchange. An advantage of this assay is that mitochondria that may have been also permeabilized by digitonin do not contribute to ATP consumption by the exposed F_1F_0 -ATPase, due to its sensitivity to BeF_3^- and Na_3VO_4 . With this assay, ADP-ATP exchange rate mediated by the ANT in permeabilized cells is measured for the entire range of mitochondrial membrane potential titrated by stepwise additions of an uncoupler, and expressed as a function of citrate synthase activity per total amount of protein.

Keywords

bioenergetics; adenine nucleotide translocator; adenine nucleotide carrier; C_2C_{12} myotubes; systems biology

INTRODUCTION

The adenine nucleotide translocase (ANT) catalyzes the reversible exchange of ADP for ATP with a 1:1 stoichiometry across the inner mitochondrial membrane [19;29]. We have previously developed a technique to measure mitochondrial ADP-ATP exchange rates by exploiting the specific feature of ANT to transport only the free ADP and ATP forms, unbound to Mg^{2+} [19;21]. The rate of ATP appearing in the medium following addition of ADP to energized isolated mitochondria, is calculated from the measured rate of change in free extramitochondrial $[Mg^{2+}]$ reported by the membrane-impermeable $5K^+$ salt of the Mg^{2+} -

© 2010 Elsevier Inc. All rights reserved.

Address correspondence to: Dr. Christos Chinopoulos, Weill Medical College, Cornell University, 1300 York Avenue, New York, NY, 10065, USA. Tel: +212 746 6707, Fax: +212 746 8276. chinopoulos.christos@eok.sote.hu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

sensitive fluorescent indicator, Magnesium Green, using standard binding equations [11]. Changes in free extramitochondrial $[Mg^{2+}]$ were attributed exclusively to the ANT because they exhibit virtually 100% sensitivity to carboxyatractyloside (cATR) in the submicromolar range [11;25], while all other known nucleotide transporters are insensitive to inhibition by cATR [14;15;28;33;37]. In isolated mitochondria, the only other reaction that interconverts adenine nucleotides in the experimental volume is that catalyzed by adenylate kinase, residing in the intermembrane space of mitochondria [11], but this is effectively inhibited by P^1, P^5 -di (adenosine-5') pentaphosphate (AP_5A) [23]. A creatine kinase isoform that also resides in the intermembrane space remains inoperable for as long as there is no creatine or its phosphate derivatives present in the medium, and is sensitive to inhibition by iodoacetamide. However, in permeabilized cells there are a number of additional reactions that interconvert adenine nucleotides, such as the Na^+/K^+ ATPase, the plasmalemmal and endoplasmic Ca^{2+} ATPase, and in contractile cells the myosin ATPase, in addition to a gamut of phosphorylases, phosphatases and kinases. Reactions interconverting adenine nucleotides other than the ANT invalidate the binding equations that are applied on recordings of free $[Mg^{2+}]$ for calculating ADP-ATP exchange rates of mitochondria [11]. In order to apply the method described in [11] to permeabilized cells, one must inhibit all competing adenine nucleotide interconverting reactions, except the ANT. BeF_3^- and vanadium compounds has been successfully used for over 30 years in many applications as inhibitors of ADP and/or ATP utilizing reactions [4;7;13;17;26;32;38]. Hereby we demonstrate a modification of the original method developed for measuring ADP-ATP exchanges in isolated mitochondria so that it can be applied in permeabilized cells, using BeF_3^- and Na_3VO_4 .

MATERIALS AND METHODS

Culturing of myoblasts and preparation of myotubes

Mouse C_2C_{12} myoblasts [5] were cultured in DMEM containing 10% fetal bovine serum and antibiotic-antimycotic solution at 37°C in 5% CO_2 . Cells were plated at 140,000 cells/well in 6 well tissue culture plates. To induce differentiation of myoblasts into myotubes, culture medium was replaced with differentiation medium containing DMEM and 2% horse serum when the cells reached >90% confluence. Fusion of myoblasts into elongated and multinucleated myotubes was evident in 3 to 4 days in differentiation medium. Myotubes were processed on the 6th day for all subsequent experiments. Myotubes were washed once in phosphate-buffered saline and harvested with 0.1 ml of 0.25% trypsin-EDTA, inactivated by 0.9 ml calf serum, followed by centrifugation at 1,100 g for 2 minutes. Cells were washed once in a buffer containing, in mM: KCl 8, K-gluconate 110, NaCl 10, Hepes 10, KH_2PO_4 10, EGTA 0.005, mannitol 10, $MgCl_2$ 0.5-1.5 (where indicated) and 0.5 mg/ml bovine serum albumin (fatty acid-free), pH 7.25 without disturbing the pellet, prior to resuspension in 0.2 mL of the same buffer including, in mM: glutamate 5, malate 5, AP_5A 0.1, iodoacetamide 0.25, NaF 5, $BeSO_4$ 0.2 and Na_3VO_4 0.1.

