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Publication Date

2018-07-01

DOI

10.1016/j.ab.2017.09.019

Peer reviewed

HHS Public Access

Author manuscript

Anal Biochem. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

Anal Biochem. 2018 July 01; 552: 60–65. doi:10.1016/j.ab.2017.09.019.

In situ measurements of mitochondrial matrix enzyme activities using plasma and mitochondrial membrane permeabilization agents

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Abstract

Activities of enzymes localized to the mitochondrial matrix of mammalian cells are often critical regulatory steps in cellular metabolism. As such, measurement of matrix enzyme activities in response to genetic modifications or drug interventions is often desired. However, measurements in intact cells are often hampered by the presence of other isozymes in the cytoplasm as well as the inability to deliver enzyme substrates across cellular membranes. Classic approaches to liberate matrix enzymes utilize harsh treatments that disrupt intracellular architecture or require significant starting material to allow mitochondrial isolation prior to sample extraction. We describe a method using permeabilization reagents for both the plasma and mitochondrial membranes to allow *in situ* measurement of matrix enzyme activities. It is applied to adherent cell monolayers in 96-well plates treated with perfringolysin O to permeabilize the plasma membrane and alamethicin to permeabilize the mitochondrial inner membrane. We present three examples validated with inhibitor sensitivity: (i) Complex I-mediated oxygen consumption driven by NADH, (ii) ATP hydrolysis by the F_1F_O complex measuring pH changes in an Agilent Seahorse XF Analyzer, and (iii) Mitochondrial glutaminase (GLS1) activity in a coupled reaction monitoring NADH fluorescence in a plate reader.

1. INTRODUCTION

Studying cellular bioenergetics often requires balancing physiological relevance with mechanistic depth [1]. Whole cells, for example, preserve changes in cell signaling, mitochondrial dynamics, and endogenous rates of energy metabolism. Although valuable for understanding global bioenergetic parameters, such as the efficiency of oxidative

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phosphorylation and maximal capacity for substrate oxidation, a mechanistic analysis is often prohibited in intact cells because of an inability to directly control substrate provision to mitochondria [1,2].

Experiments with isolated mitochondria, of course, provide such control; mitochondria can be offered specific oxidizable substrates to test maximal capacities of specific metabolic pathways [3]. However, not only do these experiments lack a wider cellular context, they often require large amounts of starting material and are prone to sub-selection during the isolation procedure. Furthermore, mitochondrial isolation from complex tissues such as the brain results in a heterogeneous mixture of mitochondria from multiple cell types. In many cases where sample size is limited, such as primary or genetically modified cells, isolation of mitochondria for mechanistic analysis is prohibitive if not impossible [1,4].

A solution to this challenge is use of plasma membrane permeabilized cells [2,4– 6]. Forming large pores in the plasma membrane of adherent cells dilutes cytoplasmic contents to the experimental medium and allows the unrestricted delivery of substrates and co-factors to *in situ*, intact mitochondria. This enables a substrate-specific, mechanistic analysis typical for isolated mitochondria to be conducted in adherent cell monolayers. The approach can therefore identify the precise pathway or mechanism underlying a global bioenergetic change observed in intact cells without having to isolate mitochondria [7–12].

In much the same way the plasma membrane hinders the study of mitochondrial function, an analogy can be drawn to the experimental constraints imposed by the mitochondrial inner membrane in the study of matrix enzymes. Activity of matrix enzymes in permeabilized cells or isolated mitochondria is often dependent on activity of upstream and downstream processes in a metabolic pathway including the facilitated import and further catabolism of substrates, which can act as rate-controlling steps for the desired enzyme activity. Additionally, the availability of most cofactors cannot be readily controlled, and readouts such as respiration or NADH fluorescence are ambiguous as they reflect the net activity of a pathway rather than a specific matrix enzyme. Historically, these challenges were overcome by toluene permeabilization of freshly isolated mitochondria [13,14]. In medium controlled for isotonicity with polyethylene glycol, matrix enzymes were retained within toluene-treated mitochondria, enabling the direct, *in situ* study of matrix enzymes without detergent solubilization or a freeze-thaw cycle that can diminish enzyme activity. Notably, this approach was used to help define regulation of TCA cycle dehydrogenases in mammalian mitochondria by divalent cations, ATP/ADP, and NADH/NAD^+ ratios [15,16].

