Aug 29, 2019

Spectrophotometric assay of the mitochondrial F1F0 ATP synthase

PLOS One

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1 Works for me dx.doi.org/10.17504/protocols.io.5gng3ve

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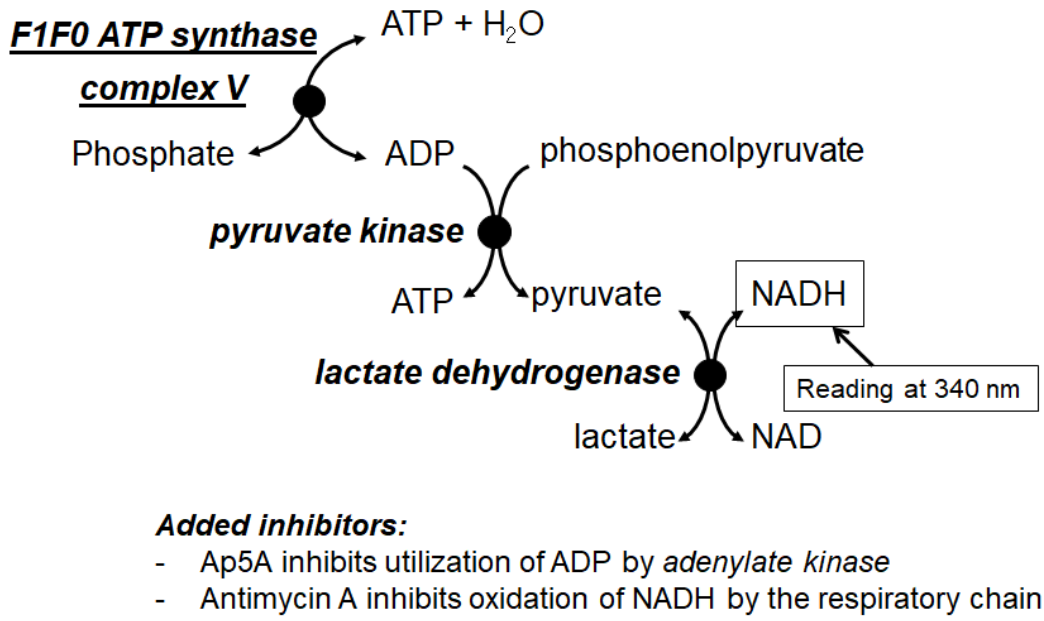
ABSTRACT

F_1F_0 ATP synthase, complex V of the oxidative phosphorylation pathway, is the enzyme that phosphorylates ADP into ATP using the electrochemical energy of the proton gradient generated by the respiratory chain. It produces the vast majority of cellular ATP.
We describe an improved spectrophotometric assay for its activity, measured in its reverse reaction i.e. ATP hydrolysis. Using this procol in four different murine organs: brain, heart, muscle and liver, we obtained organ-dependent, reproducible and stable complex V specific activities, similar with fresh and frozen organs. Similar inhibition by oligomycin and exogenous IF1 demonstrated tight coupling between F_1 and F_0 domains.
The activity was stable for several hours. It was linear with protein amount.

A schematic representation of the assay is provided below.



EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0221886

MATERIALS TEXT

reagent name	vendor	catalog#	CAS#
manitol	Sigma	M4125	69-65-8

sucrose	Sigma	S7903	57-50-1
Tris	Sigma	T1503	77-86-1
EDTA	Sigma	3690	60-00-4
Complete antiprotease cocktail	Roche	1836153	
fatty acid free bovine serumalbumin	Sigma	A6003	9048-46-8
KCl	Sigma	P3911	7447-40-7
EGTA	Sigma	E3889	67-42-5
MgCl2	Sigma	M1028	7786-30-3
NADH	Sigma	N8129	606-68-8
antimycin	Sigma	A8674	1397-94-0
phosphoenolpyruvate (PEP)	Sigma	P7127	4265-07-0
lacticodehydrogenase (LDH)	Roche	1,0127E+10	
pyruvate kinase (PK)	Sigma	P7768	
dodecylmaltoside (DDM)	Sigma	D4641	69227-93-6
P1,P5-Di(adenosine-5')pentaphosphate (Ap5A)	Sigma	D4022	4097-04-5
ATP	Sigma	A8937	34369-07-8
oligomycin	Sigma	O4876	1404-19-9
IF1	made by peptide synthesis company		

Inhibitory factor 1 (IF1) corresponds to a partial sequence of yeast IF1 (SEGSTGTPRGSGSEDSFVKRERATEDFFVRQREKEQLRHLKEQLEKQRKKID) where the underlined phenylalanine residue (F28) was replaced by a tryptophan to increase the peptide absorbance at 280 nm and facilitate its purification. This mutation did not alter its inhibitory properties (PubMed PMID: 20951672). Alternatively, one can also use a synthetic commercial peptide.

