Biochemical and Biophysical Research Communications 380 (2009) 591–596

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A direct spectrophotometric γ-glutamyltransferase inhibitor screening assay targeting the hydrolysis-only mode

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ARTICLE INFO

Article history:
Received 19 January 2009
Available online 27 January 2009

Keywords:
γ-Glutamyltransferase
γ-Glutamyl transpeptidase
Glutathione
High-throughput inhibitor screening
Ellman's reagent
Enzyme kinetics
Continuous assay
α-Phenylthio-peptide derivative
Thiophenol

ABSTRACT

γ-Glutamyltransferase (GGT, E.C. 2.3.2.2) catalyzes the hydrolysis and transpeptidation of extracellular glutathione. Due to its central role in maintaining mammalian glutathione homeostasis, GGT is now believed to be a valuable drug target for a variety of life-threatening diseases, such as cancer. Unfortunately, however, effective tools for screening GGT inhibitors are still lacking. We report here the synthesis and evaluation of an α-phenylthio-containing glutathione peptide mimic that eliminates thiophenol upon GGT-catalyzed hydrolysis of the γ-glutamyl peptide bond. The concurrent, real-time spectrophotometric quantification of the released thiophenol using Ellman's reagent creates a GGT assay format that is simple, robust, and highly sensitive. The versatility of the assay has been demonstrated by its application to the kinetic characterization of equine kidney GGT, and enzyme inhibition assays. The ability of the glutathione mimic to behave as an excellent donor substrate (exhibiting Michaelis–Menten kinetics with a K_m of 11.3 ± 0.5 μM and a k_{cat} of 90.1 ± 0.8 nmol mg^{-1} min^{-1}), coupled to the assay's ability to study the hydrolysis-only mode of the GGT-catalyzed reaction, make our approach amenable to high-throughput drug screening platforms.

γ-Glutamyltransferase (GGT; E.C. 2.3.2.2), a cell surface heterodimeric glycoprotein, catalyses the first step in glutathione (γ-L-glutamyl-L-cysteinylglycine) catabolism by hydrolyzing its γ-glutamyl bond. High GGT levels have been detected in kidney tubules, biliary epithelium and brain capillaries [1,2]. Also, soluble GGT can be released by such cell types in blood, where it forms GGT/lipoprotein complexes which may be of potential significance as a diagnostic/prognostic marker in cancer [3], as well as in other disease conditions such as liver dysfunction, coronary heart disease, Type 2 diabetes, stroke and atherosclerosis [4–7]. Moreover, GGT converts leukotriene C4 into leukotriene D4 [8] and has therefore been implicated in various inflammatory pathologies.

During the last decade, GGT has emerged as a complex molecular discriminant in tumor cell biology (reviewed in Ref. [9]). It is generally recognized that GGT-mediated catabolism of extracellular glutathione represents an important secondary source of cysteine—and probably also of glutamate [10]—for the intracellular resynthesis of glutathione as well as for general protein synthesis [1,11]. Inside cells, glutathione plays a central role in a number of detoxification pathways for electrophilic and oxidative metabolites and xenobiotics. Hence, several tumors of GGT-positive tissues exhibit an increase in GGT expression, probably because GGT-mediated cysteine-recovery confers growth and survival advantages [12,13]. While GGT clearly has been implicated in cellular antioxidant defenses [14], lines of evidence indicate that GGT plays a role in generating reactive oxygen species at the level of the cell surface as well [15,16], and, therefore, expression (also in GGT-negative cells) of GGT may play a role in human neoplasia and tumor progression [13]. In this context, GGT has emerged as an attractive target for anticancer chemotherapy, and research in this direction is currently ongoing [17,18].

The GGT-catalyzed hydrolysis of the *in vivo* donor substrate glutathione is outlined in Fig. 1A. GGT catalyses the transfer of the γ-glutamyl moiety to an active site nucleophile to form an acyl-enzyme intermediate (acylation step of the catalytic cycle) [19,20]. This intermediate reacts subsequently with water (hydrolysis), or with an α-amino acid or dipeptide (transpeptidation), or with a new glutathione molecule (autotranspeptidation) to recover the resting GGT (deacylation step), concomitantly releasing glutamate or a γ-glutamyl compound [19]. Until recently, GGT activity was studied primarily using chromogenic [21,22] or fluorigenic [23,24] substrate analogs, such as L-γ-glutamyl-*p*-nitroanilide, from which the intensely yellow colored *p*-nitroaniline is liberated as a reaction product [21]. Although satisfactory for determining serum GGT levels, these kinds of assays should be avoided for enzyme mechanistic or drug screening studies, since hydrolyses, transpeptidations and autotranspeptidations occur concurrently

