New Insights into the Bioenergetics of Mitochondrial Disorders Using Intracellular ATP Reporters

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Mutations in mitochondrial DNA (mtDNA) cause impairment of ATP synthesis. It was hypothesized that high-energy compounds, such as ATP, are compartmentalized within cells and that different cell functions are sustained by different pools of ATP, some deriving from mitochondrial oxidative phosphorylation (OXPHOS) and others from glycolysis. Therefore, an OXPHOS dysfunction may affect different cell compartments to different extents. To address this issue, we have used recombinant forms of the ATP reporter luciferase localized in different cell compartments—the cytosol, the subplasma membrane region, the mitochondrial matrix, and the nucleus—of cells containing either wild-type or mutant mtDNA. We found that with glycolytic substrates, both wild-type and mutant cells were able to maintain adequate ATP supplies in all compartments. Conversely, with the OXPHOS substrate pyruvate ATP levels collapsed in all cell compartments of mutant cells. In wild-type cells normal levels of ATP were maintained with pyruvate in the cytosol and in the subplasma membrane region, but, surprisingly, they were reduced in the mitochondria and, to a greater extent, in the nucleus. The severe decrease in nuclear ATP content under “OXPHOS-only” conditions implies that depletion of nuclear ATP plays an important, and hitherto unappreciated, role in patients with mitochondrial dysfunction.

INTRODUCTION

The concept that high-energy compounds are compartmentalized in cells was proposed more than 20 years ago (Erickson-Viitanen *et al.*, 1982a, 1982b; Saks *et al.*, 1994). For example, it was suggested that in normal smooth muscle cells the contractile functions are supported by mitochondrial ATP derived from the respiratory chain and oxidative phosphorylation (OXPHOS), whereas the plasma membrane proton pumps are supported by ATP from anaerobic glycolysis (Ishida *et al.*, 1994). In addition, recent studies showed that the import of histones into the nuclei of neonatal cardiomyocytes is strictly dependent on a concerted interaction between mitochondrial ATP synthesis and the trafficking of high-energy phosphoryls (Dzeja *et al.*, 2002).

In a technical advance, targeted luciferase has been used as an ATP sensor to investigate the kinetics of the variation of ATP concentration beneath the plasma membrane, in the mitochondria, and in the cytosol of pancre-

atic β -cells in response to glucose stimulation (Kennedy *et al.*, 1999). These experiments demonstrated that in response to the administration of glucose and potassium, ATP levels increased in the plasma membrane of β -cells in concert with that in mitochondria, whereas cytosolic ATP showed only a transient increase. On the other hand, studies using the ATP-dependent potassium channel as an ATP sensor showed that in *Xenopus* oocytes and in cultured mammalian cells there was no gradient between bulk cytosolic ATP and subplasma membrane ATP, suggesting that ATP diffuses freely between these two cell compartments (Gribble *et al.*, 2000).

Despite a growing body of evidence that high-energy molecules such as ATP and phosphocreatine may be compartmentalized in cells, we still do not fully appreciate what modifications occur in intracellular ATP pools of cells affected by defects in energy metabolism, such as those caused by mutations in mitochondrial DNA (mtDNA). These mutations, which are associated with a heterogeneous group of sporadic or maternally inherited metabolic disorders (DiMauro *et al.*, 1998), cause impairment of OXPHOS, resulting in reduced mitochondrial ATP synthesis.

The question of which cell compartments are more prone to become depleted of ATP, and whether specific ATP-

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dependent functions are affected differentially in mitochondrial diseases, remains essentially unresolved. Clearly, a better understanding of the mechanisms by which cells cope with impaired mitochondrial ATP synthesis, and which specific ATP-dependent cellular functions are preserved, down-regulated, or abolished in these conditions, could help us better understand the pathogenesis of mitochondrial diseases.

