Bioelectrochemistry 101 (2015) 46–51

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Electrochemical evaluation of glutathione S-transferase kinetic parameters

Teodor Adrian Enache, Ana Maria Oliveira-Brett *

Department of Chemistry, University of Coimbra, 3004-535 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form 8 July 2014
Accepted 13 July 2014
Available online 19 July 2014

Keywords:
Glutathione
1-Chloro-2,4-dinitrobenzene
Glutathione S-transferase
Electrochemistry
Oxidation

ABSTRACT

Glutathione S-transferases (GSTs), are a family of enzymes belonging to the phase II metabolism that catalyse the formation of thioether conjugates between the endogenous tripeptide glutathione and xenobiotic compounds. The voltammetric behaviour of glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione S-transferase (GST), as well as the catalytic conjugation reaction of GSH to CDNB by GST was investigated at room temperature, T = 298.15 K (25 °C), at pH 6.5, for low concentration of substrates and enzyme, using differential pulse (DP) voltammetry at a glassy carbon electrode. Only GSH can be oxidized; a sensitivity of 0.14 nA/μM and a LOD of 6.4 μM were obtained. The GST kinetic parameter electrochemical evaluation, in relation to its substrates, GSH and CDNB, using reciprocal Michaelis–Menten and Lineweaver–Burk double reciprocal plots, was determined. A value of K_M ~ 100 μM was obtained for either GSH or CDNB, and V_{max} varied between 40 and 60 μmol/min per mg of GST.

1. Introduction

The metabolism of cancer cells is controlled by oncogene signalling and by dysregulation of metabolic enzymes. The resulting altered metabolism supports cellular proliferation and survival but leaves cancer cells dependent on a continuous supply of nutrients. A primary cause of cancer treatment failure and patient relapse is an acquired or intrinsic resistance to anticancer therapies. Acquisition of drug resistance can be attributed to various factors that include avoidance of apoptotic cell death, altered expression of multidrug resistance-associated proteins, altered drug metabolism or uptake, and/or overexpression of phase II biotransformation enzymes [1,2].

Many metabolic enzymes, such as those belonging to the phase II metabolism, have been investigated. Glutathione S-transferases (GSTs), one of the major phase II detoxifications, are a family of enzymes that catalyse the formation of thioether conjugates between the endogenous tripeptide glutathione (GSH) and xenobiotic compounds, Scheme 1, [3]. They are abundant throughout most life forms [2], being involved in the metabolism of xenobiotics and play an important role in cellular protection against reactive and toxic electrophile species that arise through normal metabolic processes [4].

From the structural point of view, two distinct superfamilies of GSTs have been described: the soluble cytosolic classes (Alpha, Mu, Pi, Kappa and Theta) and a microsomal family, designated as MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) [5,6], and the non-enzymatic functions of GSTs involve in the interaction with proteins. Soluble GSTs and MAPEG are widely distributed throughout the body and are found in the liver, kidney, brain, pancreas, testis, heart, lung, small intestine, skeletal muscles, prostate and spleen [3]

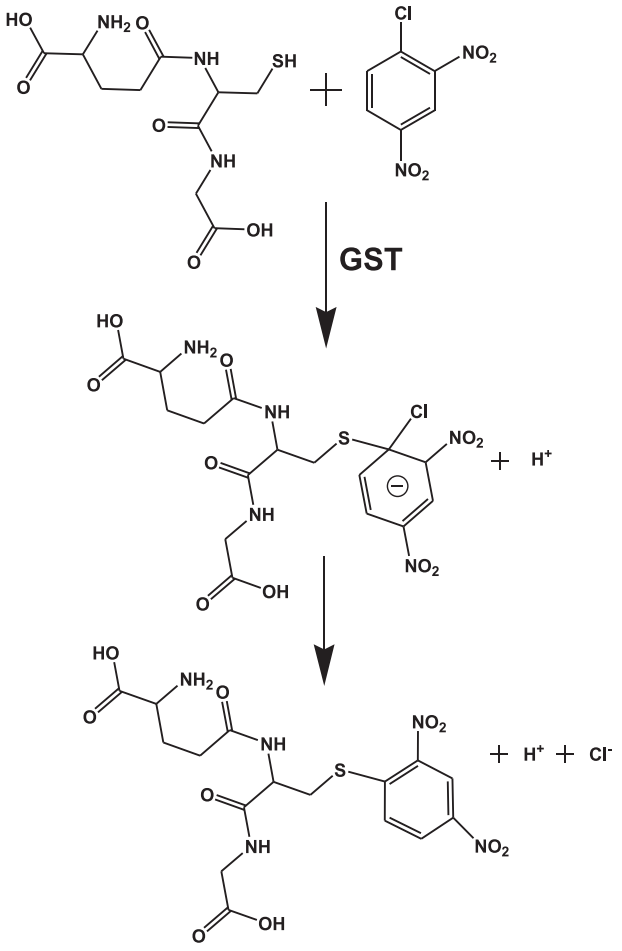
GSTs can catalyse a large number of reactions including nucleophilic aromatic substitutions, Michael additions, isomerizations and reduction of hydroperoxides, and play a major role in the detoxification of epoxides derived from polycyclic aromatic hydrocarbons and alpha-beta unsaturated ketone, quinones, sulfoxides, esters, peroxides and ozonides, and many endogenous compounds such as prostaglandins and steroids are also metabolized via a glutathione conjugation reaction [3,7,8].

