The role of matrix substrate-level phosphorylation during anoxia

PhD thesis booklet

Gergely Kiss

Semmelweis University
János Szentágothai Doctoral School of Neurosciences
Functional Neurosciences Program

Supervisor:	Christos Chinopoulos, PhD
Official reviewers:	Ferenc Gallyas Jr, DSc
	Tamás Kardon, PhD
Chairman of the final examination board:	József Mandl, DSc
Members of the final examination board:	Erzsébet Ligeti, DSc
	Olivér Ozohanics, PhD

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“Man’s quest for knowledge is an expanding series whose limit is infinity, but philosophy seeks to attain that limit at one blow, by a short circuit providing the certainty of complete and inalterable truth. Science meanwhile advances at its gradual pace, often slowing to a crawl, and for periods it even walks in place, but eventually it reaches the various ultimate trenches dug by philosophical thought, and, quite heedless of the fact that it is not supposed to be able to cross those final barriers to the intellect, goes right on.”

Stanisław Lem: His Master’s Voice (1968)

INTRODUCTION

Impaired mitochondrial function is involved in trauma, neurodegeneration, ischemia/reperfusion or excitotoxicity. Depolarized mitochondria are thought to precipitate cell death by depleting cytosolic ATP pools. In several pathological settings, mitochondria indeed revert to ATP consumption, aggravating an existing cellular pathology. However, for as long as the inner mitochondrial membrane remains intact and ATP production is substantiated via matrix substrate-level phosphorylation by SUCL without eliciting the need of oxidative phosphorylation, mitochondrial consumption of cytosolic ATP is hindered. KGDHC provides succinyl-CoA for SUCL, thus supporting matrix substrate-level phosphorylation. Although a link has been establish between the decline in its activity and brain pathology, the molecular mechanisms underlying the propensity for neurodegeneration have not been published so far. Here it is proposed that as a result of KGDHC deficiency, the decreased provision of succinyl-CoA diminishes matrix substrate-level phosphorylation, resulting in impaired mitochondrial ATP output and consumption of cytosolic ATP by respiration-impaired mitochondria. Mindful of the reaction catalyzed by KGDHC converting α-ketoglutarate, CoASH, and NAD^+ to succinyl-CoA, NADH, and CO_2, the question arises as to the source of NAD^+, under conditions of a dysfunctional respiratory chain. It is a textbook definition that NADH generated in the citric acid cycle is oxidized by complex I, resupplying NAD^+ to the cycle. In the absence of oxygen or when respiratory complexes are not functional, an excess of NADH in the matrix is expected. Yet,

previous reports of our group demonstrated that without NADH oxidation by complex I of the respiratory chain, substrate-level phosphorylation was operational and supported by succinyl-CoA, implying KGDHC activity. Alternative pathways for NADH oxidation during anoxia or pharmacologic blockade of complex I were therefore investigated, which are able to supply NAD^+ to KGDHC, what in turn yields succinyl-CoA, thus supporting substrate-level phosphorylation.

During respiratory arrest, proton extrusion to the extra-mitochondrial compartment by the reversed F_OF_1-ATP synthase contributes to the generation of $\Delta\Psi m$ at the expense of ATP hydrolysis. It has been reported recently by another group that CYPD binds to the F_OF_1-ATP synthase, resulting in a decrease in ATP synthesis and hydrolysis rates. Genetic ablation of CYPD or its inhibition by cyclosporin A led to a disinhibition of the complex, resulting in accelerated ATP synthesis and hydrolysis rates. However, these effects were demonstrated in either submitochondrial particles or alamethicin-permeabilized mitochondria, representing conditions under which there is direct access to the F_OF_1-ATP synthase. In intact mitochondria, changes in ATP synthesis or hydrolysis rates by the F_OF_1-ATP synthase do not necessarily translate to changes in ATP efflux or influx rates as a result of the presence of the ANT. The extent of contribution of CYPD on the rates of ADP and ATP fluxes towards the extramitochondrial compartment was therefore addressed in intact mitochondria to confirm and extend the result of the recent literature.

