FEBS Letters 585 (2011) 341–345

journal homepage: www.FEBSLetters.org

The extended catalysis of glutathione transferase

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ARTICLE INFO

Article history:
Received 27 October 2010
Revised 7 December 2010
Accepted 8 December 2010
Available online 14 December 2010

Edited by Stuart Ferguson

Keywords:
Glutathione transferase
Non-typical GST substrate
Inverted kinetics
Kinetics simulation
Detoxification
Glutathione

ABSTRACT

Glutathione transferase reaches 0.5–0.8 mM concentration in the cell so it works in vivo under the unusual conditions of, [S] ≪ [E]. As glutathione transferase lowers the pK_a of glutathione (GSH) bound to the active site, it increases the cytosolic concentration of deprotonated GSH about five times and speeds its conjugation with toxic compounds that are non-typical substrates of this enzyme. This acceleration becomes more efficient in case of GSH depletion and/or cell acidification. Interestingly, the enzymatic conjugation of GSH to these toxic compounds does not require the assumption of a substrate–enzyme complex; it can be explained by a simple bimolecular collision between enzyme and substrate. Even with typical substrates, the astonishing concentration of glutathione transferase present in hepatocytes, causes an unusual "inverted" kinetics whereby the classical trends of *v* versus E and *v* versus S are reversed.

1. Introduction

Glutathione transferases (GSTs) are a superfamily of detoxifying enzymes widely distributed in animals, plants and microorganisms [1,2]. Seven gene-independent cytosolic GST classes have been identified in mammals, *i.e.* Alpha, Mu, Pi, Omega, Sigma, Theta and Zeta [2]. A prominent role of these enzymes is to promote the conjugation of glutathione (GSH) to a variety of hydrophobic compounds with an electrophilic centre [1,2] but several additional functions have been discovered [2]. The Zeta and Omega class GSTs exhibit also a thiol transferase activity reminiscent of thioredoxin and glutaredoxin activities [3,4]. Interestingly, in some cells like rat hepatocytes GSTs account for 5–8% of all soluble proteins so their total cytosolic concentration is about 0.5–0.8 mM [5]. There does not appear to be any reason why the levels of this enzyme need to be so high since it may be calculated, based on the urinary output of glutathione conjugates, that in the liver the intracellular flux of toxic GST substrates is below 0.1 μM/min and that a single molecule of enzyme performs only one catalytic cycle every 5 days [5]. Glutathione transferase represents one of the few enzymes that work in vivo at [S] ≪ [E] in addition to some non-regulatory enzymes involved in glycolytic metabolism. The present study investigates this peculiar kinetic scenario and proposes a possible functional role arising from the high concentration of the enzyme. GSH, which saturates in vivo GSTs (K_m = 0.1–0.4 mM), is the most abundant thiol in the cell, reaching 8–10 mM in hepatocytes [6]. Its role is the maintenance of a proper redox status inside the cell and the elimination of dangerous compounds like alkylating compounds, disulfides and peroxides. Some of these reactions are catalyzed by specific enzymes (e.g. glutathione transferase, glutaredoxin and glutathione peroxidase), but many of them probably occur spontaneously inside the cells. However, only the deprotonated form of GSH is reactive towards these compounds. Since the pK_a of GSH is about 9.0, only 1% of the free GSH (0.08–0.1 mM) is present as the thiolate species at physiological pH values so the un-catalyzed reactions may be quite slow in vivo. However, glutathione transferase is able to lower the pK_a of the

Abbreviations: GSH, glutathione; GST, glutathione transferase; DTNB, 2,2′-dithionitrobenzoic acid; TNB^-, 2-thionitrobenzoate; BSA, bovine serum albumin; IAA, iodoacetic acid; DTOH, dithiodiethanol; CDNB, 1-choro-2,4-dinitrobenzene

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.12.009

sulfydryl group of GSH from 9.0 to 6.2–6.6 [7] and is expressed at millimolar levels. We demonstrate here that this enzyme acts as a chemical machine that increases the effective concentration of the reactive GSH thiolate and speeds its interaction with many compounds even if they are non-typical and poor substrates of GSTs ($K_m \gg 10^{-2}$ M). Surprisingly, we found that in this extended catalytic role, the assumption of a classical enzyme–substrate complex is unnecessary.