$[Mg^{2+}]_{free}$ determination from Magnesium Green fluorescence in the experimental volume containing permeabilized C_2C_{12} cells and conversion to ADP-ATP exchange rate

C_2C_{12} cells from a single 3.5 cm diameter well (~600,000 cells or ~0.3 mg protein) resuspended in 0.2 ml of a buffer as detailed above were added to a single flat-bottom well of a white opaque 96 well plate. Digitonin (3 μ l of 2.5 mM dissolved in bi-distilled water) and Magnesium Green $5K^+$ salt (1 μ M) were subsequently added. Our digitonin powder stocks were purchased as “approximately 50% estimated by thin layer chromatography”; therefore we cannot be certain of the exact concentration of digitonin present in the well. Optimum digitonin amount added to the well was empirically determined by measuring oxygen consumption of the cells, as detailed below. The entire study was performed using the same digitonin stock solution. Magnesium Green (MgG) fluorescence was recorded in a Spectramax M5 plate reader

(Molecular Devices, Sunnyvale, CA 94089, USA) at a 0.33 Hz acquisition rate (one acquisition every 2 sec plus 1 sec for mixing in between each acquisition), using 505 and 535 nm excitation and emission wavelengths, respectively. Experiments were performed at 37 °C. Mitochondrial phosphorylation was started by the addition of 2 mM ADP. At the end of each experiment, minimum fluorescence ($F_{\min}$) was measured after addition of 5 mM EDTA, followed by the recording of maximum fluorescence ($F_{\max}$) elicited by addition of 10 mM MgCl_2 . Free Mg^{2+} concentration ($[\text{Mg}^{2+}]_f$) was calculated from the equation: $[\text{Mg}^{2+}]_f = (\text{Kd}(\text{F} - \text{F}_{\min}) / (\text{F}_{\max} - \text{F})) - 0.068$ mM, assuming a Kd of 0.9 mM for the MgG-Mg^{2+} complex [22]. The correction term -0.068 mM is empirical, and possibly reflects chelation of other ions by EDTA that have an affinity for MgG , and alter its fluorescence. ADP-ATP exchange rate was estimated using the recently described method by Chinopoulos et al [11] based on a concept developed previously by Silverman et al [34] and Leyssens et al [22], exploiting the differential affinity of ADP and ATP to Mg^{2+} . The rate of ATP appearing in the medium following addition of ADP to energized mitochondria (or *vice versa* in case of sufficiently deenergized mitochondria), is calculated from the measured rate of change in free extramitochondrial $[\text{Mg}^{2+}]$ using the following equation:

$$[\text{ATP}]_t = \left(\frac{[\text{Mg}^{2+}]_t}{[\text{Mg}^{2+}]_f} - 1 - \frac{[\text{ADP}]_t(t=0) + [\text{ATP}]_t(t=0)}{K_{\text{ADP}} + [\text{Mg}^{2+}]_f} \right) / \left(\frac{1}{K_{\text{ATP}} + [\text{Mg}^{2+}]_f} - \frac{1}{K_{\text{ADP}} + [\text{Mg}^{2+}]_f} \right) \quad (\text{Eq. 1}).$$

Here, $[\text{ADP}]_t$ and $[\text{ATP}]_t$ are the total concentrations of ADP and ATP, respectively, in the medium, and $[\text{ADP}]_t(t=0)$ and $[\text{ATP}]_t(t=0)$ are $[\text{ADP}]_t$ and $[\text{ATP}]_t$ in the medium at time zero. For the calculation of $[\text{ATP}]$ or $[\text{ADP}]$ from free $[\text{Mg}^{2+}]$, the apparent Kd values are identical to those in [11] due to identical experimental conditions ($K_{\text{ADP}} = 0.906 \pm 0.023$ mM, and $K_{\text{ATP}} = 0.114 \pm 0.005$ mM). The presence of NaF, BeSO_4 or Na_3VO_4 did not alter these Kd values, nor the Kd of Mg^{2+} for MgG , or the MgG fluorescence signal itself (not shown). Likewise, no effect of any of these compounds were observed on safranin O fluorescence (not shown). $[\text{Mg}^{2+}]_t$ is the total amount of Mg^{2+} present in the media. In some experiments we used 0.5 mM $[\text{Mg}^{2+}]_t$ and in some we used 1.5 mM $[\text{Mg}^{2+}]_t$. Although the lower concentration of $[\text{Mg}^{2+}]_t$ (for the same amount of $[\text{ADP}]_t$ or $[\text{ATP}]_t$) yielded higher ADP-ATP exchange rates for the reasons elaborated in [11], in permeabilized cells we recommend using 1.5 mM $[\text{Mg}^{2+}]_t$. This is because at the greater $[\text{Mg}^{2+}]_t$, $[\text{Mg}^{2+}]_{\text{free}}$ will yield a high MgG fluorescence, sufficiently higher than $F_{\min}$, that will result in better confidence of the $[\text{Mg}^{2+}]_{\text{free}}$ to $[\text{ATP}]_{\text{free}}$ conversion. Equation 1 is available for download as an executable file at: <http://tinyurl.com/ANT-calculator>.