AIMS AND OBJECTIVES

The outlined experiments were performed in order to find an answer to the following questions in particular:

1. Does the CYPD bind to and modulate the F_OF_1-ATP synthase in intact mitochondria?
2. Does the modulation of F_OF_1-ATP synthase by CYPD affect the ANT-mediated ADP-ATP exchange rates of intact mitochondria?
3. Does the decreased succinyl-CoA provision by an impaired KGDHC affect the ANT-mediated ADP-ATP exchange rates of intact mitochondria respiring on substrates supporting substrate-level phosphorylation?
4. Does the decreased succinyl-CoA provision by an impaired KGDHC diminish the matrix ATP/ADP ratio during respiratory failure enough for the ANT to reverse?
5. Does the KGDHC-SUCL axis of the citric acid cycle operate even under conditions when complex I is unable to provide NAD^+ for the KGDHC?
6. Do mitochondrial diaphorases in the matrix contribute to the alternative regeneration of NAD^+ for KGDHC during respiratory arrest?
7. What other pathways exist during respiratory failure for NADH oxidation in the mitochondrial matrix?
8. How does complex III block affect NAD^+ provision of KGDHC by diaphorase activity during respiratory arrest?

MATERIALS AND METHODS

Animals

Mitochondria were prepared either from $CYPD^{-/-}$, $DLD^{+/-}$, $DLST^{+/-}$, $DLD^{+/-}/DLST^{+/-}$ and heart-specific superoxide dismutase 2 overexpressing transgenic or WT mice (*Mus musculus*) or from pigeons (*Columba livia domestica*).

Preparation of isolated mitochondria

Nonsynaptic brain mitochondria from mice were isolated by Percoll gradient purification. Mitochondria from the hearts and livers of all animals were isolated via differential centrifugation. The mitochondrial protein content was determined using the bicinchoninic acid assay, calibrated with bovine serum albumin standards, using a Tecan Infinite® 200 PRO series plate reader.

Isolation of synaptosomes from mouse brain

Synaptosomes were prepared from mice by differential centrifugation using Percoll gradient. Protein content was determined using the bicinchoninic acid assay described above.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) of isolated mitochondria was estimated using fluorescence quenching of the cationic dye safranin O, due to its accumulation inside the negatively charged matrix of energized mitochondria. Fluorescence was recorded in either a PTI Deltascan, or a Hitachi F-4500 or F-7000 fluorescence spectrophotometer. To

convert safranin O fluorescence into millivolts, a voltage-fluorescence calibration curve was constructed.

Alternatively, safranin O fluorescence was also detected – in parallel with oxygen consumption – using the O2k-Fluorescence LED2-Module of the OROBOROS Oxygraph-2k.

Estimation of ΔΨm from tetraphenylphosphonium (TPP^+) ion distribution was measured using a custom-made TPP^+-selective electrode. The electrode was calibrated by sequential additions of TPP^+Cl^-.

ΔΨm of *in situ* mitochondria of synaptosomes was qualitatively estimated fluorimetrically by loading synaptosomes with the potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM). Fluorescence was recorded in a Hitachi F-4500 fluorescence spectrophotometer.

ΔΨm of *in situ* mitochondria of neurons was qualitatively determined by wide-field fluorescence imaging, or confocal imaging, using the fluorescence quenching of TMRM. All ΔΨm determinations of isolated, *in situ* synaptic and neuronal somal mitochondria were performed at 37°C.

$[Mg^{2+}]_{free}$ determination from Magnesium Green™ fluorescence and its conversion to ADP-ATP exchange rate

Isolated mitochondria were added to a cytosol-like incubation medium containing the Mg^{2+}-sensitive fluorescent indicator Magnesium Green™ (MgG) and magnesium. Changes in the $[Mg^{2+}]_{free}$ of the medium were followed by measuring MgG fluorescence in a PTI Deltascan fluorescence spectrophotometer at 37°C. At the end of each experiment,

minimum and maximum fluorescence were recorded and used to convert the signal to $[Mg^{2+}]_{free}$ by means of the Grynkiewicz equation. The ADP-ATP exchange rate was estimated using a method described by our group, exploiting the differential affinity of ADP and ATP to Mg^{2+}. The rate of ATP appearing in the medium after the addition of ADP to energized mitochondria (or *vice versa* in the case of de-energized mitochondria) was calculated from the measured rate of change in the extramitochondrial $[Mg^{2+}]_{free}$ using standard binding equations, combined in an executable file available for download at *http://www.tinyurl.com/ANT-calculator*.