To begin to address those issues, we have investigated the fate of intracellular ATP pools in intact living cells harboring pathogenic mtDNA mutations. We used targeted luciferase constructs to assess free ATP levels in the cytosolic, mitochondrial, intranuclear, and subplasma membrane compartments, an approach that has already been used successfully by other investigators to detect ATP in living normal cells (Maechler *et al.*, 1998; Jouaville *et al.*, 1999; Kennedy *et al.*, 1999; Porcelli *et al.*, 2001).

We chose to analyze two known pathogenic mutations. The first one is a T→G transversion at position 8993 in the ATPase 6 gene (Anderson *et al.*, 1981), encoding a subunit of the F_0 portion of ATP synthase. This mutation is associated with two related syndromes, NARP (neuropathy, ataxia, and retinitis pigmentosa; Holt *et al.*, 1990) and MILS (maternally inherited Leigh syndrome; Santorelli *et al.*, 1993). Importantly, in cytoplasmic hybrid (cybrid) cells containing homoplasmic levels of the mutation, mitochondrial ATP synthesis is reduced by ~50–80% (Manfredi *et al.*, 2002b), but the remainder of the respiratory chain is relatively unaffected. The second mutation is an A→G transition at position 3243 in the gene encoding tRNA^{Leu(UUR)}. This mutation is associated with MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; Goto *et al.*, 1990). The mutation results in globally impaired mitochondrial protein synthesis and affects all respiratory chain complexes containing mtDNA-encoded subunits (i.e., complexes I, III, IV, and V; King *et al.*, 1992). We used cells harboring wild-type mtDNA (i.e., without known pathogenic mutations) as a positive control, and ρ^0 cells, which are devoid of mtDNA and therefore have no residual OXPHOS function (King and Attardi, 1989), as a negative control.

MATERIALS AND METHODS

Luciferase Constructs

Wild-type firefly luciferase from *Photinus pyralis* (accession number AB062786) is a 550-amino acid protein containing the canonical peroxisomal targeting sequence Ser-Lys-Leu at its C terminus (Gould *et al.*, 1987). To generate cytosolic luciferase (Luc_c), we replaced the leucine at position 550 with a valine by in vitro mutagenesis, thereby destroying the natural peroxisomal targeting sequence (Gould *et al.*, 1989) and allowing this modified luciferase to reside free in the cytoplasm. For the mitochondrial luciferase (Luc_m), Luc_c was fused downstream of the sequence encoding the mitochondrial targeting signal (MTS) of subunit VIII of cytochrome *c* oxidase (accession number NM004074). The COX VIII targeting sequence has been used successfully in other mitochondrial targeting experiments (Rizzuto *et al.*, 1992; Rizzuto *et al.*, 1995), and a similar COX VIII-luciferase construct was shown to be targeted appropriately to the mitochondrial matrix (Jouaville *et al.*, 1999; Kennedy *et al.*, 1999; Porcelli *et al.*, 2001). For the subplasma membrane luciferase (Luc_{pm}), Luc_c was fused downstream of the second external arm of the Ht1a receptor (accession number X57829), a G-linked protein; the Ht1a sequence was shown to target a reporter gene to the plasma mem-

brane of eukaryotic cells (Singer 1990). For the nuclear luciferase (Luc_n), we fused Luc_c downstream of the SV-40 large T antigen (accession number NC001669) nuclear localization signal (NLS); this NLS was shown to target recombinant luciferase to the nucleus (Michels *et al.*, 1995). All the chimeric Luc constructs were inserted into the mammalian expression vector pCDNA3.0 (Invitrogen Inc., Carlsbad, CA), as described (Manfredi *et al.*, 2002b).