Here we provide a method in which adherent cell monolayers in 96-well format are “double-permeabilized” to allow the study of *in situ* mitochondrial matrix enzymes. To permeabilize the plasma membrane, cells are treated with recombinant, mutant perfringolsyin O (rPFO), a cholesterol-dependent bacterial cytolysin engineered to form large pores (~250 Å) selectively in the plasma membrane [7] allowing large soluble enzymes to be released [6]. As the cholesterol content of the mitochondrial inner membrane is extremely low, the inner membrane remains largely intact following permeabilization with cholesterol-dependent pore-forming agents [4–7]. In comparison to sterol glycosides such as digitonin or saponin, the much steeper, all-or-none dependence of rPFO pore formation on cholesterol

concentration provides greater selectively for plasma membrane permeabilization [17,18]. To permeabilize the mitochondrial membranes to ions, substrates, and cofactors while retaining matrix enzymes, cells are treated with alamethicin [19]. Alamethicin is an α-helical, antimicrobial peptide that forms non-selective pores permeable to solutes up to 3 kDa (~10–20 Å depending on membrane lipid composition) [20,21,22]. The majority of matrix enzymes will be retained, although this pore size is sufficient to allow large amplitude swelling resulting in the outer membrane rupture and release of small soluble proteins such as cytochrome c from the intermembrane space [19,22]. The method is suitable for any matrix enzyme for which activity can be coupled to a plate-based readout and the cellular material remains in an adherent monolayer. Three examples are provided to demonstrate use of this approach with cultured mammalian cells: measurement of (i) NADH-driven respiration in the absence of added substrate using an Agilent Seahorse XF96 analyzer (ii) the hydrolase activity of the F_1F_O ATP synthase measured by pH changes with an XF96 analyzer [2,23], and (iii) mitochondrial glutaminase (GLS1) activity measured in a fluorescent plate reader with an enzyme-linked detection system [24].

2. MATERIALS AND METHODS

2.1 Reagents

Recombinant, mutant perfringolysin O (rPFO) was obtained from Agilent Technologies (XF Plasma Membrane Permeabilizer, XF PMP) and kept as a frozen stock at −20°C. Alamethicin was purchased from Enzo Life Sciences and kept as a 20 mg/mL stock solution in ethanol at −20°C. All other reagents were purchased from Sigma unless otherwise specified.

2.2 Tissue culture

HepG2 and A549 cell lines were purchased from American Type Culture Collection. HepG2 cells were cultured in MEM medium (Gibco 1109510) and A549 cells were cultured in DMEM/F12 (Gibco 11330). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM GlutaMAX, 100 U/mL penicillin, and 100 μg/mL streptomycin. Seahorse XF Analyzer-based pH measurements as well as plate reader-based fluorometric measurements were conducted in Agilent Seahorse XF 96-well plates. All cells were plated for 48 hr prior to the assay and seeded at an initial density of either 2.5×10^4 cells/well (HepG2) or 1.5×10^4 cells/well (A549).

2.3 Plasma Membrane Permeabilization

At the time of the assay, cell culture medium was aspirated, and the monolayers were quickly washed twice with MAS medium (220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, pH 7.2 at 37°C). MAS medium with 3 nM rPFO and other medium components (150 μL total volume) as described later in the text were then added to each well. We have previously compared the use of rPFO to sterol glycosides, such as digitonin, as permeabilization reagents for cell monolayers in Seahorse plates [7]. In the cell types tested, we find that rPFO is less likely to induce cell detachment from the plate surface. Unlike with digitonin, we also find no evidence of cytochrome c loss from the mitochondrial intermembrane space with rPFO when conducting assays in which

only the plasma membrane is permeabilized. This is likely attributable to the shift in cholesterol dependence of this engineered peptide [7,17,18].