The rationale for using this particular buffer composition is elaborated in [11]. Glutamate and malate as mitochondrial substrates were chosen on the basis of the fact that they support mitochondrial substrate-level phosphorylation, and as such they contribute to greater ATP efflux rates [10]. The ATP-ADP exchange rate mediated by the ANT technique has been validated in [11], especially in the context of the contribution of the $\text{ATP-Mg}^{2+}/\text{P}_i$ carrier [2] and a homologue of the Mrs2 protein originally described in yeast that mediates an electrophoretic uptake of Mg^{2+} in mitochondria [20].

Mitochondrial membrane potential ($\Delta\psi_m$) determination in situ mitochondria of permeabilized C_2C_{12} cells

$\Delta\psi_m$ was estimated using fluorescence quenching of the cationic dye safranin O due to its accumulation inside energized mitochondria [1]. C_2C_{12} cells were treated exactly as described for free $[\text{Mg}^{2+}]$ determination, except that MgG was replaced by 5 μM safranin O. Fluorescence was recorded in a Spectramax M5 plate reader at a 0.33 Hz acquisition rate (one

acquisition every 2 sec plus 1 sec for mixing in between each acquisition), using 495 and 585 nm excitation and emission wavelengths, respectively. Experiments were performed at 37 °C.

Citrate synthase

Citrate synthase activity was measured as described by Srere [35], with minor modifications. Briefly, 20 μ l aliquots (~30 μ g of protein) from the 0.2 ml cell suspensions that have been freeze-thawed were added to a 0.18 ml medium containing 20 mM Hepes pH 7.8, 0.5 mM oxaloacetate and 0.1 mM dithionitrobenzoic acid. The reaction was started after 3 min preincubation time by adding 0.36 mM acetyl-CoA. Changes in the absorbance at 412 nm due to 5-thio-2-nitrobenzoic acid formation were monitored in a Spectramax M5 plate reader at 25 °C. Activity was calculated as nmol/min/mg protein assuming an extinction coefficient for 5-thio-2-nitrobenzoic acid, of $\epsilon M = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$. The light path for 0.2 ml volume in the well of a 96 plate is 0.5 cm. Protein content was measured by the bicinchoninic acid assay using bovine serum albumin protein as standards and calibrating by a 3 parameter power function, $f = y_0 + a \cdot x^b$, where y_0 is background absorbance in the absence of protein, a and b are constants, and x is the amount of protein in the unknown samples.

Oxygen Consumption

Mitochondrial respiration was recorded at 37 °C with a Clark-type oxygen electrode (Hansatech, UK). C₂C₁₂ cells from two 3.5 cm diameter wells were resuspended in 0.5 ml of buffer containing, in mM: KCl 8, K-gluconate 110, NaCl 10, Hepes 10, KH₂PO₄ 10, EGTA 0.005, mannitol 10, MgCl₂ 0.5, Ap5A 0.1, and 0.5 mg/ml bovine serum albumin (fatty acid-free), pH 7.25, and added in the chamber. Subsequently, 2 mM of succinate was added, followed by 2 mM ADP to the medium. Increments of 1 μ l of 2.5 mM digitonin were added as indicated, while recording oxygen consumption.