2. Materials and methods

2.1. Enzyme purification

Human GSTA1-1 and GSTM2-2 were expressed in *Escherichia coli* and purified as reported previously [8].

2.2. Reaction with disulfides

Reactions of GSH with cystamine, L-cystine and dithiodiethanol (DTOH) were carried out at 25 °C in 0.1 M potassium phosphate buffer, pH 6.0 or 7.1. The GSH concentration ranged from 1 mM to 10 mM while the disulfide concentration was varied from 0.1 to 2 mM. The reduction process by GSH was followed spectrophotometrically at 340 nm by including 0.2 mM NADH and 10 U of glutathione reductase in the incubation mixture. The effect of hepatic GSTs (Alpha and Mu class enzymes) on these reactions was evaluated by adding 0.23 mM of GSTA1-1 and 0.27 mM GSTM2-2 (about 10 mg/ml) to the standard incubation mixture. Non-specific kinetic effects due to the presence of large amounts of protein were evaluated by replacing the GST with 10 mg/ml of bovine serum albumin (BSA).

2.3. Reaction with alkylating compounds

Reactions of GSH with iodoacetate were carried out at 25 °C in 0.1 M potassium phosphate buffer, pH 6.0 or 7.1. The GSH concentration ranged from 1 to 10 mM while the concentration of the alkylating compounds was varied from 0.5 to 5 mM. Kinetics of the alkylation reaction by GSH were followed at fixed times on the basis of GSH disappearance. In a typical experiment, 0.02 ml aliquots of the incubation mixture were reacted with 0.1 mM 2,2′-dithionitrobenzoic acid (DTNB) in 1 ml (final volume) of 0.1 M potassium phosphate buffer, pH 8.0. The amount of GSH was evaluated on the basis of an extinction coefficient of the 2-thionitrobenzoate (TNB^-) of 13 600 $M^{-1}\,cm^{-1}$ at 412 nm. The effect of the hepatic GSTs on these reactions was evaluated by adding 0.23 mM of GSTA1-1 and 0.27 mM GSTM2-2 (about 10 mg/ml) to the standard incubation mixture. Non-specific kinetic effects due to the use of high amounts of protein were evaluated by replacing the GST with 10 mg/ml of BSA.

2.4. Kinetic simulations

Simulations were carried out with the software package COPASI 4.4.27 [9]. The software simulates the time courses of Scheme 1 by means of numerical integration of the ordinary differential equations. The kinetics of the reaction of 1-chloro-2,4-dinitrobenzene (CDNB), with GSH catalyzed by human glutathione transferase P1-1 isoenzyme involves several intermediates [10]. However, we have used the simplified Michaelis–Menten steady-state model to simulate the overall dependence of the rate of product formation on either substrate (S) or enzyme (E) concentrations (Scheme 1)

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_3} E + P$$

Scheme 1.

Table 1
Effect of the hepatic GSTs on kinetics ($t_{1/2}$) of GSH with selected non-substrates.

	pH 7.1		
	GSH 10 mM $t_{1/2}$ (min)	GSH 10 mM + BSA 10 mg/ml $t_{1/2}$ (min)	GSH 10 mM + GST 0.5 mM $t_{1/2}$ (min)
Cystamine[a]	5.8	3.6	0.16
DTOH	8.0	5.3	0.16
Cystine	5.3	4.3	0.20
Iodoacetate	265	147	5.3
	GSH 1 mM	GSH 1 mM + BSA 10 mg/ml	GSH 1 mM + GST 0.5 mM
Cystamine	56	38	0.18
DTOH	83	55	0.15
Cystine	52	43	0.22
Iodoacetate	2500*	1400*	5.8
	pH 6.0		
	GSH 10 mM $t_{1/2}$ (min)	GSH 10 mM + BSA 10 mg/ml $t_{1/2}$ (min)	GSH 10 mM + GST 0.5 mM $t_{1/2}$ (min)
Cystamine	46	29	0.47
DTOH	69	43	0.55
Cystine	46	36	0.68
Iodoacetate	2500*	1500*	15
	GSH 1 mM	GSH 1 mM + BSA 10 mg/ml	GSH 1 mM + GST 0.5 mM
Cystamine	450*	288	0.51
DTOH	700*	400*	0.60
Cystine	450*	350*	0.74
Iodoacetate	26 000*	14 000*	16

[a] The experiments were performed at pH 6.0 and 7.1 (see Section 2). Values with the asterisk were not measured experimentally as these reactions were too slow. Instead the values were estimated by extrapolating from experiments performed at higher reagent concentrations or at higher pH values, assuming a simple bimolecular interaction.