2.4 NADH-driven respiration

MAS medium was supplemented with 2 mM NADH (Calbiochem 481913), 4 mM ADP, and 10 μM cytochrome c (Sigma C2506) immediately prior to the measurements. Medium is supplemented with exogenous cytochrome c since, upon membrane pore formation by alamethicin, it is lost from the intermembrane space thereby limiting the respiratory rate [19]. No other oxidizable substrates were added to support electron transport chain activity, and ADP was present to relieve respiratory control. Alamethicin was titrated to determine an optimal concentration for permeabilization of the mitochondrial inner membrane of HepG2 cells using injection ports A and B of the XF96 analyzer (Fig. 1b). From port C, 2 μM (final concentration) rotenone was added to quantify the rate of oxygen consumption dependent on Complex I of the electron transport chain under these conditions.

2.5 ATP Hydrolysis

After washing with MAS buffer, HepG2 cells were assayed in MAS medium supplemented with 3 nM rPFO, 20 mM ATP, 1 μM FCCP, 1 μM rotenone, and 2 μM antimycin A in a total volume of 150 μL. Consideration should be given in selecting the assay conditions depending upon the experimental objective. It may be preferable to use an ATP-regenerating system, and levels of Mg^{2+} in excess of the ATP concentration to ensure ample and constant provision of Mg^{2+}-ATP, the substrate for the F_1Fo-ATPase [25]. Where indicated, cells were also treated with 10 μg/mL alamethicin and incubated for 5 min at 37°C in a hydrated, non-CO_2 incubator. Oligomycin and carboxyatractyloside (CAT) were added acutely via the injector port in a volume of 25 μL of the Agilent Seahorse XF Analyzer at 7X the final concentration. Proton production rates were calculated by fitting to a pH standard curve generated by serial injections of HCl or H_2SO_4 from the XF cartridge ports in a set of wells dedicated for this purpose that lacked cells [26]. Liver mitochondria were isolated and plated according to established protocols [3] with 2 μg mitochondrial protein plated per well. Medium composition and injections were identical to experiments using double-permeabilized cells.

2.6 Glutaminase activity

Mitochondrial glutaminase activity was assessed using an enzyme-linked detection system that generates NADH and can be kinetically measured in a fluorometric plate reader. A549 cells were washed twice with MAS buffer and assayed in MAS supplemented with 3 nM rPFO, 10 μg/mL alamethicin, 2 mM glutamine, 5 mM NAD^+, 200 μM ADP, and 40 U/mL glutamate dehydrogenase (Sigma G2626) in a total volume of 100 μL. Where indicated in the data, 5 μM BPTES (bis-2-(5-phenylacetamido-1,3,4-thiazol-2-yl)ethyl sulfide), an inhibitor of GLS1-type glutaminases [24,27], was present at the start of the assay. As controls, alamethicin or glutamine were individually omitted from the experimental medium, with all other medium components present. NADH production was measured by fluorescence (ex./em. = 340/460 nm) with a reading taken each minute over the course of 30 min at room temperature on a Tecan Infinite M200.

3. RESULTS

We describe assays for mitochondrial matrix enzyme activity in adherent monolayers following permeabilization of both the plasma and mitochondrial membranes (the mitochondrial outer membrane is porous to small solutes). Use of double-permeabilized cells enables direct control over substrate/cofactor concentrations for matrix enzyme activity. As depicted in Fig 1a, adherent cell monolayers are treated with rPFO to form large pores in the plasma membrane to dilute cytoplasmic contents and release soluble proteins <500 kDa into the experimental medium. Plasma membrane permeabilization does not entirely remove cytoplasmic contents from the experimental medium, but reaction intermediates and cofactors are sufficiently diluted such that multistep and/or cofactor-dependent reactions occur at a negligible rate. To access matrix enzymes without the need for facilitated transport across the inner membrane, alamethicin is used to form small (10–20 Å) pores permeable to molecular species up to 3 kDa to allow unrestricted delivery of substrates and release of products.

3.1 Measurement of NADH-driven oxygen consumption

Proof of principle for this approach can be demonstrated by measuring NADH-driven respiration in double-permeabilized HepG2 cells (Fig. 1b). The inner membrane of mammalian mitochondria is largely impermeable to NADH [28], so the mitochondrial respiratory rate is marginal when plasma membrane permeabilized cells are offered only NADH. However, upon injection of a titrated amount of alamethicin from the ports of the XF96 analyzer, NADH crosses the inner membrane and can be oxidized by respiratory complex I to drive oxygen consumption. The NADH-driven respiration is entirely sensitive to rotenone, and demonstrates that alamethicin can be successfully used to deliver small solutes to matrix enzymes and measure their activity in adherent cell monolayers.