Preparation of sodium orthovanadate (Na₃VO₄) and BeF₃⁻

A 25 mM Na₃VO₄ solution was prepared in bi-distilled water. The pH was set to 8.7 with HCl, upon which the solution turned to yellow. This solution was boiled until colorless and allowed to cool to room temperature. pH was reassessed, and readjusted to pH 8.7 with HCl, upon which the solution returned again to yellow color. This solution was boiled again and this cycle was repeated until the solution remained colorless and at pH 8.7 after boiling and cooling. Finally, it was brought up to the initial volume with bi-distilled water and stored in aliquots at -80 °C. This treatment removes all decavanadate ions present in the Na₃VO₄ solution. Decavanadate induces mitochondrial membrane depolarization in addition to inhibiting oxygen consumption [3]. Orthovanadate inhibits the oxidation of only disrupted mammalian mitochondria [6]. Likewise, fluoroberyllium nucleoside diphosphate complexes inhibit only the exposed F₁F₀-ATPase [18]. BeSO₄ and NaF are prepared as aqueous solutions of 0.2 M and 0.5 M respectively, without any additional modifications, and kept at +4 °C. BeF₃⁻ (among other combinations) is formed immediately in solution upon mixing BeSO₄ and NaF, provided that NaF is in excess. Vanadate, beryllium and fluoride salts are highly toxic to tissues and to the environment, and must be handled and disposed properly. The combination of orthovanadate and BeF₃⁻ will inhibit kinases, mutases, phosphatases, and ATPases [12;31]. Some kinases though, will remain uninhibited, such as pyruvate kinase [24]. In this respect, upon permeabilization of the cells one has to totally separate pyruvate kinase from its substrate, phosphoenol pyruvate, i.e. there must be no glucose present in the medium prior to permeabilization, and a few minutes lag time must be allowed prior to ADP-ATP exchange rate measurements in order for the remaining reactions by kinases to 'die-out'. The effect of 10 nM cATR blocking completely ADP-ATP exchange rates signifies that all adenine nucleotide interconverting reactions have been rendered inoperable (see below).

Reagents

Standard laboratory chemicals, P¹,P⁵-Di(adenosine-5') pentaphosphate (AP₅A), safranin O and digitonin were from Sigma (St. Louis, MO, USA). Magnesium Green 5K⁺ salt was from Invitrogen, (Carlsbad, CA, USA). Carboxyatractyloside was from Calbiochem (San Diego, CA, USA). SF 6847 was from Biomol (catalogue number EI-215, BIOMOL GmbH, Hamburg, Germany). Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA, USA) and all other tissue culture reagents were purchased from Invitrogen. Mitochondrial substrate stock solutions were dissolved in bi-distilled water and titrated to pH=7.0 with KOH. ADP was purchased as a K⁺ salt of the highest purity available and titrated to pH=6.9 with KOH to a stock of 0.2 M. Concentration of the ADP stock solution was corrected by measuring absorbance at 260 nm using an extinction coefficient $\epsilon M = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$.

Statistics

Data are presented as mean $\pm$ SEM; $n \geq 3$ for all experiments; significant differences between groups of data were evaluated by one way ANOVA followed by Tukey's posthoc analysis, with $p < 0.05$ considered significant. Wherever single graphs are presented, they are representative of at least 3 independent experiments.

RESULTS AND DISCUSSION

Gaining access to *in situ* ANT while inhibiting other adenine nucleotide interconverting reactions

In order to gain access to the cell interior and deliver known amounts of ADP, Mg²⁺, the membrane-impermeable 5K⁺ salt of the Mg²⁺-sensitive fluorescent indicator, Magnesium Green, BeF₃⁻, Na₃VO₄, AP₅A and mitochondrial substrates (the creatine kinase inhibitor iodoacetamide is membrane-permeable) without compromising the inner mitochondrial membrane integrity, the following experiment was performed, as shown in figure 1A: Oxygen consumption of C₂C₁₂ cells was recorded in cytosol-mimicking media (the composition of which is described in Materials and Methods) and using succinate as a mitochondrial respiratory substrate (2 mM) and ADP (2 mM), both being impermeable to the cell membrane. Stepwise additions of digitonin increased oxygen consumption rates, signifying permeabilization of the cell membrane and provision of entry points for succinate and ADP to the *in situ* mitochondria. Upon reaching a sufficiently high concentration of digitonin, the detergent decreased oxygen consumption rates, indicative of either disrupting the i) inner mitochondrial membrane integrity and/or ii) outer mitochondrial membrane integrity causing a leak of cytochrome c. The concentration of digitonin that produced the highest rate of oxygen consumption was chosen for all further experiments.