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doi:10.1016/j.bbrc.2009.01.129

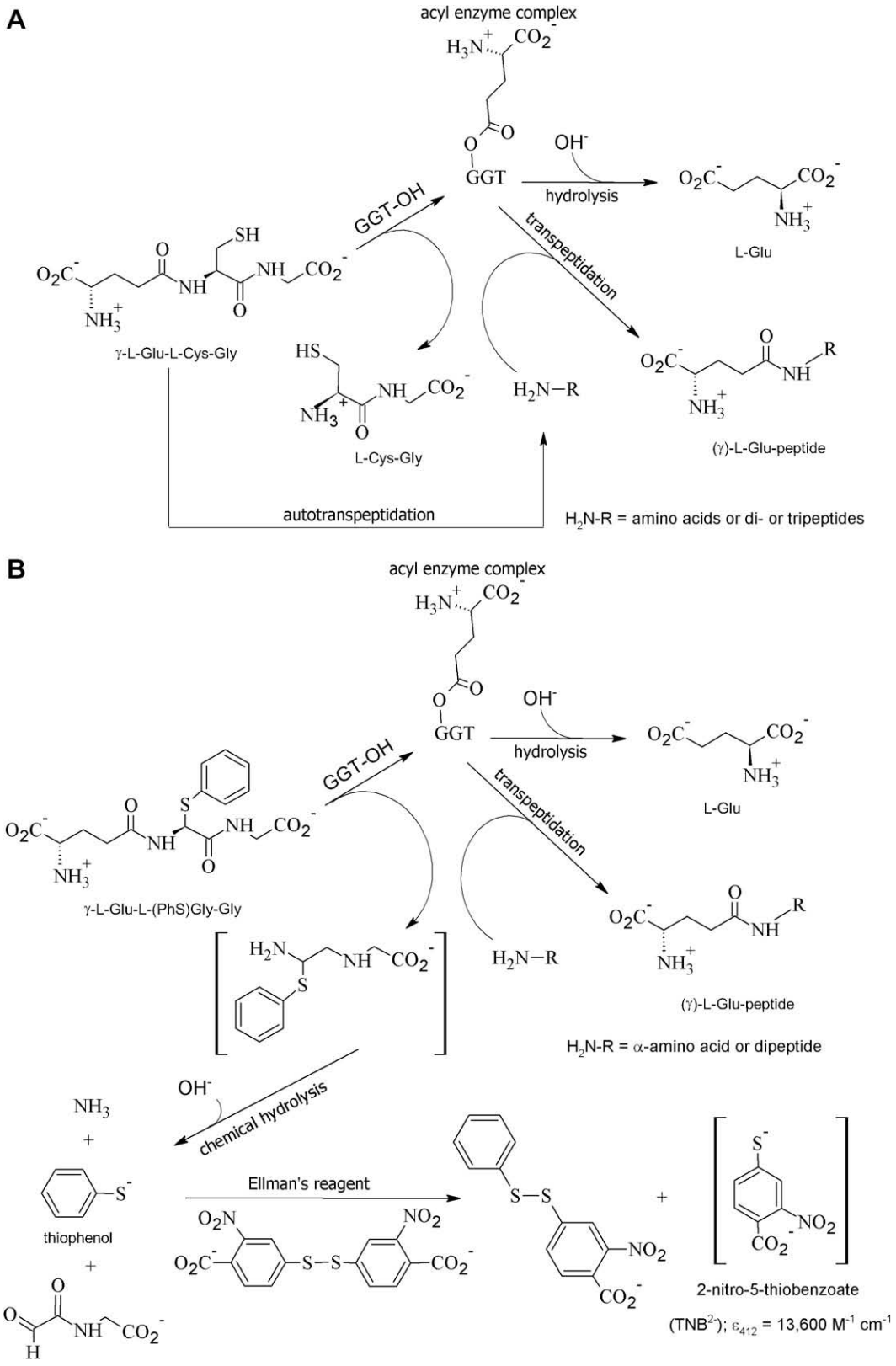

Fig. 1. Overview of the proposed catalytic mechanism of GGT (A) and the reactions involved in the direct GGT assay method (B).

[19]. For example, the formation of *p*-nitroaniline measured as a function of substrate concentration results in non-linear (highly pH-dependent) double-reciprocal plots, due to a "substrate activation"-type mechanism, in which the donor substrate L-γ-glutamyl-*p*-nitroanilide accelerates the deacylation step in a concentration-dependent manner by also acting as an acceptor substrate in an autotranspeptidation reaction [25,26]. Alternatively, some methods have been described which make use of the physiological