Published in final edited form as:

Adv Exp Med Biol. 2016 ; 899: 113–120. doi:10.1007/978-3-319-26666-4_7.

Measuring the Impact of Microenvironmental Conditions on Mitochondrial Dehydrogenase Activity in Cultured Cells

Ramon C. Sun,

Center for Environmental and Systems Biochemistry, Mackey Cancer Center, University of Kentucky Lexington, KY 40536, USA

Department of Radiation Oncology, Ohio State University Wexner School of Medicine and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, USA

Albert Koong,

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305, USA

Amato Giaccia, and

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305, USA

Nicholas C. Denko

Department of Radiation Oncology, Ohio State University Wexner School of Medicine and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, USA

Ramon C. Sun: ramon.sun@uky.edu; Albert Koong: albert.koong@stanford.edu; Amato Giaccia: amato.giaccia@stanford.edu; Nicholas C. Denko: Nicholas.denko@osumc.edu

Keywords

Enzymatic activity; Colorimetric detection; Hypoxia; Hypoglycemia; Dehydrogenase

7.1 Introduction

Mitochondria are powerhouses of a cell, producing much of the cellular ATP. However, mitochondrial enzymes also participate in many cellular biosynthetic processes. They are responsible for helping to maintain NAD(P)/H and redox balance, supplying metabolic intermediates for cell growth, and regulating several types of programmed cell death. Several mitochondrial enzymes have even been shown to participate in the oncogenic process such as isocitrate dehydrogenase, succinate dehydrogenase, and fumarate hydratase. Recent advances have identified significant metabolic changes in the mitochondria that are regulated by malignant transformation and environmental stimuli. Understanding the biological activity and regulation of mitochondrial enzymes can provide insight into how they participate in the process of oncogenic transformation and work to sustain malignant growth. This chapter describes a technique to measure mitochondrial dehydrogenase activities that is faster and more cost effective which can also be scaled up for high throughput.

7.2 General Method

Conventional methods of measuring dehydrogenase activity involve biochemical isolation of whole enzyme complexes. Crude preparations can be made by subcellular fractionation and disruption of intracellular compartments with weak ionic detergent. Isolated enzyme complexes can either be further purified via size or affinity-based fractionation methods for more accurate measurement of specific activity or used as a crude complex mixture containing other enzymes. Depending on the extent of purification, the results can be more qualitative rather than quantitative. Recently, antibodies have been employed for even more specific immune capture and purification of enzymes. For example, the PDH assay sold by Mitoscience purifies the entire core PDH complex, but does not retain regulatory kinases and phosphatases.

Conventional methods have been used for the past century as a cornerstone of many reports that built our understanding of fundamental biochemical processes. However, significant limitations to the approach exist. Due to their low abundance and low recovery, it often takes large amounts of starting material (in some cases kilograms of tissue). Biochemical purification also requires technical skill and time for high activity preparations. Procedures can take days with multiple steps of centrifugation and dialysis. On a more theoretical note, it is hard to guarantee the integrity of the enzymatic complex. Critical components of the active complex could be lost in purification, or regulatory elements that are specific to the growth conditions (i.e., modification of critical regulatory residues, or associated regulatory kinases or phosphatases).

Here, we describe a method-of measuring mitochondrial dehydrogenase activities that overcomes many of these limitations. First, the method requires only a few thousand adherent cells. Second, it keeps the enzyme complex in its native environment with only minimal disruption of the cell by permeabilization of the membranes to facilitate substrate entry. Finally, and perhaps most important is that it can be performed in a 96-well format to allow real time, replicate comparison of multiple treatment conditions. This technique is based on P. Kugler's microscopic histochemistry method to detect dehydrogenase activity in brain tissue slices [1]. This method uses a two-step biochemical reaction that produces a colorimetric detection of dehydrogenase activity which is much more sensitive than the traditional absorbance- based assays. In the first reaction the dehydrogenase carries out its dehydrogenation with the concurrent conversion of NAD/NADP to NADH/NADPH. In the second reaction NAD(P)H is oxidized via phenazine methosulfate which in turn donates the electron to nitroblue tetrazolium to produce a blue-stained water-insoluble formazan (Fig. 7.1) [2–4]. The dark formazan is then solubilized with 10% SDS in 0.01 N HCl overnight in a CO₂ incubator and the optical density measured at 540 nm in a plate reader. The optical density is a measure of NAD(P)H produced, which is a function of the amount of enzyme activity because substrates are in excess.

7.2.1 List of Stock Reagents

HBSS—See below for recipe, once made stable for up to 3 months at 4 °C.

Assay buffer-50 mM Tris-Cl, 0.05 mM EDTA, pH 7.8, stable for up to 3 months at room temperature.