In the subsequent experiment shown in figure 1B, digitonin-permeabilized C₂C₁₂ cells were incubated in cytosol-mimicking media in the presence of BeF₃⁻, Na₃VO₄, AP₅A and iodoacetamide. Addition of ADP resulted in a gradual emergence of ATP in the medium. Subsequent stepwise additions of 2 nM cATR resulted in a complete halt of ATP rise in the media after 5 additions, amounting to 10 nM cATR. That attests to the fact that the ANT was the only entity mediating ADP-ATP exchanges in these permeabilized cells using this cocktail of inhibitors. Although using this methodology the amount of ANT can be estimated [36], this amount must be considerably higher than the K_i of cATR for the transporter [36]. Since this K_i is in the 1-10 nM range, which is similar to the amount of cATR required to block ADP-ATP exchanges completely, the estimation of the amount of ANT for the amount of cells/*in situ* mitochondria that are present in the well would be overestimated. For the same reason, the molecular turnover number of the ANT cannot be estimated using such a low amount of cells/*in situ* mitochondria. During the digitonin permeabilization, it is conceivable that a small (due to digitonin titration during oxygen consumption experiments) but undetermined fraction of

in situ mitochondria would also be permeabilized. This should have exposed the hydrolytic part of the F_1F_0 -ATPase that in deenergized-permeabilized mitochondria would result in vigorous ATP hydrolysis. To address this possibility, a large bolus of digitonin (10 times higher amount than the optimal concentration for selective cell membrane permeabilization) was added to already permeabilized cells in which their mitochondria have been allowed to phosphorylate 0.285 mM of ADP to ATP, followed by inhibiting their ANT with 1 μ M cATR. As seen in figure 1C (black circles), addition of 0.375 mM digitonin that is expected to permeabilize all *in situ* mitochondria, resulted in an initial gradual decrease in ATP that halted within 30-40 seconds. We interpret this lag as the time required for BeF_3^- and orthovanadate to interrupt the ATP hydrolysis cycle of the F_1F_0 -ATPase by binding in place of the released hydrolysis product, inorganic phosphate. Accordingly, if oligomycin (olgm) was added after cATR (open circles), there was no gradual decrease in ATP upon subsequent addition of 0.375 mM digitonin. This experiment demonstrates that the gradual decrease in ATP that halted within 30-40 seconds was sensitive to oligomycin, thus it was attributed to the hydrolytic action of the exposed F_1F_0 -ATPase.

Estimation of ADP-ATP exchange rates in permeabilized cells as a function of mitochondrial membrane potential ($\Delta\psi_m$)

Among many bioenergetic parameters elaborated in [25], mitochondrial ADP-ATP exchange rate depends steeply on $\Delta\psi_m$ [19;25]. It is therefore imperative to provide ADP-ATP exchange rates mediated by the ANT as a function of $\Delta\psi_m$. In figure 2 panel A, we show the experiment where permeabilized cells were incubated in cytosol-mimicking media in the presence of BeF_3^- , Na_3VO_4 , AP_5A and iodoacetamide, and Magnesium Green fluorescence was recorded over time, calibrated to free $[Mg^{2+}]$, and calculated to extramitochondrial ATP, $[ATP]_e$. Addition of ADP resulted in a gradual emergence of $[ATP]_e$. Subsequent stepwise additions of the uncoupler SF 6847 (10 nM each) resulted in a progressive decrease in the rate of $[ATP]_e$, that leveled off upon addition of the fourth SF 6847 pulse. At this point, the ANT operated at its “reversal potential”, E_{rev_ANT} , a $\Delta\psi_m$ value during which there is no net transport of adenine nucleotides across the inner mitochondrial membrane [10]. Further additions of the uncoupler dropped $\Delta\psi_m$ to a sufficiently low level that resulted in reversal of the ANT, and conversion of mitochondria to ATP consumers [9;10]. In panel B, permeabilized cells were incubated in cytosol-mimicking media using the same cocktail of inhibitors, and $\Delta\psi_m$ was estimated by using fluorescence quenching of the cationic dye safranin O which accumulates inside energized mitochondria [1]. As shown, addition of ADP caused a moderate depolarization. Subsequent stepwise addition of the uncoupler SF 6847 (10 nM each) caused a stepwise dissipation of $\Delta\psi_m$. In isolated mitochondria, safranin O fluorescence can be calibrated to $\Delta\psi_m$ by applying the Nernst equation assuming a matrix $[K^+]=120$ mM and recording safranin O fluorescence in the presence of 2 nM valinomycin and stepwise additions of $[K^+]$ in the 0.2-120 mM range, [1]. This technique is not reproducible in permeabilized cells. Hereby, we have adopted three assumptions, in order to arbitrarily convert safranin O fluorescence to mV: i) minimum fluorescence was considered as -180 mV, ii) maximum fluorescence was considered as 0 mV, and iii) safranin O fluorescence increases linearly upon dissipation of $\Delta\psi_m$. Although these assumptions are in good agreement with a large body of literature, we cannot overemphasize that this is only an arbitrary approximation. Nevertheless, using this approximation, E_{rev_ANT} falls well within calibrated values for isolated mitochondria [10].