Mitochondrial respiration

Oxygen consumption was monitored polarographically using an OROBOROS Oxygraph-2k. Experiments were performed at 37°C. Oxygen concentration and flux were recorded and calculated using the DatLab software.

Determination of extramitochondrial pH changes

pH of the incubation medium of isolated mouse liver mitochondria was recorded by connecting a combination pH microelectrode to a BNC connector of the OROBOROS Oxygraph-2k. The medium had only minimal buffering capacity in order to allow the detection of the minor pH changes caused by mitochondrial CO_2 production. The voltage signal output of the electrode was converted to pH by calibrating with MeterLab® certified standard solutions. Experiments were performed at 37°C; therefore temperature compensation was applied during voltage-pH calibration according to data provided by the manufacturer.

Mitochondrial matrix pH (pH_i) determination

The pH_i of isolated liver mitochondria from WT and CYPD KO mice was estimated fluorimetrically by loading mitochondria with the ratiometric fluorescent dye BCECF-AM. Fluorescence of hydrolyzed BCECF trapped in the matrix was measured in a Hitachi F-4500 fluorescence spectrophotometer in a ratiometric mode, at 37°C. The BCECF signal was calibrated using buffers of known pH values, and by equilibrating matrix pH to that of the incubation medium by adding uncoupler plus nigericin. For converting BCECF fluorescence ratio to pH, an exponential function was fitted.

Determination of NADH fluorescence

Autofluorescence of reduced mitochondrial pyridine nucleotides was recorded in a Hitachi F-7000 fluorescence spectrophotometer at 37°C.

Determination of KGDHC activity

KGDHC activity was estimated fluorimetrically, detecting the autofluorescence of appearing NADH in the medium, due to NAD^+ reduction by the complex at 30°C. The experiment was performed in a Hitachi F-4500 fluorescence spectrophotometer.

Determination of SUCL activity

ATP- and GTP-forming SUCL activities were estimated in the direction of succinyl-CoA to succinate reaction. The release of CoASH from succinyl-CoA was monitored spectrophotometrically on its reaction with DTNB at 30°C in a GBC UV/VIS 920 spectrophotometer.

Cross-linking, co-precipitation and western blotting

To demonstrate the interaction between CYPD and F_OF_1-ATP synthase, intact mitochondria were incubated with the membrane-permeable cross-linker DSP in the absence or presence of cyclosporin A. Mitochondrial proteins were extracted with digitonin, immunoprecipitated with anti-complex V sera, separated by SDS-PAGE, transferred to a methanol-activated polyvinylidene difluoride membrane and finally, immunocaptured proteins were tested for the presence of CYPD using the β-subunit of the F_OF_1-ATP synthase as loading control.

Immunoreactivities of the SUCL subunits were demonstrated by using frozen mitochondrial and synaptosomal pellets, thawed on ice and separated by SDS-PAGE. Separated proteins were transferred to a methanol-activated polyvinylidene difluoride membrane. Immunoblotting was performed as recommended by the manufacturers of the antibodies. Immunoreactivity was detected in all cases by using the appropriate peroxidase-linked secondary antibodies and enhanced chemiluminescence detection reagent.

RESULTS

Modulation of F_O-F_1 ATP synthase activity by CYPD regulates matrix adenine nucleotide levels

ANT-mediated ADP-ATP exchange rates were measured in intact isolated WT and CYPD KO mouse liver mitochondria, both in the presence and absence of cyclosporin A and were compared with those obtained by direct ATP hydrolysis rates by the F_OF_1-ATP synthase in alamethicin-permeabilized mitochondria. There was no difference in the ATP efflux-$\Delta\Psi m$ profile of the WT compared to CYPD KO mice, whereas ANT was operating in the forward mode. Similarly, when mitochondria were completely depolarized, no statistically significant difference was observed between mitochondria isolated either from WT or CYPD KO mice during ATP influx, irrespective of the presence of cyclosporin A in the medium. However, if mitochondria were subsequently permeabilized by alamethicin, mitochondria isolated from CYPD KO mice exhibited a 30.9 ± 1.3% faster ATP hydrolysis rate compared to WT littermates.