Cell Culture and Transfection

We used four human cell lines: 1) 143B osteosarcoma cells containing wild-type mtDNA (i.e., ρ^+ cells); 2) 143B206 cells that were completely devoid of mtDNA, due to long-term treatment of the 143B cells with ethidium bromide (i.e., ρ^0 cells; King and Attardi, 1989); 3) transmitochondrial cell hybrids (cybrids) consisting of 143B206 cells repopulated with mitochondria containing 100% 8993-G mtDNA (i.e., homoplasmic mutation in ATPase 6) from a patient with NARP (Manfredi *et al.*, 1999), and 4) cybrids of 143B206 cells repopulated with mitochondria containing 100% 3243-G mtDNA (i.e., homoplasmic mutation in tRNA^{Leu(UUR)}) from a patient with MELAS (King *et al.*, 1992). Cells were transfected with pCDNA3.0 containing the engineered Luc genes, using the transfection reagent FuGENE6 (Roche Applied Sciences, Indianapolis, IN) as described by the manufacturer. Cells were grown in 100-mm culture dishes in DMEM containing high glucose (4.5 mg/ml), 2 mM L-glutamine, 110 mg/l sodium pyruvate supplemented with 10% fetal bovine serum (FBS), and 50 μ g/ml uridine. For stable transfections, osteosarcoma-derived cells were selected in 500 μ g/ml of the neomycin analog Geneticin (Invitrogen Inc.).

Intracellular localization of the chimeric luciferases was assayed by immunocytochemistry in transiently transfected human 293T-HEK cells as described (Kennedy *et al.*, 1999), using rabbit polyclonal antiluciferase antibodies (Promega, Madison, WI) followed by antirabbit Cy-2-conjugated secondary antibodies (Molecular Probes, Eugene, OR). The intracellular localization of the luciferase constructs was tested by staining transfected cells with cell compartment-specific fluorescent dyes (Molecular Probes): Mitotracker Red CMX-ROS was used for mitochondria, FM 4-64 for the plasma membrane, and Hoechst 33342 for the nuclei.

Calibration of the Luciferase System

To assess the range of luciferase expression in transfected cells, the luminescence derived from 2×10^5 cells was measured with a luciferase assay kit (Promega) and was compared with that obtained from a set of purified firefly luciferase standards (Roche Applied Sciences), ranging from 1 pg to 1 ng.

To determine the dependence of light production on luciferin concentration, we constructed a luciferin dose-response curve using a fixed amount of luciferase. Typically, 100 pg, a value comparable to that found in lysates of 2×10^5 stably transfected cells, was dissolved in a 25 mM tricine, 150 mM NaCl buffer, pH 7.4, in the presence of increasing ATP concentrations, ranging from 31.2 to 500 μ M.

To ascertain the kinetic properties of the targeted luciferases, we constructed ATP-luminescence curves both with purified firefly luciferase and with lysates from cells expressing the different targeted luciferases. Cells (2×10^5) were solubilized in 20 μ l of a lysis buffer containing 20 mM HEPES and 0.1% Triton X-100, pH 7.2. Purified firefly luciferase (100 pg) was also dissolved in 20 μ l of the same lysis buffer. Luminescence was measured on the soluble cell fractions diluted in 80 μ l of a buffer that mimicked physiological intracellular conditions (physiological buffer), containing 20 mM HEPES, 140 mM KCl, 10.2 mM EGTA, 6.7 mM CaCl₂, 2 mM luciferin, 20 μ g/ml digitonin, 0.5 mM MgCl₂, and 0.01 mM CoA, as described (Jouaville *et al.*, 1999; Kennedy *et al.*, 1999) and increasing concentrations of ATP ranging from 0 to 1000 μ M. To correct for variable luciferase expression levels, luminescence was normalized to the "total potential lumines-

Figure 1. Subcellular localization of recombinant chimeric luciferases in 293T cells. Maps of the engineered luciferase genes are shown at the top. (A) Cells transfected with *Luc_C* and immunostained with an antiluciferase antibody (green), showing a cytosolic, diffuse staining. (B) Cells transfected with *Luc_{pm}* and immunostained with antiluciferase antibodies (green, top panel). Cells were counterstained with FM 4–64, specific for the cytoplasmic membrane (red, middle panel). The overlay showed colocalization of the two fluorochromes (yellow-orange, bottom panel). (C) Cells transfected with *Luc_n* and immunostained with antiluciferase antibodies (green, top panel). Cells were counterstained with Hoechst 33342, specific for DNA (purple, middle panel). The overlay showed colocalization of the two fluorochromes (cyan, bottom panel). (D) Cells transfected with *Luc_m* and immunostained with antiluciferase antibodies (green, top panel). Cells were counterstained with Mitotracker Red CMXRos, specific for mitochondria (red, middle panel). The overlay showed colocalization of the two fluorochromes (yellow-orange, bottom panel).

cence” measured in lysates from an identically sized aliquot of transfected cells, diluted in 50 μ l of the luciferase assay buffer (Promega) in the presence of excess (>1 mM) ATP.

