In vitro enzymatic activity assay for ENOLASE in mammalian cells in culture

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Method Article

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Abstract

Enolase \(EC 4.2.1.11) is the second to last step in glycolysis and the third step in gluconeogenesis, catalyzing the reversible conversation of 2-phosphoglycerate and phosphoenolpyruvate. Enolase is a key enzyme in energy metabolism and measuring its enzymatic activity is of interest to investigators in diverse fields, including those studying cancer cell metabolism. We describe a simple and rapid in vitro protocol to measure Enolase activity in native lysates from mammalian cells in culture.

Introduction

Enolase \(EC 4.2.1.11) is the second to last step in glycolysis and the fourth step in gluconeogenesis, catalyzing the reversible conversation of 2-phosphoglycerate and phosphoenolpyruvate. Enolase is a key enzyme in energy metabolism and measuring its enzymatic activity in vitro is of interest to investigators in diverse fields, including those studying cancer cell metabolism. A variety of transition state analogues have been synthesized, some of which exhibit potent inhibitory activity, such as phosphonoacetohydroxamate \(1,2). The enzymatic assay described here diverges little from that used in biochemistry laboratories since the 1960's \(1,2) and derives largely from the protocol found in the Sigma protocol archives http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/2/enolase.Par.0001.File.dat/enolase.pdf). It has been used by our lab to measure Enolase activity in lysates from blood, mouse tissues and cells in culture. In principal, any lysis buffer that leaves Enolase proteins in their native state can be used, provided that fluoride is not present in excessive amounts in the final assay since the later ion can act as an Enolase-inhibitor \(1). The present Enolase assay measures NADH changes linked to Enolase by lactate dehydrogenase and pyruvate kinase. The general principle of the assay is as follows \(illustrated below). Excess phosphoglycerate, pyruvate kinase, ADP and lactate dehydrogenase are provided in the assay, with the conversion of phosphoglycerate into phosphoenolpyruvate being rate limiting and performed by Enolase present in the biological samples to be assayed. Phosphoenolpyruvate is then converted to pyruvate by pyruvate kinase, and is used by lactate dehydrogenase to oxidize NADH. Changes in NADH are then followed either spectrophotometrically \(when the absolute activity, in Units/mg protein is required) or more efficiently, fluorometrically \(where knowledge of relative levels of Enolase activity is sufficient). The rate of NADH oxidation \(decrease in absorbance at 340 nm or decrease in fluorescence excitation/emission 360/460 nm) is the actual read-out of the enolase activity assay. In fluorescent reading format, the assay is readily adaptable to 96-well format. Phosphonoacetohdroxamate is a highly potent inhibitor and can be used as a negative control \(1). PRINCIPLE Enolase: 2-Phosphoglycerate + H2O —> Phosphoenolpyruvate Pyruvate Kinase: Phosphoenolpyruvate + ADP -> Pyruvate + ATP L-Lactate Dehydrogenase: Pyruvate + β-NADH ————> L-Lactate + β-NAD+ ADP: Adenosine 5'-Diphosphate ATP: Adenosine 5'-Triphosphate β-NADH: Reduced β -Nicotinamide Adenine Dinucleotide β-NAD+: Oxidized β -Nicotinamide Adenine Dinucleotide

Reagents

REAGENTS Lysis buffer 20 mM Tris, pH 7.5 1mM EDTA, 1 mM β-mercaptoethanol Reaction buffer 100 mM Triethanolamine, pH 7.4 \(Sigma product number T1502) 2.25 mM 2-Phosphoglycerate \(2PG; prepared in water from 2-Phosphoglyceric Acid, Sigma product # P0257.) 0.2 mM β -NADH, \(Dissolved in water Sigma N8129) 30 mM Magnesium Sulfate 120 mM Potassium Chloride Solution 1.75 mM ADP \(Sigma # A2754) 10 units pyruvate kinase and 15 units L-lactic dehydrogenase \(mixed enzyme solution, Sigma # P0294) Clear Bottom, Black walled 96-well plates \(we use Costar Fisher# 07-200-567)