The CYPD-F_OF_1-ATP synthase interaction was demonstrated in intact isolated mitochondria using the membrane-permeable cross-linker DSP, followed by co-precipitation using an antibody for F_OF_1-ATP synthase as bait. Cyclosporin A was found to diminish the binding of CYPD on the F_OF_1-ATP synthase.

Mathematical predictions afforded the assumption that a 30% increase in F_OF_1-ATP synthase activity would lead to an insignificant increase (1.38–1.7%) in the ANT-mediated ADP-

ATP exchange rate in maximally polarized (forward mode of both ANT and F_OF_1-ATP synthase) and maximally depolarized (reverse mode of both ANT and F_OF_1-ATP synthase) mitochondria.

Intact mouse liver mitochondria were de-energized by substrate deprivation in the presence of the specific complex I inhibitor rotenone, followed by the addition of ATP. $\Delta\Psi m$ was recorded in order to compare WT ± cyclosporin A *vs.* CYPD KO mice. Under these conditions, and as a result of the sufficiently low $\Delta\Psi m$ values before the addition of ATP, ANT and F_OF_1-ATP synthase operated in reverse mode. Provision of ATP led to its influx to mitochondria, followed by its hydrolysis by the reversed F_OF_1-ATP synthase, which in turn extruded protons to the extramitochondrial compartment, thus generating $\Delta\Psi m$. In this setting, the ability of the F_OF_1-ATP synthase to pump protons out of the matrix represented the only component opposing the action of an uncoupler. Mitochondria isolated from the livers of CYPD KO mice resisted the uncoupler-induced stepwise depolarization more than those obtained from WT littermates. Cyclosporin A also exhibited a similar effect on WT mitochondria but not on KO mice.

The negative impact of KGDHC deficiency on matrix substrate-level phosphorylation

To label a respiration-impaired mitochondrion as an extramitochondrial ATP provider or consumer, the effect of ANT inhibitors on $\Delta\Psi m$ during ADP-induced respiration was examined. Adenine nucleotide exchange through the ANT is electrogenic, since 1 molecule of ATP^{4-} is exchanged for 1 molecule of ADP^{3-}. Therefore, during the forward mode of

ANT, abolition of its operation by an ANT inhibitor, such as carboxyatractyloside (cATR) or bongkrekate, will lead to a gain of $\Delta\Psi m$, whereas during the reverse mode of ANT, abolition of its operation by the inhibitor will lead to a loss of $\Delta\Psi m$. This *biosensor test* (*i.e.*, the effect of cATR on isolated, or bongkrekate on *in situ* mitochondria obtained from WT, $DLD^{+/-}$, $DLST^{+/-}$ and $DLD^{+/-}/DLST^{+/-}$ mice) was used to address the directionality of ANT during respiratory inhibition.

If respiratory substrates added to the medium supported matrix substrate-level phosphorylation, addition of cATR to respiration-impaired isolated liver or brain mitochondria obtained from WT mice resulted in the gain of $\Delta\Psi m$, indicating that the ANT was operating in forward mode. On the other hand, addition of cATR to respiration-impaired isolated liver or brain mitochondria obtained from $DLD^{+/-}$, $DLST^{+/-}$ and $DLD^{+/-}/DLST^{+/-}$ mice resulted in the loss of $\Delta\Psi m$, implying that the ANT was operating in reverse mode despite the presence of substrate-level phosphorylation supporting substrates.

ATP efflux rates of isolated liver and Percoll-purified brain mitochondria obtained from WT, $DLD^{+/-}$, $DLST^{+/-}$ and $DLD^{+/-}/DLST^{+/-}$ mice were determined and compared. In KGDHC-deficient mitochondria, those substrates supporting substrate-level phosphorylation yielded lower ATP efflux rates than WT littermates; but for those substrates that support only weakly or not at all substrate-level phosphorylation, no statistically significant difference was found in the ATP efflux rates.