In Scheme 1, E represents the enzyme in complex with GSH (K_m = 0.1 mM; the intracellular GSH concentration being up to 10 mM) and S the co-substrate having $K_m \approx 1$ mM. The following rate constants according to Scheme 1, $k_1 = 10^3\ M^{-1}\ s^{-1}$; $k_2 = 1\ s^{-1}$; $k_3 = 0.1\ s^{-1}$ were used. These rate constants are close to those for the overall reaction of the classical co-substrate CDNB, with GSH catalyzed by human GST P1-1 [10].

3. Results

3.1. Effect of GST on the reaction of GSH with selected non-typical substrates

Glutathione transferases from rat liver are able to conjugate GSH to many hydrophobic toxic compounds. Small and hydrophilic disulfides like cystine, cystamine and dithioethanol, and hydrophilic alkylating compounds like iodoacetate, are not known as typical substrates of this enzyme. As expected, we did not find any trace of activity with these compounds in the standard activity conditions, *i.e.* [S] = 1 mM, [GSH] = 1 mM and using high enzyme concentrations (up to 5 μM Alpha or Mu GSTs) (not shown). Actually, only the Omega class GST and a peculiar lens GST display a moderate thiol transferase activity with dithioethanol while the liver Alpha and Mu GSTs have been reported to be completely ineffective [4,11]. In the absence of specific enzymes all these hydrophilic compounds react with GSH spontaneously, following a pH-dependent bimolecular mechanism (not shown). At a pH value and GSH concentration similar to those found in the liver cytosol (pH 7.1, GSH 10 mM) these reactions are quite slow, showing apparent $t_{1/2}$ values ranging from 3 min to about 4 h (Table 1). When the same reactions were carried out in the presence of