Measurements of Cellular ATP

For luciferase assays, aliquots of 2×10^5 transfected cells were incubated in DMEM containing the following combinations of substrates and inhibitors (20 replicate samples for each condition): 1) glucose (4.5 mg/ml) plus 110 mg/l pyruvate; 2) glucose plus 1 μ g/ml the ATP synthase inhibitor oligomycin; 3) pyruvate alone (110 mg/l); 4) pyruvate plus 5 ng/ml oligomycin; and 5) pyruvate plus 10 ng/ml oligomycin. The ρ^0 cells were also assayed with glucose plus 1 μ g/ml oligomycin in the presence of 110 mg/l pyruvate. Luminescence was measured as described (Manfredi *et al.*, 2002b). Briefly, cells were placed in noncoated 24-well plastic plates and incubated with gentle rocking (to prevent cell attachment) for 1 h at 37°C in 5% CO₂. Cells were collected by aspiration, pelleted immediately by centrifugation, and resuspended in 90 μ l buffer containing 25 mM tricine and 150 mM NaCl, pH 7.4. Beetle luciferin (Promega) was added to the cell suspension (final concentration, 2 mM), and light emission was measured in a luminometer (MGM Instruments, Camden, CT) at 5-s intervals until the maximum value of luminescence was reached. To correct for the variability of luciferase expression in each sample, the relative luminescence values in each cell compartment were normalized to the “total potential luminescence” as described above.

To assess the total ATP content in cells incubated in the same conditions as above, we used an HPLC-based method (Manfredi *et al.*, 2002b), using appropriate ATP standards. ATP content measured in total cell lysates was expressed as nmoles/mg cellular proteins. Proteins were measured with a DC protein assay kit (Bio-Rad) as recommended by the manufacturer.

Statistical analyses of differences between ATP concentration values were performed by unpaired two-tailed Student’s *t* test.

RESULTS

Expression of Targeted Luciferase Constructs

We obtained light production in lysates of cells transfected with all four luciferase constructs, whereas the mock transfection of pCDNA3.0 without insert gave a negative assay.

To verify the correct subcellular localization of the chimeric luciferases, the transfected 293T-HEK cells were treated with specific fluorescent dyes, which stained the intracellular compartment of interest, and then were immunostained with antiluciferase antibodies. The overlay of images of the same field of cells photographed using appropriate light filters demonstrated a good degree of colocalization of the fluorochromes in cells transfected with *Luc_m*, *Luc_{pm}*, and *Luc_n* (Figure 1, B–D). Cells transfected with *Luc_C* showed a diffused staining compatible with cytosolic localization (Figure 1A).

Kinetics of Chimeric Luciferases and Calibration of the System

A standard dilution curve of firefly luciferase plotted vs. luminescence showed that light production was linearly proportional to the amount of luciferase added (Figure 2A). On the basis of this luciferase standard curve, we estimated that aliquots of 2×10^5 stably or transiently transfected cells typically expressed 50–150 pg of luciferase.

