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Kinetic measurement by LC/MS of γ-glutamylcysteine ligase activity

Karim Chik [a], Françoise Flourie [a], Khelifa Arab [a,b], Jean Paul Steghens [a,b,*]

[a] *UF 21455 Stress Oxydant et Vitamines, Fédération de Biochimie, Biochemistry Federation, Hôpital E. Herriot, 5 Place d'Arsonval, F-69437 Lyon, France*
[b] *EA 3090 Claude Bernard University, Lyon 1, France*

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Abstract

γ-Glutamylcysteine ligase (GCL) combines cysteine and glutamate through its gamma carboxyl moiety as the first step for glutathione (GSH) synthesis and is considered to be the rate-limiting enzyme in this pathway. The enzyme is a heterodimer, with a heavy catalytic and a light regulatory subunit, which plays a critical role in the anti-oxidant response. Besides the original method of Seelig designed for the measurement of a purified enzyme, few endpoint methods, often unrefined, are available for measuring it in complex biological samples. We describe a new, fast and reliable kinetic LC/MS method which enabled us to optimize its detection. L-2-Aminobutyrate is used instead of cysteine (to avoid glutathione synthetase interference) as triggering substrate with saturating concentrations of glutamate and ATP; the γ glutamylaminobutyrate formed is measured at $m/z = 233$ at regular time intervals. Reaction rate is maximum because ATP is held constant by enzymatic recycling of ADP by pyruvate kinase and phosphoenolpyruvate. The repeatability of the method is good, with CV% of 6.5 and 4% for catalytic activities at, respectively 0.9 and 34 U/l. The affinities of rat and human enzymes for glutamate and aminobutyrate are in good agreement with previous published data. However, unlike the rat enzyme, human GCL is not sensitive to reduced glutathione and displays a more basic optimum pH.

Keywords: γ-Glutamylcysteine ligase; Glutathione metabolism; Oxidative stress; Catalytic activity; LC/MS

1. Introduction

Glutathione (GSH), the most abundant antioxidant in cells, plays an important role in the defense against oxidative stress [1–2]. GSH is synthesized from its constituent aminoacids in two sequential enzymatic reactions catalyzed by γ-glutamylcysteine ligase (GCL, EC 6.3.2.2) and GSH synthetase (EC 6.3.2.2). GCL, also known as γ-glutamylcysteine synthetase, is a heterodimer consisting of heavy (GCL-hs, 73 kD) and light (GCL-ls, 27 kD) subunits. The heavy subunit, which is up-regulated during oxidative stress, exhibits the catalytic activity of the enzyme whereas the light subunit plays a regulatory role by modulating the affinity of the heavy subunit for substrates and inhibitors. Its catalytic activity is redox dependent and this enzyme is considered to be the rate-limiting enzyme in de novo GSH synthesis [3–8]. In view of its main role in GSH homeostasis, GCL could be a target for new drugs in different fields such as cancer or parasitosis. Many results report on the transcriptional regulation of the protein, but the catalytic activity is rarely checked.

In fact, few assays of GCL activity have been described. The original method by Seelig and Meister [9] described for purified enzyme, relied on a spectrophotometric detection of NADH formed in enzymatic coupled reactions with pyruvate kinase and lactate dehydrogenase. Recently, HPLC methods with fluorometric or electrochemical detection have been described [10–13]. However, these methods are hampered by a tedious and time-consuming sample preparation (gel or membrane filtration) which is necessary to decrease the numerous metabolic side reactions due to low molecular weight compounds such as amino acids and their relatives (as recently recalled by Wu et al. [14]). Furthermore, these HPLC meth-

* Corresponding author. Tel.: +33 4 72 11 06 23; fax: +33 4 72 11 06 75.
E-mail address: jean-paul.steghens@chu-lyon.fr (J.P. Steghens).

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ods, except for that of Nardi and Cipollaro [10], estimated catalytic activity on a single measurement of the reaction product formed by the enzyme. Another method involved a fluorescence-based microtiter plate assay with a single measurement point after naphtalenedicarboxaldehyde derivatisation but in fact it needed a blank measurement, at least for GSH and cysteine correction [15].

As GCL is a key enzyme in glutathione homeostasis and is regulated at numerous levels, the need for a precise measurement of its activity is important and explains why we describe a new method for its kinetic measurement, based on an HPLC–ESI-MS technique: L-2-aminobutyrate is used instead of cysteine as triggering substrate with saturating concentrations of glutamate and ATP, and the γ glutamylaminobutyrate formed is measured at $m/z = 233$ at regular time intervals. The reaction rate is maximum because ATP is held constant by enzymatic recycling of ADP by pyruvate kinase and phosphoenolpyruvate.