3.2 Measurement of ATP hydrolase activity of the F_1Fo ATP synthase

Next, we measured the hydrolase activity of the F_1F_O-ATP synthase using pH measurements with the Agilent Seahorse XF analyzer [2,23]. When the mitochondrial electron transport chain generates a membrane potential during NADH or $FADH_2$ oxidation, the ATP synthase utilizes this electrochemical potential to drive phosphorylation of ADP (ATP synthase activity in the “forward” direction). Conversely, when the membrane potential collapses, such as occurs during anoxia or electron transport chain inhibition, the enzyme operates as an ATP hydrolase (ATP synthase activity in the “reverse” direction), hydrolyzing ATP into ADP and inorganic phosphate while pumping protons into the intermembrane space to maintain a mitochondrial membrane potential.

Both the ATP synthase and hydrolase activities can be indirectly measured by pH changes in the experimental medium. Phosphorylation of ADP alkalinizes the experimental medium as it consumes H^+, and similarly, hydrolysis of ATP generates H^+ and acidifies the experimental medium [2]. Therefore, in isolated mitochondria or rPFO-treated cells, activity of the ATP synthase can be measured by pH changes under conditions where the enzyme operates in the forward or reverse directions. Fig. 2a specifically depicts conditions for measurement of hydrolase activity. Addition of rotenone and antimycin A (to block the

electron transport chain), FCCP (to dissipate the membrane potential), and ATP will drive the enzyme to consume ATP and acidify the medium. Sensitivity to oligomycin, can confirm that the pH changes are directly attributable to the ATP synthase when assessing either synthase or hydrolase activity. As shown in Fig. 2b, medium acidification is negligible in double-permeabilized HepG2 cells, but upon injection of ATP, the rate of H^+ production sharply increases due to ATP hydrolysis.

As proof of principle, we first demonstrate hydrolase activity attributable to the ATP synthase with isolated liver mitochondria (Fig. 2c), where there is only marginal ATP hydrolysis apart from the ATP synthase. Without alamethicin, both oligomycin and carboxyatractyloside (CAtr), an inhibitor of the adenine nucleotide translocator, completely block medium acidification when FCCP, respiratory chain inhibitors, and ATP are present. When alamethicin is added to the experimental medium, however, ATP hydrolysis remains sensitive to oligomycin but becomes insensitive to CAtr, as transport of ATP by adenine nucleotide translocator is rendered unnecessary upon inner membrane permeabilization. Furthermore, the rate of hydrolysis-mediated acidification upon alamethicin treatment substantially increases, showing that adenine nucleotide exchange across the inner membrane is rate-controlling for this assay and providing another positive control for the effect of alamethicin. Indeed, the adenine nucleotide translocator has also been reported to rate-limit ATP synthesis by the F_1F_O complex [29].

In double-permeabilized HepG2 cells (Fig. 2d), activity of the mitochondrial F_1F_O-ATP synthase accounts for roughly 30% of the total ATP hydrolysis activity, as demonstrated by partial sensitivity to oligomycin. This fraction is insensitive to CAT, demonstrating sufficient permeabilization of the inner membrane by alamethicin. The difference in results between isolated mitochondria and permeabilized cells highlights a difference between the two systems: while most other cellular content is lost during the mitochondrial isolation procedure, integral membrane and membrane-bound ATPases are still present in a permeabilized cell preparation. Further, unless the soluble cytoplasmic contents are removed after plasma membrane permeabilization, soluble ATPases have merely been diluted to the experimental medium and may remain active. Accordingly, and because ATP hydrolysis is thermodynamically favorable, there is a high background rate of reaction that is insensitive to oligomycin from other cellular ATPases in double-permeabilized cells. Additional experiments using inhibitors of other ATPases (e.g. vanadate for P-type ATPases or ouabain for the Na^+/K^+-ATPase) could be done to determine the contribution of other ATP hydrolases to the rate of acidification. Nonetheless, the data show that alamethicin can be used with either isolated mitochondria or rPFO-permeabilized cells to directly measure activity of the ATP synthase.