Figure 2. Calibration of the luciferase ATP reporter system. (A) Luciferase titration curve. Light emission was measured with increasing amounts of luciferase. Light emission was directly proportional to the amount of luciferase added, within the limits of detection of the luminometer (i.e., $0\text{--}2 \times 10^6$ relative light units [RLU]). Values are average of two replicate samples. (B) Luciferin titration curve. The dependence of light emission on luciferin concentration was assayed in the presence of a fixed amount of firefly luciferase (100 pg) and the indicated concentrations of ATP. For all ATP concentrations, maximal luminescence was achieved with 2 mM luciferin. Values are the average of two replicate samples. (C) Kinetics of the affinity of recombinant chimeric luciferases for ATP in "physiological buffer." Luminescence with the indicated recombinant luciferases was measured in aliquots of 2×10^5 lysed transfected cells resuspended in physiological buffer (see MATERIALS AND METHODS) or in physiological buffer containing 100 pg of purified firefly luciferase in the presence of different ATP concentrations. The RLU is the ratio of luminescence obtained from the lysed cells in physiological buffer to the "total potential luminescence" obtained from an identical aliquot of lysed cells measured with a luciferase detection kit in the presence of excess ATP (see MATERIALS AND METHODS). Bars represent standard deviations of six replicate samples.

Therefore, the kinetic properties of the chimeric luciferases were compared with those of 100 pg of firefly luciferase.

A luciferin standard curve (Figure 2B) was obtained using a fixed amount of firefly luciferase in the presence of increasing concentrations of luciferin (between 100 μ M and 3 mM) and ATP (between 31.2 and 500 μ M). Maximal light emission was reached for all luciferin concentrations with 500 μ M ATP; the absolute luminescence values were highest with 2 mM luciferin. This suggested that luciferin concentrations between 1 and 2 mM did not inhibit luciferase, whereas lower concentrations (i.e., <0.5 mM) were unable to achieve maximum luminescence in the range of luciferase present in transfected cells. Furthermore, in prokaryotes, free diffusion of luciferin inside cells at physiological pH is limited by its negative charge (Di Tomaso *et al.*, 2001). Therefore, it is possible that also in mammalian cells the intracellular concentration of luciferin may be lower than that in the buffer. For these reasons, we used 2 mM luciferin for all our assays.

To measure luciferase activity in an environment that closely reflects the intracellular one, the apparent K_m values for ATP of the chimeric luciferases were determined in a buffer that mimicked physiological intracellular conditions and compared with that of purified firefly luciferase. In this buffer, the apparent K_m of native firefly luciferase (Figure 2C) was ~ 70 μ M ATP, which was slightly above the values previously reported for *Photinus pyralis* luciferase (~ 60 μ M ATP; DeLuca and McElroy 1974; Lemasters and Hackenbrock 1977). The K_m values of the four chimeric luciferases in soluble cell fractions of transfected cells were all higher than that of firefly luciferase. Whereas Luc_m , Luc_c , and Luc_{pm} were very similar to each other, Luc_n had a substantially higher K_m than the other three (Figure 2C). These kinetic differences were presumably due to conformational changes resulting from the addition of the targeting peptides. The polynomial equations (unpublished data) describing the curves in Figure 2C were used to extrapolate the absolute ATP levels detected by targeted luciferases in intact cells.

Total Cellular ATP Content in Wild-type and Mutant Cells

We measured total cellular ATP content by high-pressure liquid chromatography (HPLC) of cell lysates from wild-type and mutant (i.e., NARP, MELAS, and ρ^0) cells after incubation in medium containing different combinations of substrates and inhibitors (Figure 3). We first measured total ATP levels in medium containing both glucose and pyruvate (i.e., substrates for glycolysis and OXPHOS, respectively). In this "complete" medium, all cell types had similar ATP contents, ranging from 17 to 26 nmol/mg cell protein.

In "glycolysis-only" medium (containing glucose but lacking pyruvate, and containing 1 μ g/ml oligomycin, a concentration that completely inhibits mitochondrial ATP synthase), not only the wild-type cells, but also the NARP and MELAS cells maintained almost unchanged ATP levels, suggesting that glycolysis was sufficient to provide the required cellular ATP. However, ρ^0 cells, which rely exclusively on glycolysis for ATP synthesis (as they do not possess a functioning respiratory chain) showed a statistically significant reduction in total ATP content in glucose alone compared with complete medium (41% reduction; $p < 2 \times 10^{-6}$). Because these cells also lack the oligomycin-binding subunit

Figure 3. Quantification of total cellular ATP. Wild-type, NARP, MELAS, and ρ^0 cells were incubated for 2 h in medium containing the indicated combinations of substrate and inhibitors. Cells were harvested and lysed immediately in perchloric acid. Total cellular ATP content in cell lysates was measured by HPLC and expressed as nmol ATP/mg cellular proteins. The reduction of ATP content in NARP cells in OXPHOS medium was accentuated by low doses of oligomycin (double-headed arrows). Bars represent standard deviations of four replicate samples. P values that were particularly relevant to the discussion of the results are indicated.