SAFETY WARNINGS

Antimycin and oligomycin are poisons and should therefore be handled with gloves

BEFORE STARTING

Before starting, cool the centrifuge at 4 degrees centigrade and prepare a box with crushed ice to cool all microtubes, tissue grinders, and homogenization buffer.

1 **Reagents preparation**

1- Homogenization buffer (kept at 0-4°C for few weeks or at -20°C for longer period of time)= 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 10 mM Tris HCl pH 7.2

Anti-protease cocktail at the working concentration given by the manufacturer should be added before use

2- Assay buffer (kept at 0-4°C for few weeks or at -20°C for longer period of time) = 250 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml BSA, 50 mM Tris pH 8.25

3- Stock solutions of reagents (kept frozen at -20°C)

0.4 M NADH (diluted in water)
1 mM antimycin A (diluted in dimethylsulfoxide)
250 mM phosphoenopyruvate (diluted in water)
10% w/w dodecylmaltoside (diluted in water)
3 mM P1,P5-Di(adenosine-5')pentaphosphate (AP5A) (diluted in water)
50 mg/mL fatty acid free bovine serumalbumine (diluted in water)
100 mM ATP (diluted in water, adjust pH to 7.5)
1 mM oligomycin (diluted in dimethylsulfoxide)
1 mM IF1

4- Stock solution of enzymes lacticodehydrogenase and pyruvate kinase (kept at 0-4°C)

2 **Preparing assay medium**

Prepare as many mL as samples to analyze

In assay buffer, add

0.4 mM NADH (1 µL stock solution per mL of assay buffer)
1 µM antimycin A (1 µL stock solution per mL of assay buffer)
1 mM PEP (4 µL stock solution per mL of assay buffer)
10 units LDH (volume depending on the batch)
25 units PK (volume depending on the batch)
0.01% w/w DDM (1 µL stock solution per mL of assay buffer)
3 µM AP5A (1 µL stock solution per mL of assay buffer)

Make sure that the spectrophotometer response is linear for at least 2.5 units. Otherwise, use NADH concentration of 0.25 mM instead of 0.4 mM

3 **Samples preparation**:

1- Fragments of muscle, liver, heart or brain have to be kept at -80°C until their utilization for the assay

2- Use Potter Elvehjem Tissue Grinder with rough glass pestle for muscle and heart. Use either rough glass or smooth polytetrafluoroethylene pestle for liver and brain.

3- Label one homogenizer and two 1.5 mL Eppendorf tubes per sample and keep them in ice.

4 - Use 20 to 50 mg tissue fragment obtained by lightly smashing the fragment in a mortar cooled by liquid nitrogen.

5 - Add 9 volumes of homogenization buffer (i.e. 270 µL for 30 mg fragment), manually homogenize while keeping the homogenizer in ice, stop as soon as the solution appears homogeneous, avoid over-homogenizing with subsequent heating of the solution. 10-20 passes are often sufficient

6- Using a Pasteur pipette, transfer the homogenate into one pre-cooled 1.5 mL Eppendorf tube, centrifuge at 650 g and 0-4°C during 10 min, transfer the supernatant (also called post-nuclear supernatant) into the second 1.5 mL Eppendorf tube

7- Take an aliquot for protein measurement (supernatant from these 10% homogenates usually contain between 4 to 7 mg protein/mL; a dilution 1/10 is often used to fit the concentration with the standards used in the protein assay)

8- Add bovine serum albumin fatty acids-free to a final concentration of 5 mg/mL, the post-nuclear supernatant is then stable for hours when kept at 0-4°C

4 **Spectrophotometric assay**

1-- Warm to 37°C 1 mL assay medium in 1 mL spectrophotometric cuvettes, either under stirring using a Suprasil ^{TM} 109004F-10-40 cuvette (Hellma, Germany) or without stirring using a regular plastic cuvette.

2- Add post-nuclear supernatant. The amount should be at least 20 µg protein for brain, 30 µg for heart and muscle and 40 µg for liver preparations.

3- In the absence of stirring, close the cuvette with thumb using a piece of Parafilm ^{TM} and rapidly shake it (at least four reversals).

4- Continuously measure absorbance at 340 nm with a 1 cm optical pathway.

5- After 3-5 minutes of incubation in the spectrophotometer, trigger the reaction by adding 1 mM ATP (10 µL of 100 mM solution)

6- After 10-15 minutes, add 5 µM oligomycin to measure ATP hydrolysis independent from complex V (5 µL of 1 mM solution).

7- To test the coupling between F_0 and F_1 domains, add 3 µM IF1 (Inhibitory Factor 1) (3 µL of 1mM solution).

5 **Analysis**

Analyze the kinetics of ATP hydrolysis using εM = 6220 M-1 cm-1.

To obtain the specific complex V ATP hydrolysis activity, subtract the slope obtained after oligomycin addition and after oligomycin + IF1 addition from that obtained after ATP addition.