MITOCHONDRIAL SUBSTRATE-LEVEL PHOSPHORYLATION: FROM METABOLIC INTERFERENCE TO SMALL MOLECULE INTERACTIONS

Thesis booklet

Dávid Bui

Semmelweis University Doctoral School
János Szentágothai Neurosciences Division

Supervisor:
Dr. Chinopoulos Christos, MD, D.Sc

Official reviewers:
Dr. Gallyas Ferenc, D.Sc
Dr. Kardon Tamás, MD, Ph.D

Head of the Complex Examination Committee:
Dr. Rónai Zsolt, MD, D.Sc

Members of the Complex Examination Committee:
Dr. Sarnyai Farkas, Ph.D
Dr. Gallyas Ferenc, D.Sc

Budapest
2025

1. Introduction

In eukaryotic cell, mitochondria are compartmentalized hubs of primary metabolism. Carbohydrate, amino acid, isoprene and fatty acid metabolic pathways both end and start from them. Mitochondria's ability to perform their role in the cell is tightly intertwined with cellular respiration owing to the mitochondrial electron transport chain. Consequently, oxygen deprivation has a direct and immediate effect on mitochondrial processes. Hypoxic conditions may be found in solid tumours far from sufficient vasculature, in various pathological ischemic conditions or even in physiological niches. Tumour hypoxia has been negatively correlated to patient prognosis and treatment responsiveness. Many cancers acquire metabolic adaptations to hypoxic environments, which they retain even with adequate oxygen supply as a selective advantage. Under hypoxic conditions

mitochondria must maintain a delicate balance between ATP availability and membrane potential. In severe hypoxia, mitochondria cannot maintain their membrane potential using the energy of electron transfer reactions. ATP synthase expends the energy available in ATP to act as a hydrogen ion pump while ANT facilitates the transport of ATP into mitochondria. The energetic dependence on cytosolic ATP can be prevented if another substrate-level process steps up and provides the membrane potential and/or the ATP in the mitochondrial matrix. The mitochondrial substrate-level phosphorylation by succinate-CoA ligase (STK) is the primary candidate for this role. STK can use the chemical energy in the thioester bond to phosphorylate ADP into ATP. Glutamine is by far the most widely available amino acid for cells and the hydrolytic cleavage of the amide group provides glutamate without additional reductive burden. In transformed cells, enhanced glutaminolysis has been

one of the characteristic metabolic phenotypes. Glutamate is converted to 2-oxoglutarate either by transamination or by glutamate dehydrogenase. The 2-oxoglutarate with the use of NAD and CoA can then be oxidised further by KGDHC to produce the thioester bond carrying succinyl-CoA. Proline, histidine and ornithine could also contribute, but their metabolism takes several additional steps and in the case of proline and ornithine, an additional oxidation step by delta-1-pyrroline-5-carboxylate dehydrogenase is also required. A major hindrance for KGDHC during hypoxia comes from the availability of NAD. This reducing equivalent carrier coenzyme relies on regeneration by complex I (NADH:ubiquinone oxidoreductase) and the electron transport chain under normal conditions. However, in hypoxia this is no longer possible, and anything that affects the NAD/NADH ratio can indirectly suppress or promote mitochondrial substrate-level phosphorylation.

2. Objectives

1. 2-oxobutyrate is another oxocarboxylic acid that can be converted to mitochondrial succinyl-CoA. Overall, the pathway to succinyl-CoA from 2-oxobutyrate is energetically very similar to that from 2-oxoglutarate, except for an additional ATP consuming carboxylation step. We sought to investigate the interaction between the two pathways when mitochondria are dependent on the ATP made by substrate-level phosphorylation.

2. We searched for various possibilities to overcome the NAD limitation of KDGHC under hypoxic conditions and thereby support substrate-level phosphorylation. We tested whether complex I could continue its function as the NAD regenerator by channelling electrons through ubiquinone to succinate dehydrogenase (SDH) and ultimately reduce fumarate to succinate.

We also investigated whether NADH oxidation by malate dehydrogenase (MDH2) alone, with an inhibited SDH would also be sufficient to support substrate-level phosphorylation.