Specific substrates of GSTs have been already described [9]. Ethacrynic acid has been shown to be a very specific substrate for GST-P1 [10] and trans-stilbene oxide is a diagnostic substrate for GST-M1 [11]. Relatively small molecules, e.g. methylene chloride, ethylene dibromide or isoprene derivate have been shown to be conjugated by GST-T [12]. The 1-chloro-2,4-dinitrobenzene (CDNB) has been described as a universal GST substrate [13,14], except for theta-class enzymes which lack activity with this substrate [15].

Chemotherapeutic-resistant tumour cell lines have been shown to overexpress GST isozymes. This overexpression leads to an accelerated detoxification of drug substrates and thus an acquired resistance [1]. As a particular case, glutathione S-transferase Pi (GST-P) is a marker protein in many cancers (ovarian, non-small cell lung, breast, liver, pancreas, colon, and lymphomas) and high levels are linked to drug resistance, even when the selecting drug is not a substrate [16,17]. Therefore, GSTs have emerged as a promising therapeutic target because specific isozymes are overexpressed in a wide variety of tumours

* Corresponding author at: Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3004-535 Coimbra, Portugal. Tel./fax: +351 239 827703.
E-mail address: brett@ci.uc.pt (A.M. Oliveira-Brett).

http://dx.doi.org/10.1016/j.bioelechem.2014.07.002
1567-5394/



Scheme 1. The conjugation reaction of GSH with CDNB by GST.

and may play a role in the aetiology of other diseases, including neurodegenerative diseases, multiple sclerosis, and asthma [17]. Consequently, there are many studies regarding GST substrate conjugation or inhibition reactions, most of them based on spectroscopic techniques, which require high concentrations, above millimolar, of protein and/or substrate [18–26].

However, no electrochemical assay for the determination of the kinetic parameters of GST was developed. The electrochemical techniques offer sensitivity and selectivity [27], making them very attractive tools for protein investigation [28–30]. Differential pulse voltammetry is recognised to be the most sensitive voltammetric method when the analyte is irreversibly oxidized or reduced, and the glassy carbon electrode has excellent detection limits and high sensitivity, together being excellent tools for the redox behaviour of biologic compound investigation. In this research the catalytic conjugation reaction of CDNB and GSH catalysed by GST at micromolar concentrations, using differential pulse voltammetry and a glassy carbon electrode, was investigated. The results may contribute to an advance understanding of the enzymatic reactions occurring at low concentrations, which in turn can decrease the costs of new cancer research therapies.

2. Experimental

2.1. Materials and reagents

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione S-transferase (GST) from equine liver, from Sigma–Aldrich were used without further purification. Stock solutions of 1 mM GSH and CDNB (containing 30% (v/v) ethanol) were daily prepared in ultra-pure water from a Millipore Milli-Q system (conductivity $\leq 0.1\ \mu S\ cm^{-1}$). The GST solutions of different concentrations were prepared in 5% glycerol and were stored at −22 ºC.

The supporting electrolyte was 0.1 M phosphate buffer pH = 6.5.

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co. Inc., Woburn, USA). All experiments were done at room temperature (25 ± 1 °C) in 0.1 M phosphate buffer, pH = 6.5.

2.2. Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon electrode (GCE) as a working electrode, a Pt wire as a counter electrode and an Ag/AgCl (3 M KCl) as a reference electrode. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude of 50 mV, pulse width of 70 ms and a scan rate of 5 mV s^{-1}.

The GCE (d = 1 mm) was polished using diamond spray (particle size 3 μm) before each experiment. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30 s; then it was placed in a supporting electrolyte and various DP voltammograms were recorded until a steady state baseline voltammogram was obtained.

2.3. Acquisition and presentation of voltammetric data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average with a step window of 3 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, although the peak height is in some cases reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

3. Results and discussion

An electrochemical method for evaluation of GST activity and determination of its kinetic parameters was developed. The conjugation reaction of GSH with CDNB catalysed by GST was studied by DP voltammetry in solutions incubated for different time periods and different concentrations of enzyme and substrates in 0.1 M phosphate buffer pH = 6.5.

GSH oxidation occurs at the cysteine residue and is an irreversible, diffusion-controlled, pH dependent process that involves the sulfhydryl group oxidation [28,31]. GST catalyses the proton removal from GSH to generate the thiolate anion GS^-, that is more reactive than GSH. The thiolate conjugation reaction with CDNB occurs at carbon one where chloride was bound, producing a Meisenheimer complex. This complex is unstable, chloride dissociates, and the glutathionyl-dinitrobenzene (GS-DNB) conjugate is formed in solution [32], Scheme 1.

Therefore, as GST catalyses the conjugation reaction of CDNB, less free GSH oxidizable sulfhydryl groups are available in solution to react. Consequently, the formation of the GS-DNB complex as well as the GST activity can be indirectly determined by the electrochemical evaluation of the GSH oxidation current decrease.

The DP voltammograms were recorded using a clean GCE surface and the current corresponding to GSH sulfhydryl group oxidation was measured in order to quantify the GS-DNB product. The CDNB, GST, and conjugation reaction product GS-DNB were not electroactive in the experimental conditions used.