1 M α KG made up in MilliQ water and stored at -20°C for up to 1 month.

1 M MgCl_2 made up in MilliQ water and stored at -20°C for up to 1 month.

1 M CaCl_2 made up in MilliQ water and stored at -20°C for up to 1 month.

1 M Na pyruvate made up in MilliQ water and stored at -20°C for up to 1 month.

1 M Glucose made up in MilliQ water and stored at -20°C for up to 1 month.

100 mM Isocitrate made up in MilliQ water and stored at -20°C for up to 1 month.

200 mM ThDP made up in MilliQ water and stored at -20°C for up to 1 month.

300 mM NAD made up in MilliQ water and stored at -20°C for up to 1 week.

100 mM CoA made up in MilliQ water and stored at -20°C for up to 1 week.

10 mg/ml Nitroblue tetrazolium made up in MilliQ water and stored at -20°C for up to 1 month.

31.25 mg/ml Phenazine methosulfate made up in MilliQ water and stored at -20°C for up to 1 month.

0.5 mg/ml Rotenone made up in DMSO or ethanol and stored at -20°C .

7.2.2 Method for Selected Enzymes

The following steps for α KGDH, PDH, and IDH are optimized for RKO human colorectal cancer cells (Fig. 7.2). Cell density, time of incubation, and substrate concentrations may need to be adjusted for cell lines of different origins.

1. Seed cells in a 96-well plate at a seeding density of 1×10^4 /well overnight in 200 μl of growth medium. Plates can be treated with hypoxia or other experimental conditions such as treatment with DMOG to induce HIF-1 activity.

Low-glucose DMEM with 10% FBS should be used to grow cells overnight since glucose has been shown to suppress mitochondrial oxygen consumption and enzyme activities. The even seeding of adherent cells can be achieved by allowing cells to attach on a 360° orbital (i.e. “belly dancer”) plate shaker at 37°C if possible.
2. On the day of the measurement, the medium is aspirated, and the cells are washed once with balanced salt solution (BSS) [140 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES (pH 7.4)]. To test the activity of enzymatic inhibitors in vivo, treat cells with the desired inhibitor in BSS for up to 2 h.

PBS can be used in place of BSS. BSS is crucial to maintain normal cellular homeostasis for slow-acting inhibitors.

3. At the end of the treatment, or if there is no treatment, wash the cells with 200 μ l of Hanks' balanced salt solution (HBSS) containing 0.1 % (v/v) Triton X-100.

Due to its activity as a weak detergent, Triton X-100 produces permeabilization of the cells and entry of small molecules without compromising cell or enzyme complex integrity.

4. Incubate with 200 μ l of either complete assay mixture or the assay mixture without substrate as the blank for background dehydrogenase activity, and incubate for 40 min at 37 °C.

- a. The reaction mixture for alpha-ketoglutarate dehydrogenase (α KGDH) also known as 2-oxoglutarate dehydrogenase (2oxGDH) contains 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM EDTA, 0.3 mM ThDP, 0.5 μ g/ml rotenone, 0.2% Triton X-100, 3 mM KG, 3 mM NAD⁺, 1 mM CoA, 0.75 mM nitroblue tetrazolium, and 0.05 mM phenazine methosulfate [5–12].

Nitroblue tetrazolium and phenazine methosulfate should be added immediately before the reaction. Control buffer should not contain α KG or CoA. NADH ubiquinone reductase (mitochondrial complex 1) is a main enzyme consuming NADH; therefore suppressing its activity with rotenone is important. Correct pH is also crucial at this step for optimal enzyme activity. Different cell lines have different amounts of α KGDH activity; therefore longer or shorter incubation may be required for optimal detection.

- b. Reaction buffer for pyruvate dehydrogenase (PDH) activity consists of 1 mM $MgCl_2$, 0.05 mM EDTA, 0.2% Triton X-100, 0.3 mM ThDP, 10 μ M rotenone, 10 mM pyruvate, 3 mM NAD, 1 mM Co-A, 2 mM bromopyruvate, 0.75 mM nitroblue tetrazolium, and 0.05 mM phenazine methosulfate in 50 mM Tris-HCl pH 7.8 [13–17].

Nitroblue tetrazolium and phenazine methosulfate were added immediately before the reaction. Control buffer should not contain pyruvate and CoA. In the case of PDH activity, both rotenone (complex 1 inhibitor) and bromopyruvate (glyceraldehyde phosphate dehydrogenase inhibitor) are needed to minimize background reading due to high levels of NADH and pyruvate produced from glycolysis.