3. We also addressed the possibility that the membrane potential indicator dye safranin could also act as an artificial electron acceptor. To our knowledge, nothing has been published so far regarding the electron transfer interactions between safranin and mitochondria.

4. We sought to express and purify recombinant human STK for the purpose of starting a systematic search for a new inhibitor. A 10 million compound DNA-encoded library was used to identify promising molecular candidates that show high affinity binding.

3. Methods

Mouse mitochondria preparation

Mice were from a mix of 129 Sv and C57Bl/6N strains. Liver mitochondria were isolated by standard differential centrifugation after tissue homogenisation. Non-synaptic brain mitochondria were isolated similarly, with an additional discontinuous Percoll gradient step. Protein concentrations were measured with Pierce™ bicinchoninic acid protein assay.

Fluorescence and oxygen concentration measurement

Mitochondrial membrane potential was estimated fluorimetrically with safranin or rhodamine 123. In select experiments, oxygen concentration was monitored simultaneously in the enclosed volume amperometrically using a Clark electrode.

Electrochemical measurements

Voltammetric measurements were made with a glassy carbon working, platinum auxiliary and Ag/AgCl secondary reference electrode.

Sample preparation for mass spectroscopic metabolite measurement

Initial respiratory substrates were light glutamate and light malate. L-norleucine was added as internal standard. Just before anoxia, glutamate-$^{13}C_5$ was added. After various waiting times further metabolism was terminated by addition of an inhibitor cocktail. Mitochondria were centrifuged, the supernatant removed and the pellet was extracted with methanol and chloroform. The metabolite concentrations were measured with GC-MS after derivatization.

Hydrogen peroxide measurement

Solutions of safranin and NADH were exposed to light irradiation at incremental wavelengths. A sample aliquot was acidified to degrade excess NADH. Following neutralization, Amplex UltraRed and horseradish peroxidase was added to convert

hydrogen peroxide into Amplex UltroxRed signal and its absorbance was measured.

Recombinant STK expression

STK was expressed in Escherichia coli and purified with a sequence of metal affinity, ion-exchange and streptavidin affinity chromatography. The purified enzyme concentration was estimated with UV absorbance

STK activity assay

Thioester bond formation was followed with UV spectrophotometry in a cuvette with the soluble enzyme.

Alternatively, excess CoA was measured with 5,5′-dithiobis-(2-nitrobenzoic acid) with immobilized enzyme.

Polyacrylamide gel electrophoresis

Standard Laemmli 10 % denaturing and 6 % native PAGE was used with Tris-glycine running solution.

DEL screening and sequencing

The enzyme was immobilized on magnetic metal affinity beads. The library was incubated separately with empty control beads and enzyme loaded beads. The bound enzyme-compound complexes were eluted. The DNA labels were amplified and elongated with polymerase chain reaction and the final DNA mixture was sequenced.

Partial cytochrome c oxidase purification from mitochondria

Frozen mitochondria was diluted and solubilized with deoxycholate. Proteins were differentially precipitated with $(NH_4)_2SO_4$. The resulting green pellet was dissolved and stored frozen.

4. Results

We confirmed 2-oxobutyrate's ability to enter mitochondria. This is evidenced by the polarisation and the minor increase in respiration of isolated liver

mitochondria upon addition. The combination of glutamate and malate provides a strong substrate-level phosphorylation under pseudohypoxic conditions. When 2-oxobutyrate was added, we saw a concentration dependent shift in ANT directionality. This was additive with the effect of 3-hydroxybutyrate.

When SDH is inhibited in anoxia glutamate and malate cannot prevent ANT reversal. In comparison, aspartate and 2-oxoglutarate demonstrated support for mitochondrial substrate-level phosphorylation under the same condition. This demonstrates the ability of MDH2 to regenerate NAD for KGDHC.

By using isotope tracing we measured how the amount and the isotope composition of metabolites change over time in glutamate-malate-utilizing mitochondria under anoxia. Mass balance showed an increasing malate-derived fumarate reduction and a moderate glutamate-derived 2-oxoglutarate oxidation. Overall, this shows that reverse SDH

supports residual complex I activity, which in turn supports substrate-level phosphorylation from 2-oxoglutarate. We calculated SDH turnover to be approximately 2.78 times more than KGDHC, which is enough to reach the electron neutral stoichiometry of 2 (1 electron pair for KGDHC; 1 electron pair for glutamate dehydrogenase).