Isolated nerve terminals (synaptosomes) and cultured cortical neurons were prepared from the brains of WT, $DLD^{+/-}$, $DLST^{+/-}$ and $DLD^{+/-}/DLST^{+/-}$ mice. Also, bongkrekate was used to

inhibit ANT instead of cATR, as the former can penetrate the plasma membrane, but the latter cannot. The *biosensor test* was applied to identify whether partially polarized *in situ* synaptic mitochondria – where substrate is an uncontrolled variable – consume extramitochondrial ATP. Addition of bongkrekate led to a robust repolarization in WT, $DLD^{+/-}$ and $DLST^{+/-}$ mice, implying that the ANT was still operating in the forward mode. Only in synaptosomes prepared from $DLD^{+/-}$/$DLST^{+/-}$ mice did bongkrekate cause almost no repolarization, followed by a delayed minor depolarization.

Results obtained from cultured cortical neurons from WT, $DLD^{+/-}$, and $DLST^{+/-}$ mice show that when *in situ* mitochondria were inhibited by rotenone, subsequent addition of bongkrekate led to a repolarization in WT cultures, unlike in cultures obtained from $DLD^{+/-}$ and $DLST^{+/-}$ mice where a depolarization was observed. This implied that respiration-impaired *in situ* neuronal somal mitochondria of $DLD^{+/-}$ and $DLST^{+/-}$ mice were consuming extramitochondrial ATP.

Alterations in KGDHC due to genetic manipulations could have an effect also on SUCL. Therefore, KGDHC activity and ATP- and GTP-forming activities of the two SUCL isoforms were measured in isolated liver and brain mitochondria and synaptosomes obtained from WT and transgenic mice, as well as immunoreactivities of all three subunits of SUCL. KGDHC activity of $DLD^{+/-}$ and $DLST^{+/-}$ liver and brain mitochondria was reduced by 20-48%, compared to WT littermates. KGDHC activity of $DLD^{+/-}$/$DLST^{+/-}$ liver and brain mitochondria was reduced by 62 and 50%, respectively. On the contrary, ATP-

and GTP-forming maximal activities of the two SUCL isoforms were not different between WT and transgenic mice.

Mitochondrial diaphorases as NAD^+ donors to segments of the citric acid cycle that support substrate-level phosphorylation yielding ATP during respiratory arrest

Using the recently developed O2k-Fluorescence LED2-Module of the OROBOROS Oxygraph-2k, it became possible to follow $\Delta\Psi m$ while measuring oxygen concentration in the same sample. Therefore, fundamental experiments establishing the basis of the previous chapter were repeated, hence at the time they were performed such technology was not available. The conclusion was drawn that in true anoxic conditions the ANT could be maintained in forward mode implying active matrix substrate-level phosphorylation in isolated mitochondria, similar to the paradigms with a poisoned respiratory chain.

The effect of arsenite – which inhibits PDHC and KGDHC – was investigated on the rate of acidification in weakly buffered media. The concept of this experiment relied on the fact that mitochondria are net CO_2 producers acidifying the medium as dissolving CO_2 becomes carbonic acid. Depending on the substrate(s) combined with targeted inhibition of bioenergetic entities, the role of arsenite-inhibitable targets may be deduced. With substrate combinations bypassing PDHC, arsenite caused a statistically significant decrease in acidification in mitochondria pretreated with rotenone, cATR and oligomycin. The assumption was made that in mitochondria in which complex I is blocked by rotenone, the ANT and the F_OF_1-ATP synthase are blocked by cATR and oligomycin, respectively, the arsenite-inhibitable acidification may only stem from

KGDHC generating CO_2. The CO_2 production by KGDHC in respiration-impaired mitochondria suggested the availability of NAD^+. Mindful of the above results, NAD^+ sources in mitochondria other than that produced by complex I were sought for.

To address the contribution of mitochondrial diaphorases to provision of NAD^+ for KGDHC reaction in anoxia, 4 pharmacological inhibitors were tested. First, the concentration range was established in which the compounds exhibited no appreciable uncoupling activity. The next step was to determine their effects in the *biosensor test* addressing the direction of ANT operation by recording the effect of cATR on $\Delta\Psi m$ in respiration-impaired isolated liver mitochondria. In the presence of diaphorase inhibitors, cATR induced depolarization without affecting the rate of respiration, implying ANT reversal. Likewise, in the presence of diaphorase inhibitors rotenone-treated mitochondria responded with depolarization to cATR, as compared to control or their vehicles showing cATR-induced repolarizations.