- c. Reaction buffer for NAD/NADP isocitrate dehydrogenase (IDH) activities consists of 8 mM MgCl₂, 1 mM MnCl₂, 0.05 mM EDTA, 0.2% Triton X-100, 10 μM rotenone, 2 mM NAD/0.5 mM NADP, 1.5 mM isocitrate, 10 mM citrate, 2 mM ADP, 0.75 mM nitroblue tetrazolium, and 0.05 mM phenazine methosulfate in 50 mM Tris-HCl pH 7.5 [18–21].
 - d. Reaction buffer for glutamate dehydrogenase (GDH) activity consists of 1 mM MgCl₂, 0.1 mM CaCl₂, 0.05 mM EDTA, 0.2% Triton X-100, 10 μM rotenone, 2 mM ADP, 3 mM glutamate, 3 mM NAD, 0.75 mM nitroblue tetrazolium, and 0.05 mM phenazine methosulfate in 50 mM Tris-HCl pH 7.6 [22, 23].
5. After incubation, aspirate the treatment medium, and gently wash cells with Ca²⁺ - and Mg²⁺ -free HBSS. Add 200 μl of 10% (w/v) SDS in 0.01 N HCl to solubilize the dark blue formazan overnight at 37 °C in a CO₂ incubator.

Red formazan should be fairly visible to the naked eye after wash. Plates need to be sealed with parafilm to prevent uneven evaporation of buffer. Alternatively, plates can be placed carefully in a water bath sonicator for up to 1 h for reading on the same day.
6. Measure absorbance (OD) at 540 nm on any compatible plate reader.
7. Activity is calculated by subtraction of the absorbance of the well containing the blank control (without substrate) from the absorbance of the well containing the reaction with the specific substrate.

7.3 Results and Discussion

Here we used RKO colorectal cells as a model to measure the effect of HIF1α stabilization on both αKGDH and PDH activities. Both cell density and incubation time were chosen for optimal absorbance while still being in the linear range of the reaction. For the best result, it is important to find the linear range or both parameters, shown in red in Fig. 7.2. Arrows indicate optimal cell density and incubation time for RKO cells specifically for the 96-well plate reaction, which is at 10,000 cells and 40 min, respectively. Both parameters should be determined empirically for other cell lines of interest.

In order to validate the method, we treated RKO cells overnight with 500 nM DMOG in order to stabilize HIF1α. Previous reports have shown that HIF1 activation suppresses both αKGDH and PDH activities [24, 25]. This experiment) shows approximately 50–70% inhibition of these regulatory dehydrogenases in response to HIF1 activation, consistent with reported values [24, 25]. The mechanisms of inhibition of PDH and αKGDH by hypoxia are very different, but both serve to decrease mitochondrial oxygen consumption and still provide the necessary macromolecules for growth of the tumor cells. An additional series of

RKO cells were also treated with dichloroacetate (DCA), a pyruvate mimetic that also activates PDH through the inhibition of the inhibitory pyruvate dehydrogenase kinases. Treatment of normoxic cells with DCA showed a modest 10–20% increase in activity. Normoxic PDH activity is near maximal, so this increase is in agreement with the literature.

7.4 Final Remarks

The method described above is faster, requires less starting material, has higher throughput, and most important of all measures enzyme activity while they are in their native cellular compartment. Different assay conditions and buffers should be tested for enzymes not described here. The limitations include the following: only adherent cell lines that grow as monolayers can be used for this assay, the formazan can be hard to dissolve, and background activities can be high depending on the substrate used. Appropriate inhibitors should be used in this assay to eliminate confounding activities, such as NADH dehydrogenase.