We demonstrated the reduction of safranin by mitochondria using various respiratory substrates. In anoxia this could be seen by either fluorescence decrease or increasing electrochemical current. Under aerobic conditions, reduced safranin rapidly oxidises and produces hydrogen peroxide from oxygen. Reduced safranin could also be oxidised by SDH up to a limited capacity in the presence of fumarate or malate. Safranin could be reduced in the absence of mitochondria with NADH by light irradiation in the suitable wavelength region. The required wavelength coincided with the absorption

spectrum of safranin. In intact mitochondria this mode of reduction was minimal.

We expressed the recombinant ATP-specific human STK in Escherichia coli. We purified the enzyme using a series of 3 chromatography steps to an approximate 98% purity. The short-term storage of the enzyme was determined to be best at 4°C in a pH 7.2 Tris buffer without or with very low amounts of glycerol. Long-term storage was best done by freezing in a pH 7.6 Tris buffer with higher glycerol content. Following the binding assay, PCR and DNA sequencing; we identified the 50 compounds with the greatest binding affinity. Of these compounds the 5, which showed high outlying enrichment were labelled as potential lead compounds.

5. Conclusions

In contrast to 2-oxoglutarate, the conversion of 2-oxobutyrate to succinate does not result in net ATP yield. The metabolism of 2-oxobutyrate competes with 2-oxoglutarate in isolated mitochondria and reduces ATP production under pseudoanoxic conditions, promoting ANT reversal.

Substrate-level electron acceptors are crucial for NAD regeneration to support continued operation of KGDHC under anoxia and consequently for ATP generation to prevent ANT reversal. Fumarate reduction by SDH supports complex I's residual NADH oxidising activity. Similarly, oxaloacetate reduction by MDH2 can directly regenerate NAD. Both of these options are sufficient on their own to promote substrate-level phosphorylation under anoxic conditions.

Safranin can be reduced under two separate conditions. One, continuous light excitation in

aqueous solution with NADH forms reduced safranin irrespective of mitochondria. Under aerobic conditions, reduced safranin then rapidly oxidises. This occurs only to a small extent with intact mitochondria. Two, independent from the light-induced reaction, mitochondria reduce safranin in the absence of oxygen. Since the reaction is extremely slow, using low concentrations (5 μM) is unlikely to qualitatively alter experimental results under most conditions. For substrate-level phosphorylation, it was insufficient to prevent ANT reversal in anoxia.

We expressed, purified and optimised the storage conditions for recombinant ATP-specific human STK. Using the recombinant enzyme, we screened a 10 million compound DNA-encoded library to identify the ligands with the highest affinity that will serve as leads for future drug development.

6. Bibliography of the candidate's publications

Related publications:

1. Ravasz D, Bui D, Nazarian S, Pallag G, Karnok N, Roberts J, et al. Residual Complex I activity and amphidirectional Complex II operation support glutamate catabolism through mtSLP in anoxia. Sci Rep. 2024;14(1):1729.

2. Bui D, Ravasz D, Chinopoulos C. The Effect of 2-Ketobutyrate on Mitochondrial Substrate-Level Phosphorylation. Neurochem Res. 2019;44(10):2301-2306.

Unrelated publications:

1. Doczi J, Karnok N, Bui D, Azarov V, Pallag G, Nazarian S, et al. Viability of HepG2 and MCF-7 cells is not correlated with mitochondrial bioenergetics. Sci Rep. 2023;13(1):10822.

2. Pallag G, Nazarian S, Ravasz D, Bui D, Komlodi T, Doerrier C, et al. Proline Oxidation Supports Mitochondrial ATP Production When Complex I Is Inhibited. Int J Mol Sci. 2022;23(9).
3. Szabo E, Wilk P, Nagy B, Zambo Z, Bui D, Weichsel A, et al. Underlying molecular alterations in human dihydrolipoamide dehydrogenase deficiency revealed by structural analyses of disease-causing enzyme variants. Hum Mol Genet. 2019;28(20):3339-3354.