3.3 Measurement of Mitochondrial Glutaminase Activity

In addition to using measurements of O_2 and H^+, plate-based fluorescence can also be used with adherent monolayers of double-permeabilized cells to assess matrix enzyme activities. As an example, we present a rate-based assay for matrix-localized glutaminase (referred to as GLS1, glutaminase C, or phosphate activated mitochondrial glutaminase) in human A549 lung adenocarcinoma cells. Alamethicin permeabilization of the mitochondrial inner

membrane allows delivery of glutamine to the matrix without the need for facilitated transport. The glutamate arising from glutamine deamidation can be detected with exogenously added glutamate dehydrogenase and NAD^+, which will generate NADH (Fig. 3a). Figures 3 b, c show that robust, glutamine-supported NADH production is observed under these conditions in a manner sensitive to the glutaminase inhibitor BPTES. As an important control, marginal activity is observed in the absence of alamethicin, demonstrating that the assay can distinguish between matrix-enclosed NADH and extra-mitochondrial NADH generated in the experimental medium. Notably, 3 μM BPTES lowers the rate of glutamine supported NADH fluorescence below the rate generated in the absence of alamethicin. As it is unlikely that BPTES will have an off-target effect on glutamate dehydrogenase at 3 μM [2,24], the result likely indicates that some fraction of glutaminase in A549 cells is associated with the intermembrane space, outer membrane, or the diluted cytoplasm.

4. DISCUSSION

We describe an experimental approach to assess mitochondrial matrix enzyme activities in adherent cell monolayers in multi-well plates. This approach is particularly useful when both cytoplasmic and mitochondrial matrix isoforms of an enzyme exist (such as glutaminase, isocitrate dehydrogenase, aconitase, several transaminases, and glutathione peroxidase and reductase), and there is interest in specifically characterizing matrix enzyme activity. Solubilization of cells with detergent, as is often done for measurement of enzyme activities, obviously mixes the population of isoenzymes across cellular substructures. The method outlined is particularly useful when the number of cells available for assay is limited. For example, the technique could be used on acutely isolated, short-lived cell types that are limited in number. These include subsets of hematopoietic or immune cells, which can be acutely captured on a plate using either adhesive substrates like Cell Tak or antibody-based capture.

The technique involves selective permeabilization of the plasma membrane to release soluble enzymes from the cytoplasm, for which we use rPFO as in previous work [2,4,7,10–12]. rPFO is a recombinant, mutant form of perfringilysin O, a cholesterol-dependent cytolysin. Use of rPFO improves upon detergent-based methods by eliminating the need to titrate appropriate concentrations for each cell type to avoid mitochondrial outer membrane permeabilization and the lower propensity for cell detachment [4,7]. Plasma membrane permeabilization is followed by a second permeabilization step to create pores in the mitochondrial inner membrane too small to allow release of matrix enzymes, but large enough to deliver substrates and cofactors to for the enzymatic reaction of interest to proceed. We have used alamethicin for this purpose, as others have previously with isolated mitochondria [19]. The approach obviates the need for isolation of mitochondria, which can be problematic from small cellular samples such as primary or genetically modified cells.

In principle, other permeabilization reagents can potentially be used: saponin, digitonin, or cytolysins other than rPFO can permeabilize the plasma membrane, and toluene could be used to perforate the inner membrane. That said, the ideal reagents will allow large pore formation at the plasma membrane, small pores at the mitochondrial membrane, and will not

induce cell detachment from the monolayer. The last point is critical for measurements made in the Seahorse XF analyzer, which require adherent monolayers of biological material.

Access to matrix enzyme activities can be achieved by other methods. A common approach is to offer ester-linked carboxylic acids as enzyme substrates, such as methyl pyruvate or dimethyl-α-ketoglutarate, that are membrane-permanent analogs which can be subsequently cleaved into the desired metabolite (pyruvate and α-ketoglutarate, respectively) to measure dehydrogenase or transaminase activity. The approach has several drawbacks, though, including generation of intracellular organic byproducts (e.g. methanol), rate limitations from esterase activity, as well as designing detection methods specific for the enzyme of interest. Additionally, when studying the role of intracellular metabolites as inhibitors of matrix enzymes, it can be difficult to discriminate between whether the active moiety is the (putatively) cleaved metabolite or the alkyl group.