By linear regression analysis of the ADP-ATP exchange rates of *in situ* mitochondria of permeabilized cells plotted as a function of $\Delta\psi_m$ titrated by stepwise additions of an uncoupler, we derived the “ADP-ATP exchange rate/ $\Delta\psi_m$ ” profile depicted in figure 3, showing an average plot of 3 independent experiments. By analogy of a current-voltage relationship of a channel [27] or a transporter [16], this graph depicts the rate of transfer of an adenine nucleotide

phosphorylated group per mg protein per unit time, as a function of the potential that exists across the membrane through which the phosphorylation group transfer takes place. However, if one is to compare different cell types, or samples of the same cell type but with manipulated mitochondria, comparisons must be made for the same amount of mitochondria. One intrinsic mitochondrial parameter that is representative of the amount of mitochondria in a cell is citrate synthase activity. In our hands, citrate synthase specific activity of C₂C₁₂ cells was 436 ± 13 nmol/min/mg protein. By comparison, isolated mitochondria from skeletal muscle exhibit 8-10 times higher citrate synthase specific activity [8;30;39], which is in a good agreement with 8-10 times higher ADP-ATP exchange rates in similar type of mitochondria [11].

CONCLUSIONS

The present paper describes the methodology to measure ADP-ATP exchange rates in *in situ* mitochondria of permeabilized cells. This is an extension of a previously established method measuring ADP-ATP exchange rates in isolated mitochondria, where there are no competing reactions interconverting adenine nucleotides [11]. Therefore, regarding advantages and disadvantages of the present method, the reader is referred to the earlier paper.

Acknowledgments

We are grateful to Prof. David C. Gadsby for helpful discussions and Dr. László Csanády for valuable theoretical advice. This work was supported by the National Institute of Health/National Institute of Neurological Disease and Stroke grant F31 NS054554 to H.K., the NIH grant 1R21NS065396 to A.A.S., the NIH grant R01 GM088999 to G.M., and the Országos Tudományos Kutatási Alapprogram-Nemzeti Kutatási és Technológiai Hivatal (OTKA-NKTH) grant NF68294 and OTKA NNF78905 grant and Egeszsegügyi Tudományos Tanács (ETT) grant to C.C.

Abbreviations

ANT	adenine nucleotide translocase
AP ₅ A	P ¹ ,P ⁵ -di(adenosine-5') pentaphosphate
cATR	carboxyatractyloside
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
E _{rev_ANT}	Reversal potential of the ANT
MgG	magnesium green
SF 6847	Tyrphostin 9, RG-50872, Malonaben, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile, 2,6-di-t-butyl-4-(2',2'-dicyanovinyl)phenol

REFERENCES

- [1]. Akerman KE, Wikstrom MK. Safranin as a probe of the mitochondrial membrane potential. *FEBS Lett* 1976;68:191–197. [PubMed: 976474]
- [2]. Aprille JR. Mechanism and regulation of the mitochondrial ATP-Mg/P(i) carrier. *J.Bioenerg.Biomembr* 1993;25:473–481. [PubMed: 8132487]
- [3]. Aureliano M, Crans DC. Decavanadate (V10 O28 6-) and oxovanadates: oxometalates with many biological activities. *J.Inorg.Biochem* 2009;103:536–546. [PubMed: 19110314]
- [4]. Baukrowitz T, Hwang TC, Nairn AC, Gadsby DC. Coupling of CFTR Cl- channel gating to an ATP hydrolysis cycle. *Neuron* 1994;12:473–482. [PubMed: 7512348]