2. Materials and methods

2.1. Chemicals and reagents

L-Glutamic acid, L-2-aminobutyrate and benzylamine hydrochloride were purchased from Aldrich (St Louis, MO, USA). Pyruvate kinase, phosphoenolpyruvate, ATP, Tris were from Roche.

γ Glutamylaminobutyrate was from Bachem (Bubendorf, Switzerland), L-2-aminobutyric 3,3-d2 acid from CDN Isotopes (Canada) and other common reagents from Sigma (St Louis, MO, USA).

2.2. Enzymatic assay

All the concentrations were final concentrations in the kinetic reaction (before the addition of sulfosalicylic acid).

The general mixture (without or with glutamate) was Tris (25 mM), KCl (150 mM), $MgCl_2$ (20 mM), EDTA Na_2 (2 mM), glutamate (40 mM), adjusted at pH 8.6 with NH_4OH. This mixture was aliquoted (9 ml) and frozen at −20 °C.

2.3. Reagents for the enzymatic reaction

The incubation reagent (R1) was obtained daily by adding ATP (5.6 mM), PEP (2.1 mM) benzylamine hydrochloride (5 μM) and pyruvate kinase (30 000 U/l) to the general mixture with glutamate, the pH being checked at 8.6 after addition.

The starting reagent (R2) was L-2-aminobutyrate (20 mM) in the general mixture without glutamate.

2.4. γ glutamylaminobutyrate calibration

A stock solution of γ glutamylaminobutyrate (24 mM) was prepared in water, and aliquots (100 μl) were frozen at −20 °C. Standards (24, 12, 6, 3 μM) were prepared daily by dilution of the stock solution in water. Then each calibration point was treated as a sample, i.e. 10 μl with 70 μl of R1, 20 μl R2 and 40 μl of sulfosalicylic acid (SSA, 6% in water).

2.5. Samples: human fibroblasts and rat liver extracts or microsomes

Human fibroblasts were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum containing penicillin (98 U/ml), streptomycin (98 μg/ml) and fungizone (2.5 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested by trypsinization, washed twice in phosphate buffered saline (PBS) solution, then lysed by two cycles of freezing and thawing associated with ultrasounds for 15 s and kept frozen at −20 °C until analysis.

Rat liver microsomes were prepared by tissue homogeinisation with Tris–HCl buffer 100 mM, pH 7.4, containing 100 mM KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4000 × *g* for 15 min at 4 °C. For tissue assays, aliquots of the supernatant were frozen at −80 °C until use. To complete the microsome extraction, the supernatant was centrifuged at 100,000 × *g* for 90 min at 4 °C. The resulting microsomes were washed in a volume (equal to that of the original homogenate) of 100 mM sodium pyrophosphate buffer, pH 7.4. The washed microsomes were re-suspended at a protein concentration of 40–50 mg/ml, in 50 mM KH_2PO_4 buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 20% glycerol and frozen at −80 °C until use.

2.6. GCL kinetic measurement

For the assay of the enzymatic activity, 40 μl of sample were mixed with 280 μl of R1, distributed in three Eppendorf tubes (80 μl each) and incubated for 2 min at 37 °C. The reaction was started simultaneously with 20 μl of R2 in each tube and stopped individually with 40 μl of SSA at 2, 4 or 6 min. After centrifugation at 13,000 × *g* for 2 min, the γ glutamylaminobutyrate was measured by LC/MS.

2.7. LC–MS analysis of the reaction product γ glutamylaminobutyrate

LC–MS separations were performed on a Thermo liquid chromatograph with a pump P 4000, a thermostated autoinjector AS 3000 and a Navigator Aqua mass spectrometer detector equipped with a diverting valve (between the column and the detector). The separation was carried out on a 150 mm × 2 mm UP3 HDO cartridge (octadecylsilane silica with 3 μm particle size, Interchim, Montluçon, France), kept at 45 °C in an oven, with a flow rate of 0.2 ml/min.

The mobile phase was ammonium acetate 5 mM (final concentration) adjusted to pH 2.5 with formic acid and 5% (v/v) methanol. The standards and samples were kept at 15 °C in the autosampler and the injection volume was 5 μl.