Indeed, there are some caveats to the double-permeabilized cell approach as well, including that some cell types will detach after prolonged exposure to the permeabilization reagents. In addition to the adherent monolayers used in the current examples, it should be noted that the approaches described here are readily applicable to suspensions of cells. We have previously described protocols for plasma membrane permeabilization in cell monolayers as well as suspended cells for measurements of mitochondrial oxygen consumption [4]. Alternative measures of oxygen consumption, including conventional polarography, can be applied to suspensions of double-permeabilized cells, as can alternative methods of detecting enzyme activity. However, the amount of cells required for measurements in suspension is often orders of magnitude higher than what is necessary for similar measurements with the XF Analyzer [4]. Therefore, the contents of the cell released upon permeabilization may make a greater contribution to the assay medium and potentially affect results. To avoid this, the soluble cytoplasmic contents (which can include cytoplasmic enzymes, substrates, cofactors, etc.) can be removed from suspensions of permeabilized cells following centrifugation (approximately 500*g* for 3–5 minutes). As such, the double-permeabilization method is by no means exclusive to the Agilent Seahorse XF format, although this approach likely minimizes the need for biological material.

Another consideration for the double-permeabilized cell approach is that other organelles are retained in a permeabilized cell preparation on which alamethicin will also have an effect, including the endoplasmic reticulum, peroxisomes, lysosomes, and the nucleus [30]. Metabolic intermediates, cofactors, and other small soluble constituents may therefore be released from these organelles into the experimental assay medium. It also should be noted that, as with any permeabilization approach, the method does not report the endogenous activity of an enzyme in intact cells, as this can be restricted by context-specific, intracellular regulatory mechanisms. Rather, it reports the maximal activity under the conditions of the assay medium. Nonetheless, the technique should nonetheless provide a useful means to assess matrix enzyme activities, particularly with sample-limited experimental systems such as primary or genetically modified cells.

Acknowledgments

This work was supported by US National Institutes of Health grant R01NS087611 (to ANM) and Agilent Technologies (consultancies to ANM and ASD). GWR is an employee of Agilent Technologies.

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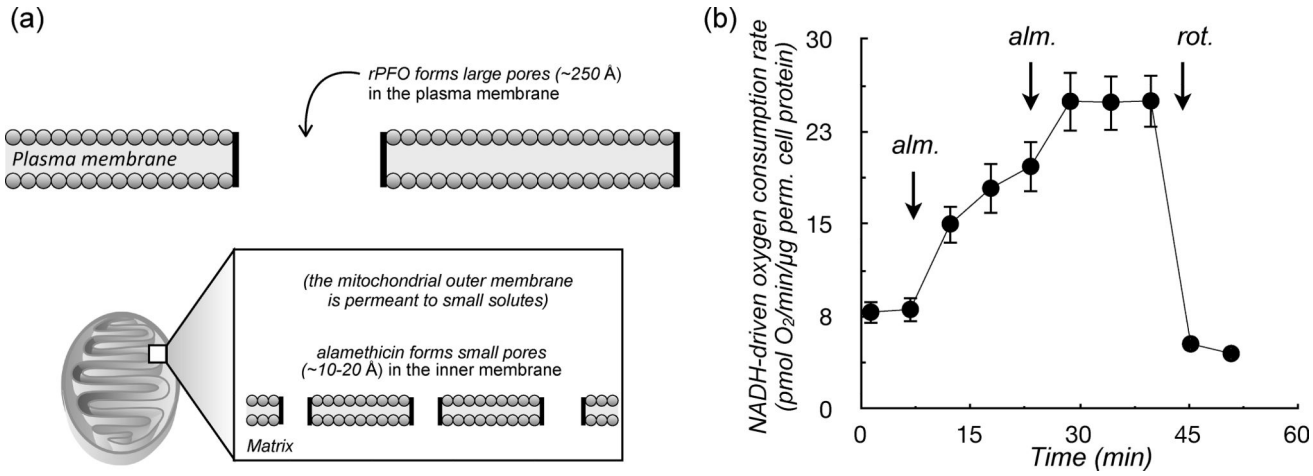

Figure 1.