- [5]. Blau HM, Chiu CP, Webster C. Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* 1983;32:1171–1180. [PubMed: 6839359]
- [6]. Byczkowski J, Zychlinski L, Tluczkiwicz J. Interaction of vanadate with respiratory chain of rat liver and wheat seedling mitochondria. *Int.J.Biochem* 1979;10:1007–1011. [PubMed: 229012]
- [7]. Cantley LC Jr, Josephson L, Warner R, Yanagisawa M, Lechene C, Guidotti G. Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. *J.Biol.Chem* 1977;252:7421–7423. [PubMed: 144127]
- [8]. Chess DJ, Khairallah RJ, O'Shea KM, Xu W, Stanley WC. A high-fat diet increases adiposity but maintains mitochondrial oxidative enzymes without affecting development of heart failure with pressure overload. *Am.J.Physiol Heart Circ.Physiol* 2009;297:H1585–H1593. [PubMed: 19767529]
- [9]. Chinopoulos C, Adam-Vizi V. Mitochondria as ATP consumers in cellular pathology. *Biochim.Biophys.Acta* 2010;1802:221–227. [PubMed: 19715757]
- [10]. Chinopoulos C, Gerencser AA, Mandi M, Mathe K, Torocsik B, Doczi J, Turiak L, Kiss G, Konrad C, Vajda S, Vereczki V, Oh RJ, Adam-Vizi V. Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation. *FASEB J* 2010;24:2405–2416. [PubMed: 20207940]
- [11]. Chinopoulos C, Vajda S, Csanady L, Mandi M, Mathe K, Adam-Vizi V. A novel kinetic assay of mitochondrial ATP-ADP exchange rate mediated by the ANT. *Biophys.J* 2009;96:2490–2504. [PubMed: 19289073]
- [12]. Climent F, Bartrons R, Pons G, Carreras J. Effect of vanadate on phosphoryl transfer enzymes involved in glucose metabolism. *Biochem.Biophys.Res.Commun* 1981;101:570–576. [PubMed: 6272762]
- [13]. Davies DR, Hol WG. The power of vanadate in crystallographic investigations of phosphoryl transfer enzymes. *FEBS Lett* 2004;577:315–321. [PubMed: 15556602]
- [14]. Dolce V, Fiermonte G, Runswick MJ, Palmieri F, Walker JE. The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. *Proc.Natl.Acad.Sci.U.S.A* 2001;98:2284–2288. [PubMed: 11226231]
- [15]. Fiermonte G, De LF, Todisco S, Palmieri L, Lasorsa FM, Palmieri F. Identification of the mitochondrial ATP-Mg/Pi transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution. *J.Biol.Chem* 2004;279:30722–30730. [PubMed: 15123600]
- [16]. Gadsby DC, Nakao M. Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J.Gen.Physiol* 1989;94:511–537. [PubMed: 2607333]
- [17]. Gordon JA. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol* 1991;201:477–482. [PubMed: 1943774]
- [18]. Issartel JP, Dupuis A, Lunardi J, Vignais PV. Fluoroaluminum and fluoroberyllium nucleoside diphosphate complexes as probes of the enzymatic mechanism of the mitochondrial F1-ATPase. *Biochemistry* 1991;30:4726–4733. [PubMed: 1827593]
- [19]. Klingenberg M. The ADP-ATP translocation in mitochondria, a membrane potential controlled transport. *J.Membr.Biol* 1980;56:97–105. [PubMed: 7003152]
- [20]. Kolisek M, Zsurka G, Samaj J, Weghuber J, Schweyen RJ, Schweigel M. Mrs2p is an essential component of the major electrophoretic Mg²⁺ influx system in mitochondria. *EMBO J* 2003;22:1235–1244. [PubMed: 12628916]
- [21]. Kramer R. Influence of divalent cations on the reconstituted ADP, ATP exchange. *Biochim.Biophys.Acta* 1980;592:615–620. [PubMed: 6251873]
- [22]. Leysens A, Nowicky AV, Patterson L, Crompton M, Duchon MR. The relationship between mitochondrial state, ATP hydrolysis, [Mg²⁺]_i and [Ca²⁺]_i studied in isolated rat cardiomyocytes. *J.Physiol* 1996;496(Pt 1):111–128. [PubMed: 8910200]
- [23]. Lienhard GE, Secemski II. P 1, P 5 -Di(adenosine-5')pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J.Biol.Chem* 1973;248:1121–1123. [PubMed: 4734335]
- [24]. Lord KA, Reed GH. Vanadyl(IV) complexes with pyruvate kinase: activation of the enzyme and electron paramagnetic resonance properties of ternary complexes with the protein. *Arch.Biochem.Biophys* 1990;281:124–131. [PubMed: 2166476]