Equipment

Sonicator \(we used several instruments, including Bioruptor XL Spectrophotometer \(we use Beckman DU 640) Fluorimeter plate-reader \(BMG FLUOstar Omega Microplate Reader)

Procedure

Preparation of biological test samples Sample preparation: Mammalian cells are grown under conventional conditions to around 90% confluence in 10-cm dishes. Cells are washed once with PBS and 0.5mL of Lysis buffer is added \(RIPA or NP-40 buffer may be used instead, but the specific Enolase activity is lower) following which cells are scraped and placed in 1.5mL Eppendorf tubes. Cells are then broken by sonication and the lysate is cleared by centrifugation at 20,000g for 30 minutes. Protein concentration is determined spectrophotometrically using the Bradford method \(Biorad #500-0006). We routinely freeze samples at -80C until further analysis. Assay procedure \(fluorescent plate reader): Two buffers are prepared, reaction buffer without 2PG \(reaction buffer A) and reaction buffer with 2X \(4.5 mM) 2PG \(solution B). The biological samples are equalized for protein concentration and are pre-mixed at dilutions from 1:500 to 1:10 with reaction buffer A \(The enolase enzymatic reaction cannot start for lack of substrate, 2PG) and a second set of samples is prepared with the addition of 2μM PhAH as a negative control. Mammalian blood \(diluted 1:1 with lysis buffer, sonicated, and centrifuged at 20,000g) can be used as a positive control. These are aliquoted at 100uL in a 96 well plate \(black walls, clear bottom); a set of wells without added biological specimen \(100uL buffer A alone) is included as a negative control. The reaction is started by the addition of 100uL of Buffer B \(adding the enolase substrate, 2PG). This is best done using a multichannel pipet and the fluorescence reading is started as quickly as possible. Fluorescence readings \(NADH excitation 360nm, emission 460nm) are recorded every 30 seconds. The slope of the time-dependent decrease in NADH fluorescence is determined for each sample, with the slope of the negative control \(no biological sample added) subtracted to account for background drift and non-Enolase mediated oxidation of NADH. The relative enolase activity may be expressed in relative fluorescent units/mg protein, or if protein loading was equalized for each sample, relative Enolase activity may simply be expressed as a ratio of the average of all samples. Assay procedure \(spectrophotometer, from Sigma protocol EC 4.2.1.11) Prepare fresh reaction buffer as described above and aliquot 0.9 mL in a 1 mL cuvette. Monitor the absorbance at 340 nm until reading is constant. The add 0.1 mL of sample and mix thoroughly by pipetting up and down. Use 0.1 mL of lysis buffer for the blank. As described above, a negative control can be prepared by adding 2μM PhAH and positive control can be done by using mammalian blood. Record the reduction in absorbance for about 5 min and measure the slope of the linear curve \(change in absorbance/min). The enzymatic activity can be calculated by the following equation: \(slope \(sample) – slope \(blank))\(dilution factor) U/mg = _____ \(6.22)\(volume of sample used)\(sample concentration in mg/mL) 6.22 is extinction coefficient of NADH at 340 nm

Timing

Sample preparation: Dependent on number of samples 2 hours for 10 samples One can freeze samples at this point. Assay: Spectrophotometric: 30 minutes of set-up and 20 minutes/sample Fluorimetric 30 minutes of set-up and 30 minutes per 96 well plate

Troubleshooting

The procedure described here offers a rapid and highly reliable procedure to measure Enolase activity. We have encountered minimal difficulties, except with very low abundance samples \(low confluence of culture plates). Although the procedure described here is designed for cells in culture, we have successfully used it for mouse tissues and mouse blood, using the same extraction reagents and procedures. The major drawback of this technique is that it is not possible to distinguish the activity contribution from the three isoforms \(ENO1, 2, 3). For this purpose, it is necessary to run a native gel, with an in-gel enzymatic assay \(3).

Anticipated Results

Mammalian glioblastoma cell lines show about 100 Units Enolase/mg of protein. The inhibition with pre-incubation of 2uM PhAH is over 95%.

References

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