6 of ATPase, which is mtDNA encoded, this reduction cannot be attributed to the effect of oligomycin. One possible explanation is that in cells lacking a functioning complex I (NADH dehydrogenase-CoQ reductase), NADH derived from glycolysis can only be reoxidized by lactate dehydrogenase, using pyruvate as substrate. Therefore, in the absence of supplemented pyruvate, lactate dehydrogenase may function more slowly and NADH may accumulate and partially inhibit glycolysis. In support of this hypothesis, ATP levels were restored to 85% of the value in complete medium when pyruvate was added back to the glycolysis-only medium (our unpublished results).

In "OXPHOS-only" medium containing pyruvate as the sole energy substrate (i.e., cells were forced to utilize OXPHOS to generate ATP), there was a decline in total ATP levels in all four cell lines (Figure 3). However, there was a 45% decline in the ATP content of wild-type cells compared with the value in complete medium, whereas NARP cells, which have a partial defect in mitochondrial ATP synthesis, showed a more marked (66%) reduction in total ATP. This difference between wild-type and NARP cells in OXPHOS-only medium was statistically significant ($p < 0.009$). Both MELAS cells, which have a more severe respiratory chain defect (due to compromised translation of mtDNA-encoded polypeptides; King *et al.*, 1992), and ρ^0 cells showed an almost complete loss of measurable ATP (≤ 1 nmol/mg protein).

To elicit a more severe ATP loss in NARP cells, which have a milder mitochondrial ATP synthesis defect than do MELAS cells, we used pyruvate medium containing the ATPase inhibitor oligomycin. We had previously demonstrated that low doses of oligomycin substantially decrease mitochondrial ATP synthesis in NARP, but not in wild-type, cells (Manfredi *et al.*, 2002a). As expected, the addition of oligomycin did not significantly affect ATP levels in wild-type cells (Figure 3), whereas NARP cells showed further

statistically significant decreases (53% [$p < 5 \times 10^{-6}$] and 76% [$p < 2 \times 10^{-6}$], in 5 and 10 ng/ml oligomycin, respectively) compared with wild-type cells treated with oligomycin. This showed that NARP mutant cells have reduced capability to maintain ATP levels when the OXPHOS machinery is put under pressure by limiting doses of inhibitors or, presumably, by increased energy demand.

Subcellular ATP Concentration in Wild-type and Mutant Cells

We measured subcellular ATP concentrations in cells incubated in different substrates, as described above. We first looked at cytosolic ATP in cells transfected with *Luc_c* (Figure 4A). ATP concentration in the cytosol of all cell types reflected closely the trend that we observed in total cell lysates. In particular, maximal ATP concentrations (between 91 and 110 μ M) were obtained in complete medium, and these values remained stable in glycolysis-only medium. In OXPHOS-only medium, wild-type cells lost 29% of their maximal ATP levels, whereas NARP cells showed a significantly greater loss of ATP compared with wild-type cells (62%; $p < 0.007$). MELAS and ρ^0 cells showed an almost complete loss of cytosolic ATP (i.e., < 1 μ M).

We then looked at cells transfected with *Luc_{pm}* (Figure 4B). ATP concentrations in the subplasma membrane region were similar to those of cytosolic ATP, suggesting that there was no real ATP "gradient" between these two cell compartments. These results are in agreement with those obtained using pancreatic β cells (Jouaville *et al.*, 1999) and cardiomyocytes (Dzeja *et al.*, 2002), where no difference in ATP concentration was found between the cytosol and the plasma membrane.