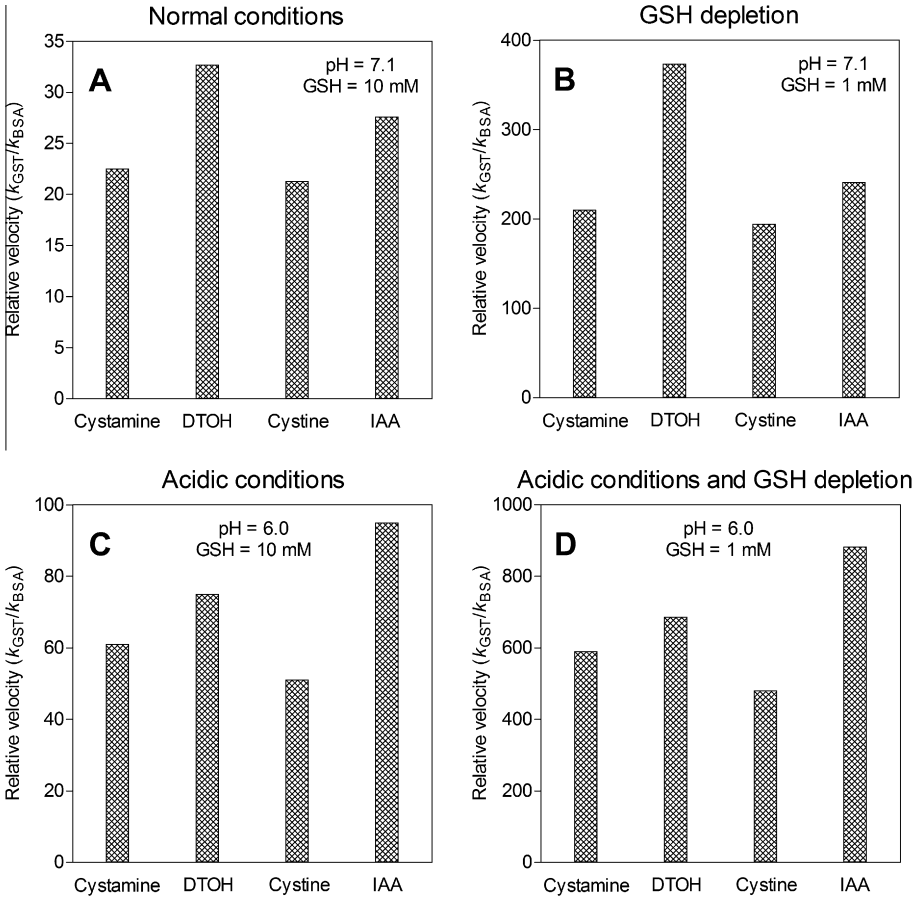

Fig. 1. Acceleration factors for the reaction of GSH with selected non-substrates under different experimental conditions. The ratio represents the observed pseudo-first order kinetic constants in the presence of the GSTs over those measured adding 10 mg/ml of BSA. (A) pH 7.1, 10 mM GSH, 0.5 mM GSTs. (B) pH 7.1, 1 mM GSH, 0.5 mM GSTs; (C) pH 6.0, 10 mM GSH, 0.5 mM GSTs; (D) pH 6.0, 1 mM GSH, 0.5 mM GSTs. IAA is iodoacetic acid.

0.5 mM GSTs, approximately with the same isoenzyme composition found in rat liver (Alpha class GST = 0.27 mM, Mu class GST = 0.23 mM [12]), they become 20–30 times faster (Table 1 and Fig. 1). A linear dependence of the velocity was observed by varying the enzyme concentration from 0.05 to 0.5 mM or by increasing the concentrations of disulfides or alkylating compounds up to 5 mM (not shown). Thus, no enzyme saturation occurs, confirming a null or very poor affinity of GSTs for these compounds ($K_m \gg 10$ mM). This conclusion is also supported by the absence of competitive inhibition (up to 5 mM) toward the classical co-substrate CDNB (data not shown). When GSTs are replaced by BSA, only a mild acceleration of the spontaneous reactions occurs, probably due to a protein crowding affect (Table 1). We checked also the individual contribution of the Alpha and Mu isoenzymes to the observed catalysis: 0.5 mM of GSTA1-1 or GSTM2-2 gave approximately the same acceleration both for disulfide reduction and for the reaction with alkylating compounds. Interestingly, the estimated amount of GS^- bound to the enzyme is 0.4–0.5 mM (about five times higher than that coming from spontaneous deprotonation of GSH). This increased concentration could be sufficient to speed up these reactions by up to five times assuming that only a simple bimolecular collision occurs between enzyme and these compounds. Given that the acceleration due to GSTs is of the same order of magnitude (Fig. 1), it follows that these reactions do not require the assumption of a ternary complex. In other words, GST may act also towards non-typical substrates with very low or almost no affinity for the enzyme (very high K_m) by increasing the amount of deprotonated GS^-. Obviously, other factors may cooperate to accelerate these reactions, *i.e.* the desolvation effect within the active site and a proper orientation of the GSH thiolate inside the active site.

3.2. Reaction of GSH with selected non-typical substrates of GST under low GSH conditions

The GSH concentration in the cell can drop down in response to protein malnutrition, oxidative stress, and many pathological conditions [13]. When the selected disulfides and alkylating compounds were reacted at pH 7.1 with 1 mM GSH (simulating a cytosolic GSH depletion of about 90%), all these reactions in the absence of GSTs displayed kinetics about 10 times slower than those observed at 10 mM GSH, as expected for reactions that follow a simple bimolecular mechanism. In contrast, in the presence of 0.5 mM GSTs, they proceed at comparable rates with either 1 or 10 mM GSH (Table 1). This fact is not surprising because similar amounts of GS^- are found in the G-site either at 1 or 10 mM of GSH, given that both these levels saturate GSTs (K_d^{GSH} values are 0.03 and 0.1 mM for Alpha and Mu GSTs, respectively [14]). These findings suggest that this enzyme acts in vivo as a 'kinetic buffer' that ensures an efficient reactivity of GSH towards toxic compounds even in cases of significant loss of this tripeptide.