(a) Permeabilization of the plasma membrane of adherent monolayers is achieved with rPFO, which forms pores sufficiently large to dilute cytoplasmic contents to the experimental medium. Alamethicin is used to perforate the mitochondrial inner membrane, allowing small solutes to freely enter the matrix without the need for facilitated transport. (b) NADH does not support respiration in plasma membrane permeabilized HepG2 cells because it is not transported across the mitochondrial inner membrane. Upon injections of alamethicin (alm., sequential injections represent final concentrations of 5 µg/mL and 10 µg/mL), however, NADH freely accesses the matrix localized arm of complex I and can be oxidized to drive rotenone-sensitive respiration.

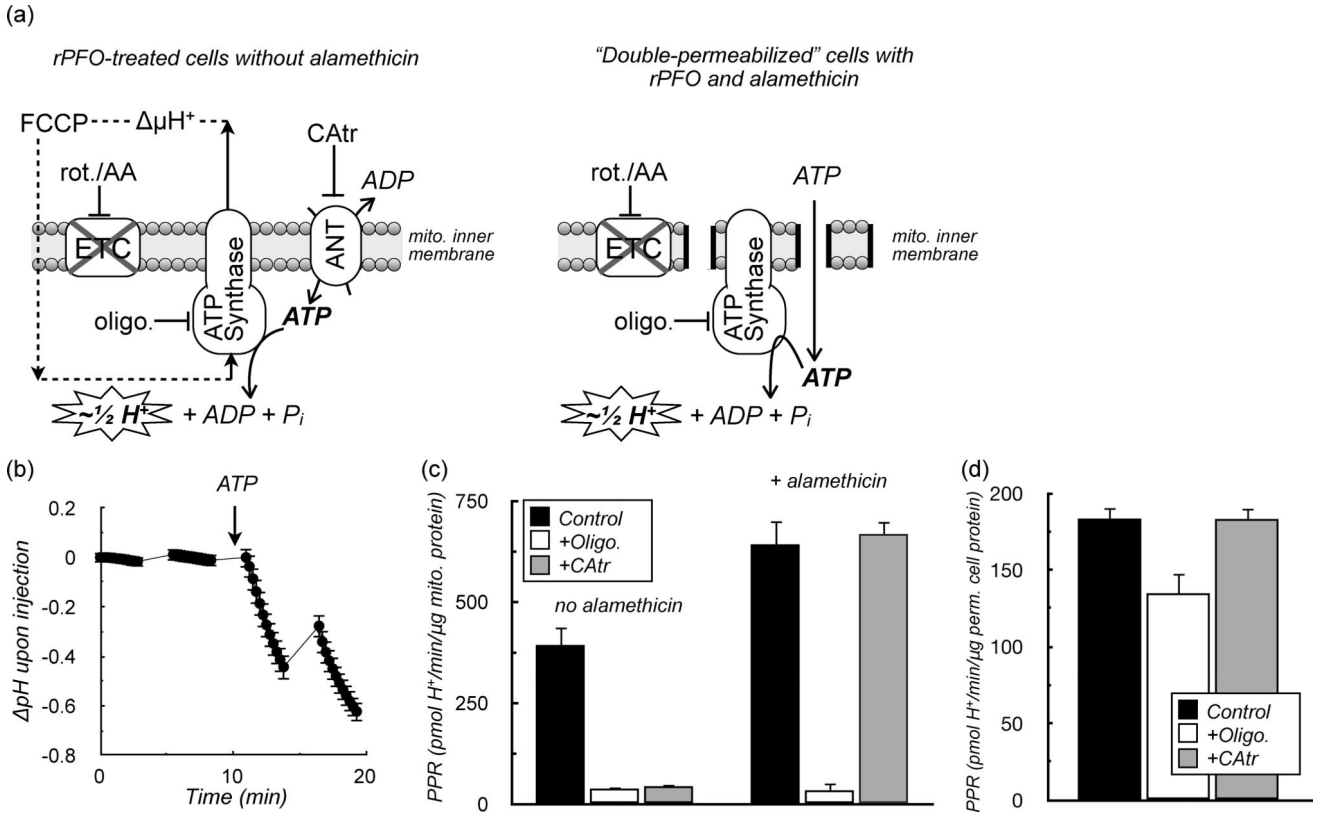

Figure 2.