Next, we measured ATP concentrations in the mitochondrial matrix using the *Luc_m* construct (Figure 4C). In complete medium, wild-type cells showed a high matrix ATP content (223 μ M) that was more than double the level observed in the cytosol (Figure 4A). On the other hand, MELAS, NARP, and ρ^0 cells all had ATP levels (101–112 μ M) that were essentially identical to the respective cytosolic concentrations. This "equilibration" of ATP concentrations between the mitochondrial and cytosolic compartments in these cells was presumably the consequence of the fact that in OXPHOS-defective cells, ATP is imported from the cytosolic compartment into the mitochondrial matrix (Buchet and Godinot 1998; Loiseau *et al.*, 2002). This interpretation is consistent with the observation that in wild-type cells in glycolysis-only medium, the ATP concentration in the mitochondrial matrix (86 μ M) was reduced to a value comparable to that of cytosolic ATP (91 μ M), because in this condition all mitochondrial ATP must be imported from the cytosol.

In ρ^0 cells grown in glycolysis-only medium, mitochondrial ATP was significantly decreased (34 μ M) compared with that in wild-type cells (86 μ M; $p < 0.002$). This was unexpected, because ρ^0 cells produce most of their ATP through glycolysis. However, as discussed above for total cellular ATP, this reduction was probably due to diminished glycolytic activity resulting from the lack of exogenous pyruvate. In fact, when pyruvate was added back to the glycolysis-only medium, ρ^0 cells were able to restore their mitochondrial matrix ATP concentration to 87% of the value in complete medium (our unpublished results).

Figure 4. ATP concentration in intracellular compartments. Wild-type, NARP, MELAS, and ρ^0 cells expressing luciferases targeted to the indicated cell compartment (A–D) were incubated in medium containing the indicated combinations of substrate and inhibitors. Luminescence was measured on aliquots of 2×10^5 intact cells in the presence of 2 mM luciferin. ATP concentrations were extrapolated based on the RLU curves obtained in the ATP titration experiment shown in Figure 2C. Bars represent standard deviations of 20 replicate samples. P values that were particularly relevant to the discussion of the results are indicated.

In OXPHOS-only medium, MELAS and ρ^0 cells had virtually no detectable mitochondrial ATP, confirming that in these cells all the mitochondrial ATP is of glycolytic origin. In NARP cells, which have reduced mitochondrial ATP synthesis (Manfredi *et al.*, 2002a), ATP in the matrix (30 μ M)

was only partially decreased compared with wild-type cells (55 μ M; $p < 0.0003$).

Finally, we analyzed nuclear ATP content with Luc_n (Figure 4D). In complete medium, nuclear ATP levels were similar in all cell lines and were only moderately decreased

in MELAS and ρ^0 cells in glycolysis-only medium, presumably because of the lack of pyruvate. In glycolysis-only medium, ρ^0 cells showed a significant decrease of ATP concentration compared with complete medium ($p < 0.004$), albeit less marked than in the mitochondrial compartment. In OXPHOS-only medium there was a striking decrease in nuclear ATP. Surprisingly, wild-type cells lost $\sim 79\%$ of nuclear ATP in OXPHOS-only medium compared with the level in complete medium ($p < 2 \times 10^{-5}$). ATP was undetectable in MELAS and ρ^0 cells, and in NARP cells there was a marked ATP decrease to 11% of the value measured in complete medium ($p < 1 \times 10^{-6}$). This value was significantly lower than in wild-type cells ($p < 3 \times 10^{-6}$). As expected, the addition of low doses of oligomycin to the OXPHOS-only medium almost completely eliminated nuclear ATP in all cell lines.