References

1. Kugler P. Quantitative enzyme histochemistry in the brain. *Histochemistry*. 1988; 90(2):99–107. [PubMed: 3068215]
2. Feinstein RN, Lindahl R. Detection of oxidases on polyacrylamide gels. *Anal Biochem*. 1973; 56(2):353–360. [PubMed: 4148790]
3. Fried R. Enzymatic and non-enzymatic assay of superoxide dismutase. *Biochimie*. 1975; 57(5):657–660. [PubMed: 171001]
4. Rajagopalan KV, Handler P. Oxidation of phenazine methosulfate by hepatic aldehyde oxidase. *Biochem Biophys Res Commun*. 1962; 8(1–2):43–47. [PubMed: 14489971]
5. Bunik VI, et al. Phosphonate analogues of alpha-ketoglutarate inhibit the activity of the alpha-ketoglutarate dehydrogenase complex isolated from brain and in cultured cells. *Biochemistry*. 2005; 44(31):10552–10561. [PubMed: 16060664]
6. Mizuno Y, et al. An immunohistochemical study on α -ketoglutarate dehydrogenase complex in Parkinson's disease. *Ann Neurol*. 1994; 35(2):204–210. [PubMed: 8109900]
7. Mastrogiacono F, Bergeron C, Kish SJ. Brain α -ketoglutarate dehydrogenase complex activity in Alzheimer's disease. *J Neurochem*. 1993; 61(6):2007–2014. [PubMed: 8245957]
8. Lai JCK, Cooper AJL. Brain α -ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. *J Neurochem*. 1986; 47(5):1376–1386. [PubMed: 3760866]
9. Patel MS. Inhibition by the branched chain 2 oxo acids of the 2 oxoglutarate dehydrogenase complex in developing rat and human brain. *Biochem J*. 1974; 144(1):91–97. [PubMed: 4462577]
10. Linn TC. Studies on the apparent instability of bovine kidney α -ketoglutarate dehydrogenase complex. *Arch Biochem Biophys*. 1974; 161(2):505–514. [PubMed: 4839044]
11. Imai K, Tomita Y. Interaction of the α ketoglutarate dehydrogenase complex with basic polypeptides. *J Biochem*. 1974; 75(4):699–705. [PubMed: 4847208]
12. Bowden JA, McArthur CL Iii, Fried M. Metabolic diseases and mental retardation. I. The chick embryo as a model system in branched-chain ketoaciduria; the effect of α -ketoisocaproic acid. *Int J Biochem*. 1974; 5(4):391–396.
13. Humphries KM, Szveda LI. Selective inactivation of α -ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. *Biochemistry*. 1998; 37(45):15835–15841. [PubMed: 9843389]
14. Bowker-Kinley MM, et al. Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J*. 1998; 329(1):191–196. [PubMed: 9405293]
15. Stanley CJ, Perham RN. Purification of 2-oxo acid dehydrogenase multienzyme complexes from ox heart by a new method. *Biochem J*. 1980; 191(1):147–154. [PubMed: 7470091]

16. Stansbie D, et al. Acute effects in vivo of anti insulin serum on rates of fatty acid synthesis and activities of acetyl coenzyme A carboxylase and pyruvate dehydrogenase in liver and epididymal adipose tissue of fed rats. *Biochem J.* 1976; 160(2):413–416. [PubMed: 12755]
17. Kerbey AL, et al. Regulation of pyruvate dehydrogenase in rat heart. Mechanism of regulation of proportions of dephosphorylated and phosphorylated enzyme by oxidation of fatty acids and ketone bodies and of effects of diabetes: role of coenzyme A, acetyl-coenzyme A and reduced and oxidized nicotinamide-adenine dinucleotide. *Biochem J.* 1976; 154(2):327–348. [PubMed: 180974]
18. Hurley JH. Catalytic mechanism of NADP⁺-dependent isocitrate dehydrogenase: implications from the structures of magnesium-isocitrate and NADP⁺ complexes. *Biochemistry.* 1991; 30(35): 8671–8678. [PubMed: 1888729]
19. Thorsness PE, Koshland DE Jr. Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J Biol Chem.* 1987; 262(22):10422–10425. [PubMed: 3112144]
20. Lai JCK, et al. Synaptic and non synaptic mitochondria from rat brain: isolation and characterization. *J Neurochem.* 1977; 28(3):625–631. [PubMed: 16086]
21. Alp PR, Newsholme EA, Zammit VA. Activities of citrate synthase and NAD⁺ linked and NADP⁺ linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates. *Biochem J.* 1976; 154(3):689–700. [PubMed: 8036]
22. Gouia H, Habib Ghorbal M, Meyer C. Effects of cadmium on activity of nitrate reductase and on other enzymes of the nitrate assimilation pathway in bean. *Plant Physiol Biochem.* 2000; 38(7–8): 629–638.
23. Benzi G, Moretti A. Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic Biol Med.* 1995; 19(1): 77–101. [PubMed: 7635361]
24. Papandreou I, et al. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab.* 2006; 3(3):187–197. [PubMed: 16517406]
25. Sun RC, Denko NC. Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. *Cell Metab.* 2014; 19(2):285–292. [PubMed: 24506869]

Fig. 7.1.
Generation of the blue formazan precipitate through the transfer of electrons from NAD(P)H to the nitroblue tetrazolium to form the purple-blue precipitate

Fig. 7.2.

(a–d) Determination of reaction parameters for adherent RKO colorectal cancer cells for both α KGDH and PDH assays. Note that cell number (a, b) and time of reaction (c, d) are both chosen to be in the linear portion of the curve. (e, f) determination of the effect of HIF1 stabilization by DMOG on α KGDH and PDH activities, and determination of the effect of the pyruvate dehydrogenase kinase inhibitor dichloroacetate on PDH activity