The *biosensor test* was performed in the presence of known diaphorase substrates in mitochondria undergoing respiratory inhibition by anoxia or rotenone. 14 diaphorase substrates were tested in concentrations exhibiting no uncoupling or other side-effects on $\Delta\Psi m$ or respiration rate. Mouse liver mitochondria were respiring on glutamate plus malate plus β-hydroxybutyrate, a substrate combination that limited the availability of NAD^+ during anoxic conditions. In this paradigm, dose-dependent addition of duroquinone during anoxia led to cATR-induced repolarization. When respiratory

inhibition was achieved by rotenone instead of anoxia, not only duroquinone, but menadione and mitoQ also resulted in a strong cATR-induced repolarization. There appeared to be a clear distinction between anoxia and rotenone-induced respiratory inhibition; in anoxia, menadione and mitoQ were not effective in conferring cATR-induced repolarization. The reason(s) for this were investigated further.

To address the above discrepancy, mitochondrial respiration was inhibited with the specific complex III inhibitor stigmatellin. The rationale behind this relies on reports pointing to complex *b* of complex III as being capable of re-oxidizing substrates that are reduced by mitochondrial diaphorases. Stigmatellin was indeed negating the beneficial effect of diaphorase substrates assisting in cATR-induced repolarization.

The diaphorase activity described for rodent and human tissues has been reported to be absent in the liver and breast muscle of pigeons. Isolated pigeon liver mitochondria were repolarized by cATR added after ADP and rotenone. The lack of diaphorase involvement in this effect was confirmed by the results that menadione failed to cause a more robust cATR-induced repolarization, and none of the diaphorase inhibitors caused cATR-induced depolarization. Accordingly, duroquinone was without an effect on cATR-induced changes in $\Delta\Psi m$ of mitochondria during anoxia. These results support the conclusion that the effects of diaphorase substrates and inhibitors observed in mouse liver mitochondria were likely mediated through genuine diaphorase activity. Furthermore, it is apparent that in the absence of a diaphorase, pigeon liver

mitochondria were still able to maintain the KGDHC-SUCL axis sustaining substrate-level phosphorylation.

Above data corroborate that mitochondrial diaphorases are not the sole providers for NAD^+ during respiratory arrest. Therefore, alternative sources for NAD^+ were considered. Heart mitochondria obtained from WT *vs.* heart-specific superoxide dismutase 2 overexpressing mice were compared by the *biosensor test*. These mice exhibit very low levels of reactive oxygen species emanation in cardiac mitochondria, in which cATR-induced a much smaller repolarization or even a depolarization, compared to WT littermates. From these results one can conclude that in the presence of rotenone, reactive oxygen species may assist in the regeneration of NAD^+ in the mitochondrial matrix.

It is possible that mitochondrial IDH isoforms may regenerate NAD^+ during respiratory arrest: IDH2 converts α-ketoglutarate plus NAD(P)H to isocitrate plus $NAD(P)^+$ and then IDH3 oxidatively decarboxylates isocitrate plus $NAD(P)^+$ to α-ketoglutarate plus NAD(P)H. To address the extent of contribution of this IDH-mediated futility to mitochondrial NAD^+ provision, it was attempted to shift the reaction towards NADH formation by manipulating the concentration of its substrates. It was reasoned that the presence of citrate in excess would negate the beneficial effect of other substrates supporting substrate-level phosphorylation in terms of cATR-induced changes in $\Delta\Psi m$ during respiratory arrest. Indeed, addition of citrate to glutamate or α-ketoglutarate reverted the cATR-induced repolarization to a depolarization.

CONCLUSION

The above results indicate that in case of submaximal uncoupling or impaired mitochondrial respiration, F_OF_1-ATP synthase may operate in reverse mode without the concomitant reversal of the ANT. Under these conditions matrix substrate-level phosphorylation plays a critical role in supplying ATP for the reversed F_OF_1-ATP synthase, setting $\Delta\Psi m$ at a level just enough to prevent ANT reversal. The resultant prevention of cytosolic ATP consumption by dysfunctional mitochondria increases the survival chances of the harboring cell.