3.3. Reaction of GSH with selected non-typical substrates of GST under acidic conditions

The cytosolic pH of hepatocytes ranges between 6.9 and 7.3 [15], but under stress conditions can drop to lower values [16]. In the case of acidification, all spontaneous reactions involving

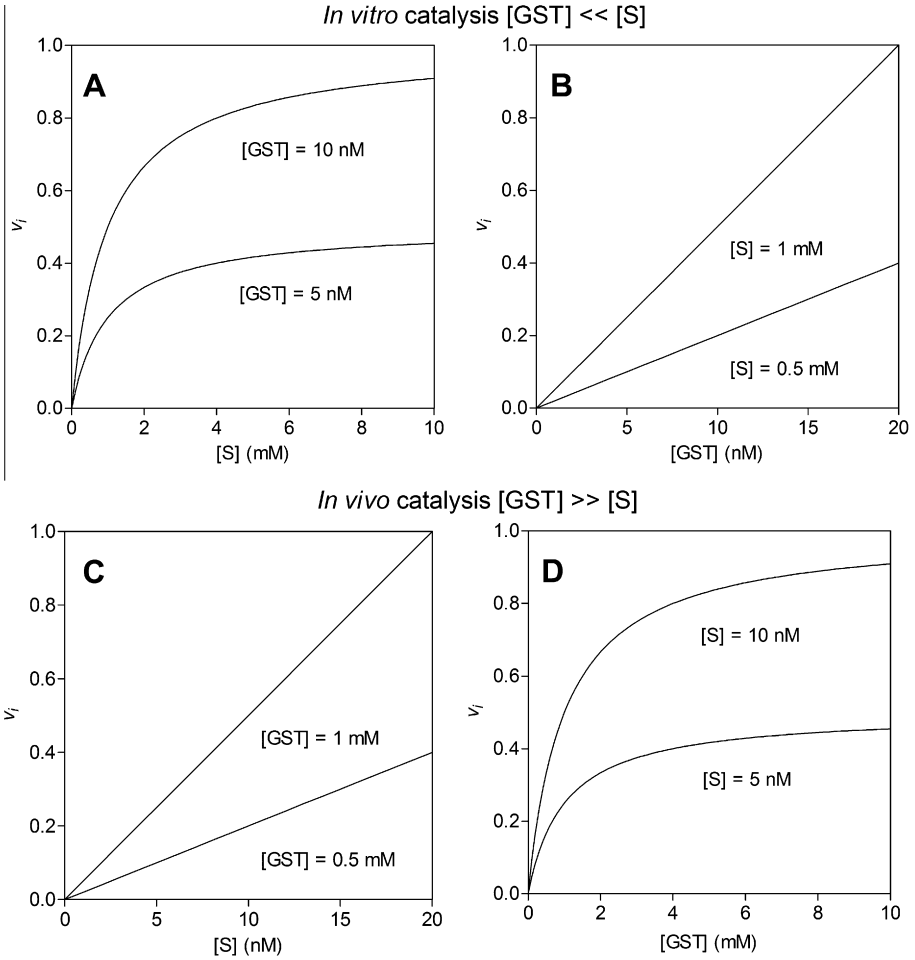

Fig. 2. Simulations of steady-state kinetics. According to the classical Scheme 1, simulations were carried out with the software COPASI 4.4.27, using the following rate constants according to Scheme 1: $k_1 = 10^3\ M^{-1}\ s^{-1}$; $k_2 = 1\ s^{-1}$; $k_3 = 0.1\ s^{-1}$ ($K_m \approx 1$ mM for CDNB). Substrate concentration range was between 0.1 mM and 1 pM and the corresponding time courses are overimposed. V_i represents the initial ($t \ll 1$ s) rate of substrate disappearance, expressed as $10^{-5}\ M\ s^{-1}$.

GS^- become slower. For example, at pH 6.0 the reaction of GSH with disulfides and alkylating compounds is about 10 times slower than at pH 7.1 (Table 1). In contrast, in the presence of 0.5 mM GSTs, the reaction rate was only half of the one measured at pH 7.1 (Table 1). This result can be explained by noting that the pK_a value of the bound GSH is 6.2–6.6. Thus at pH 6.1, about 30–40% of the bound GSH is still present as the deprotonated species. Again, this enzyme behaves as a 'kinetic buffer' which maintains a kinetic efficiency of GSH in the case of cytosol acidification. Fig. 1 summarizes the kinetic efficiency of the GST/GSH system. In the case of simultaneous GSH depletion and intracellular acidification, the effect of GSTs is much more evident and the rate of these reactions appears thousands times higher than that measured in the absence of this enzyme.