(a) Schematic of hydrolase activity by the F_1F_O complex in isolated mitochondria and plasma membrane permeabilized cells with and without alamethicin treatment. With the inner membrane intact, the hydrolysis reaction requires rotenone and antimycin A (to inhibit the respiratory chain), FCCP (to dissipate the membrane potential), and facilitated uptake of ATP by the adenine nucleotide translocase (ANT). This reaction is sensitive to both oligomycin and carboxyatractyloside (CAtr). In alamethicin permeabilized mitochondria, ATP can readily cross the inner membrane and serve as a substrate for hydrolysis. (b) Medium acidification in double-permeabilized cells is negligible in the absence of exogenous ATP, but immediately occurs upon injection of ATP (20 mM final concentration). (c) In isolated mitochondria without alamethicin treatment, inhibition of both the ATP synthase with oligomycin and the ANT with CAtr inhibits ATP hydrolysis. In alamethicin-permeabilized mitochondria, however, ATP freely crosses the inner membrane and CAtr has no effect. (d) In HepG2 cells treated with both rPFO and alamethicin, CAtr has no effect on the rate of ATP hydrolysis while oligomycin decreased the rate by roughly 30%, with residual activity likely due to contaminating, ATPases retained within a permeabilized cell preparation.

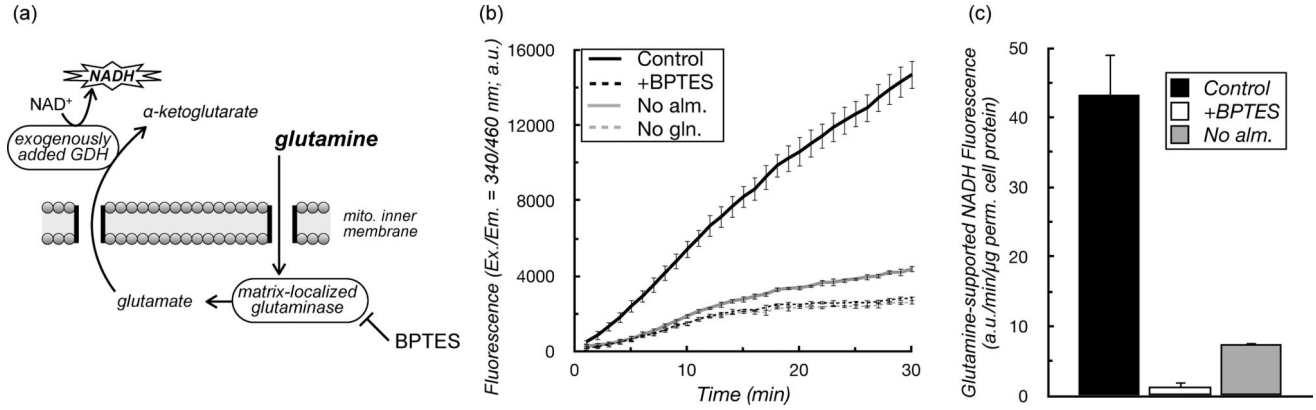

Figure 3.

(a) Schematic of assay to measure mitochondrial glutaminase activity in which glutaminase-derived glutamate is coupled to NADH production with excess, exogenously added glutamate dehydrogenase (GDH) and NAD^+. (b) Sample kinetic trace of glutaminase activity as measured by NADH fluorescence. NADH production is largely sensitive to BPTES, and is dependent on the presence of exogenously added glutamine and alamethicin to permeabilize the mitochondrial inner membrane. (c) Data from (b) is converted into a rate, with fluorescence in the absence of added glutamine considered to be background signal. Control, contains alamethicin and all glutaminase reagents required for glutaminase activity; +BPTES = 5 μM BPTES, an inhibitor of GLS1-type glutaminases [24,27], was present at the start of the assay; No alm., no alamethicin was added; No gln., No glutamine was added to the assay mixture.