DISCUSSION

We have shown that targeted luciferase can be used to assess the degree of ATP compartmentalization inside cells. The apparent K_m for ATP *in vitro* has been reported to be $63 \mu\text{M}$ (DeLuca and McElroy, 1974; Lemasters and Hackenbrock, 1977), but it was suggested previously that, when measured in an intracellular milieu, the kinetic properties of luciferase are different from those found *in vitro* (Gandelman *et al.*, 1994; Maechler *et al.*, 1998; Jouaville *et al.*, 1999; Kennedy *et al.*, 1999). In our system, the kinetic properties of the targeted luciferases were dissimilar from that of native firefly luciferase as well as from each other, suggesting that the addition of leader peptides somehow affect their biochemical properties. Therefore, ATP standard curves were generated for each construct in order to determine ATP concentrations in the cytosol, subplasma membrane region, mitochondria, and nucleus with some confidence. In principle, our results suggest that a targeted luciferase approach could also be used to measure ATP concentrations in other cell compartments. These could be compartments found ubiquitously in all cells, such as the endoplasmic reticulum and the nuclear envelope, or could be compartments specific to certain cell types, such as secretory vesicles, synaptic buttons, and the contractile apparatus of myocytes.

We have investigated here the fate of intracellular free ATP pools (or more precisely, ATP available to react in a luciferin-luciferase assay) in intact wild-type cells and in cells harboring pathogenic mtDNA mutations grown under conditions in which they used preferentially either glycolytic or oxidative metabolism for ATP synthesis. We obtained evidence that ATP distribution is not uniform throughout the cell compartments that we analyzed, suggesting that the partition of high energy phosphorylated molecules in cells does not occur simply by diffusion of ATP along concentration gradients. Clearly, each ATP pool in the cell contributes to different extents to the overall cellular ATP pool. Here, we have looked at four cell compartments and found that the changes in total cellular ATP observed in different metabolic conditions (Figure 3) were reflected in some but not in all cell compartments. For example, the cytosolic ATP pool (Figure 4A), which presumably is the largest one in the cell and contains the majority of hydrolysable ATP, was the compartment that showed a better correlation, albeit not a perfect one, with the total cellular

ATP pool. On the other hand, the nuclear pool (Figure 4D), ostensibly much smaller than the cytosolic one, showed a rather different behavior. These differences underscore the importance of developing tools such as targeted luciferases to investigate ATP concentrations within specific cellular compartments.

In healthy cells grown in “rich” conditions, when both glycolysis in the cytosol and OXPHOS in the mitochondria were active, the ATP concentration in the mitochondrial matrix was approximately double that in other cell compartments (Figure 4). In the same conditions, cells with impaired OXPHOS had apparently equilibrated their ATP concentrations among all the four cell compartments studied (including, notably, their mitochondria), suggesting that in these cells the bulk of ATP was generated by glycolysis and imported into mitochondria.

Under oxidative conditions, ATP levels were markedly decreased in all cell compartments of NARP cells, and even more so in MELAS cells. Conversely, wild-type cells were able to maintain ATP levels comparable to those measured in complete medium in the cytosol and in the subplasma membrane region, but showed reduced ATP levels in the mitochondria and, to an even greater extent, in the nucleus. These findings suggest that, even in normal cells, when mitochondrial OXPHOS is the sole source of high-energy phosphoryls, ATP is exported preferentially from the mitochondria to supply energy to other energy-intensive compartments, such as cytosol and subplasma membrane.

In oxidative conditions, particularly when ATP supplies in the cell become limited (e.g., after the addition of low doses of oligomycin), the nucleus appears to be the compartment most susceptible to ATP depletion, not only in mutant but also in wild-type cells. These findings are particularly relevant in light of those of Dzeja and colleagues (Dzeja *et al.*, 2002), who reported that the import of histone proteins into cardiomyocyte nuclei was dependent mainly on mitochondrial ATP in concert with phosphotransfer enzymes, such as adenylate kinase and creatine kinase.

The decrease in nuclear ATP content under OXPHOS-only conditions implies that depletion of nuclear ATP plays an important, and hitherto unappreciated, role in patients with oxidative energy dysfunction. In these patients, the output of ATP from mitochondria may become critically low, particularly in cells with higher metabolic demand and higher oxidative rates, such as myocytes and neurons, which are frequently the most affected by mitochondrial disorders. Loss of ATP in the nucleus of these cells may have potentially severe consequences, resulting in the impairment of crucial cellular functions such as nuclear import, DNA transcription, and replication.

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