ATP production by SUCL is not interrupted, moreover, it is activated during hypoxia. Considering the reasoning above, its adequate succinyl-CoA provision through KGDHC thus dissociates mitochondria with a dysfunctional respiratory chain from extramitochondrial ATP dependence. Relevant to this, in anoxia there is pronounced conversion of α-ketoglutarate to succinate, implying KGDHC operability. Therefore, alternative pathways providing NAD^+ for KGDHC during impaired respiration have to exist, as NADH oxidation via complex I is impracticable. The final conclusion is that among other sources, mitochondrial diaphorases are the major contributors.

CYPD binds to the F_OF_1-ATP synthase decreasing both ATP synthesis and hydrolysis rates. However, as a result of the imposing role of the ANT, this is not reflected in the extramitochondrial compartment. The modulation of F_OF_1-ATP synthase activity by CYPD rather represents a means of regulating the matrix ATP/ADP ratio, influencing both the ANT and the F_OF_1-ATP synthase.

ABBREVIATIONS

ΔΨm:	mitochondrial membrane potential
ADP:	adenosine 5'-(trihydrogen diphosphate)
ANT:	adenine nucleotide translocase
ATP:	adenosine 5'-(tetrahydrogen triphosphate)
BCECF-AM:	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
cATR:	carboxyatractyloside
CO_2:	carbon dioxide
CoASH:	coenzyme A
CYPD:	cyclophilin D
DLD:	dihydrolipoyl dehydrogenase
DLST:	dihydrolipoyl succinyltransferase
DSP:	3,3'-dithiobis(sulfosuccinimidyl propionate)
DTNB:	5,5'-dithiobis(2-nitrobenzoic acid)
GTP:	guanosine 5'-(tetrahydrogen triphosphate)
IDH(2 or 3):	isocitrate dehydrogenase, isoform 2 or 3
KGDH(C):	α-ketoglutarate dehydrogenase (complex)
MgG:	Magnesium Green™, pentapotassium salt
$NAD(P)^+$:	nicotinamide adenine dinucleotide (phosphate)
PDH(C):	pyruvate dehydrogenase (complex)
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SUCL:	succinate-CoA ligase
TMRM:	tetramethylrhodamine methyl ester
TPP:	tetraphenylphosphonium
WT or KO:	wild-type or knock-out

LIST OF PUBLICATIONS

Publications related to the thesis

1. Chinopoulos C, Gerencsér AÁ, Mándi M, Máthé K, Törőcsik B, Dóczi J, Turiák L, Kiss G, Konrád C, Vajda S, Vereczki V, Oh RJ, Ádám-Vizi V. (2010) Forward operation of adenine nucleotide translocase during F_0F_1-ATPase reversal: critical role of matrix substrate-level phosphorylation. FASEB J, 24: 2405-2416.

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2. Chinopoulos C, Konrád C, Kiss G, Metelkin E, Törőcsik B, Zhang SF, Starkov AA. (2011) Modulation of F_0F_1-ATP synthase activity by cyclophilin D regulates matrix adenine nucleotide levels. FEBS J, 278: 1112-1125.

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3. Kiss G, Konrád C, Dóczi J, Starkov AA, Kawamata H, Manfredi G, Zhang SF, Gibson GE, Beal MF, Ádám-Vizi V, Chinopoulos C. (2013) The negative impact of alpha-ketoglutarate dehydrogenase complex deficiency on matrix substrate-level phosphorylation. FASEB J, 27: 2392-2406.

I.F.: 5.704 (2012)

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9. Chinopoulos C, Kiss G, Kawamata H, Starkov AA. Measurement of ADP-ATP exchange in relation to mitochondrial transmembrane potential and oxygen consumption. In: Galluzzi L, Kroemer G. (ed.), Methods in Enzymology Vol. 542. Conceptual Background and Bioenergetic/Mitochondrial Aspects of Oncometabolism. Academic Press, Burlingon, 2014: 333-348. **I.F.: 2.002 (2012)**