- [25]. Metelkin E, Demin O, Kovacs Z, Chinopoulos C. Modeling of ATP-ADP steady-state exchange rate mediated by the adenine nucleotide translocase in isolated mitochondria. *FEBS J* 2009;276:6942–6955. [PubMed: 19860824]
- [26]. Mukherjee B, Patra B, Mahapatra S, Banerjee P, Tiwari A, Chatterjee M. Vanadium--an element of atypical biological significance. *Toxicol.Lett* 2004;150:135–143. [PubMed: 15093669]
- [27]. Offner FF. Ion flow through membranes and the resting potential of cells. *J.Membr.Biol* 1991;123:171–182. [PubMed: 1720177]
- [28]. Palmieri L, Rottensteiner H, Girzalsky W, Scarcia P, Palmieri F, Erdmann R. Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. *EMBO J* 2001;20:5049–5059. [PubMed: 11566870]
- [29]. Pebay-Peyroula E, Brandolin G. Nucleotide exchange in mitochondria: insight at a molecular level. *Curr.Opin.Struct.Biol* 2004;14:420–425. [PubMed: 15313235]
- [30]. Rasmussen UF, Krstrup P, Kjaer M, Rasmussen HN. Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. *Exp.Gerontol* 2003;38:877–886. [PubMed: 12915209]
- [31]. Ray BD, Moore JM, Rao BD. ³¹P NMR studies of enzyme-bound substrate complexes of yeast 3-phosphoglycerate kinase: III. Two ADP binding sites and their Mg(II) affinity; effects of vanadate and arsenate on enzymic complexes with ADP and 3-P-glycerate. *J.Inorg.Biochem* 1990;40:47–57. [PubMed: 2283509]
- [32]. Robinson JD, Davis RL, Steinberg M. Fluoride and beryllium interact with the (Na + K)-dependent ATPase as analogs of phosphate. *J.Bioenerg.Biomembr* 1986;18:521–531. [PubMed: 3025194]
- [33]. Shin SJ, Lee WK, Lim HW, Park J. Characterization of the ATP transporter in the reconstituted rough endoplasmic reticulum proteoliposomes. *Biochim.Biophys.Acta* 2000;1468:55–62. [PubMed: 11018651]
- [34]. Silverman HS, Di LF, Hui RC, Miyata H, Sollott SJ, Hanford RG, Lakatta EG, Stern MD. Regulation of intracellular free Mg²⁺ and contraction in single adult mammalian cardiac myocytes. *Am.J.Physiol* 1994;266:C222–C233. [PubMed: 8304418]
- [35]. Srere, PA. Citrate synthase. In: Lowenstein, JM., editor. *Methods Enzymol.* Vol. 13. Academic Press; New York: 1969. p. 3-11.
- [36]. Streicher-Scott J, Lapidus R, Sokolove PM. Use of carboxyatractylate and tight-binding inhibitor theory to determine the concentration of functional mitochondrial adenine nucleotide translocators in a reconstituted system. *Anal.Biochem* 1993;210:69–76. [PubMed: 8489026]
- [37]. Voza A, Blanco E, Palmieri L, Palmieri F. Identification of the mitochondrial GTP/GDP transporter in *Saccharomyces cerevisiae*. *J.Biol.Chem* 2004;279:20850–20857. [PubMed: 14998997]
- [38]. Werber MM, Peyser YM, Muhrad A. Characterization of stable beryllium fluoride, aluminum fluoride, and vanadate containing myosin subfragment 1-nucleotide complexes. *Biochemistry* 1992;31:7190–7197. [PubMed: 1386527]
- [39]. Winder WW, Baldwin KM, Terjung RL, Holloszy JO. Effects of thyroid hormone administration on skeletal muscle mitochondria. *Am.J.Physiol* 1975;228:1341–1345. [PubMed: 165730]

Figure 1. Validating the method for permeabilized cells upon gaining access to cell interior
A: Mitochondrial respiration of C₂C₁₂ cells; succ: succinate, 2 mM, ADP: 2 mM, Dig.: digitonin 1 μl of 2.5 mM. **B:** Time course of [ATP]_e in the medium, calculated from [Mg²⁺]_{free} as described in the main text. Effect of stepwise addition of 2 nM cATR to permeabilized cells. **C:** Time courses of [ATP]_e appearing in the medium, calculated from [Mg²⁺]_{free} as described in the main text. Effect of high concentration of digitonin on *in situ* mitochondria with inhibited ANT. In the trace formed by black circles, only ADP, cATR and digitonin were added where indicated. In the trace formed by the open circles, oligomycin (olgm) was also added where indicated. Panels **B** and **C** share the same x-axis.

Figure 2. Estimation of ADP-ATP exchange rates and $\Delta\psi_m$ in permeabilized cells

A: Time course of $[ATP]_e$ in the medium, calculated from $[Mg^{2+}]_{free}$ as described in the main text. Effect of membrane depolarization to various voltages by stepwise addition of 10 nM SF 6847. **B:** Reconstructed time course of $\Delta\psi_m$, calculated from safranin O fluorescence. Permeabilized cells were challenged initially by 2 mM ADP, followed by stepwise additions of 10 nM SF 6847.

Figure 3. ADP-ATP exchange rate/ $\Delta\psi_m$ profile of *in situ* mitochondria of permeabilized cells
 Plot of ATP-ADP exchange rate mediated by ANT versus $\Delta\psi_m$ in *in situ* mitochondria of C₂C₁₂ permeabilized cells depolarized to various voltages by increasing amounts of SF 6847; constructed from the data of 3 independent experiments performed as described in figure 2.