3.4. The 'inverted' kinetics

The astonishing abundance of GSTs in liver cells produces interesting kinetic consequences not only with non-typical and poor substrates but also with substrates that display discrete and measurable K_m values like CDNB ($K_m = 10^{-3}$ M). By using the classical kinetic Scheme 1 with the assumption $E \gg S$, simulated calculations display an inverted scenario where the classical linear trend v versus E is replaced by a hyperbolic behavior (Fig. 2). In contrast, the classical hyperbolic shape of v versus S is substituted by a linear dependence. In this context, K_m does not represent the substrate concentration but the enzyme concentration that causes $½V_{max}$ (Fig. 2). In this 'inverted' kinetics, the half time of S disappearance does not depend on its concentration like it occurs in the classical conditions $[S] \gg E$ with $[S] < K_m$.

4. Discussion

Usually, enzymes in the cell work at a substrate concentration typically exceeding a hundred to thousand times the enzyme concentration. However, glutathione transferases exhibit atypical enzymatic behavior in the liver cell. While the cytosolic concentration of these enzymes is about 0.5–0.8 mM, the amount of toxic compounds that are used by GSTs as co-substrates is very low and their metabolic flux (calculated on the basis of GSH-conjugate excretion) may be estimated below 0.1 μM/min. Furthermore, GSTs exhibit a moderate affinity for many co-substrates (0.1–1 mM). It appears that GSTs have evolved to eliminate many types of toxic compounds with the necessity of broad specificity resulting in low affinity for co-substrates but compensated by a very high enzyme concentration in the cell.

This hyperexpression and the intrinsic ability to deprotonate GSH enhances the effective concentration of GS^- inside the cell by five times thus speeding its interaction with toxic compounds that display no or very poor affinity for the enzyme ($K_m \gg 10^{-2}$ M). This peculiar property becomes more evident in case of GSH depletion and cell acidification (Fig. 1). Interestingly, these accelerations do not require the assumption of a substrate/enzyme complex to be invoked but only a simple bimolecular collision between GS^- bound to the en-

zyme and non-typical substrates. Paradoxally, in this context the ratio k_{cat}/K_m, that measures the efficiency of a specific enzyme, approximates to zero (K_m value to infinity) but the enzyme still favors an acceleration that may be essential for the cell. To our knowledge this is the first indication of the possible irrelevance of the Michaelis complex in an enzymatic catalysis. This peculiar mechanism differs from other unusual kinetics like the Theorell-Chance scheme described for alcohol dehydrogenase [17]. In that case the formation of the ternary complex is not kinetically limiting and it is not significantly populated during the reaction; nevertheless its formation in the active site is the conditio sine qua non for the catalytic event.

By looking at the crystallographic structures of Alpha and Mu GSTs, it appears that the sulfur atom of GSH in the active site is only partially exposed to the solvent and a direct interaction with target molecules is unlikely. However, natural 'breathing' motions of the G-site, previously observed for Alpha, Mu and Pi GSTs [18–20], may expose the thiol group of GSH to the solvent and thus to a productive collision with the toxic compounds: an increased reactivity of the thiolate due to the desolvation of the active site, or proper positioning of the thiolate sulfur, caused by a hydrogen bond to an active site tyrosine residue, may be additional factors that contribute to the observed extended catalysis by GSTs.

This phenomenon may not be confined to hepatocytes, although this tissue is more exposed to the insults of alkylating or oxidizing compounds. Literature data indicate that other tissues such as the kidney, brain and testes have cytosolic concentrations of GSTs between 3 and 10 times lower than the liver, but have corresponding lower levels of GSH of about 1–2 mM. Thus, the GST/GSH ratio of about 0.05 found in the liver, is not very different from that present in other organs (0.02–0.04). Assuming that Alpha or Mu classes GSTs are the prominent isoenzymes in these tissues, this ratio is high enough to accelerate these reactions several times and, more importantly, to act as 'kinetic buffer' up to 0.1 mM cytosolic GSH, a concentration that saturates most GSTs.

Acknowledgment

The authors would like to thank Prof. Andrea Bellelli for helpful discussion. M.W.P. is an Australian Research Council Federation Fellow and National Health and Medical Research Council of Australia Honorary Fellow.

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