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Forum

BIOLOGIC AND PHARMACOLOGIC REGULATION OF MAMMALIAN GLUTATHIONE SYNTHESIS

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Abstract—Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is synthesized from its constituent amino acids by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase. The intracellular GSH concentration, typically 1–8 mM, reflects a dynamic balance between the rate of GSH synthesis and the combined rate of GSH consumption within the cell and loss through efflux. The γ-GCS reaction is rate limiting for GSH synthesis, and regulation of γ-GCS expression and activity is critical for GSH homeostasis. Transcription of the γ-GCS subunit genes is controlled by a variety of factors through mechanisms that are not yet fully elucidated. Glutathione synthesis is also modulated by the availability of γ-GCS substrates, primarily L-cysteine, by feedback inhibition of γ-GCS by GSH, and by covalent inhibition of γ-GCS by phosphorylation or nitrosation. Because GSH plays a critical role in cellular defenses against electrophiles, oxidative stress and nitrosating species, pharmacologic manipulation of GSH synthesis has received much attention. Administration of L-cysteine precursors and other strategies allow GSH levels to be maintained under conditions that would otherwise result in GSH depletion and cytotoxicity. Conversely, inhibitors of γ-GCS have been used to deplete GSH as a strategy for increasing the sensitivity of tumors and parasites to certain therapeutic interventions. © 1999 Elsevier Science Inc.

Keywords—γ-Glutamylcysteine synthetase, Free radical, Oxidative stress, Transcriptional regulation, Cysteine availability, Feedback inhibition, Nitric oxide, Buthionine sulfoximine

INTRODUCTION

Glutathione (L-γ-glutamyl-L-cysteinylglycine, [GSH]) is the predominant low molecular weight thiol in mammalian cells and plays a major role in cellular defenses against oxidative and nitrosative stress and against reactive electrophiles [1–4]. Although relatively resistant to "spontaneous oxidation" [4], GSH reacts rapidly and nonenzymatically with hydroxyl radical, the cytotoxic Fenton reaction product, and with N_2O_3 and peroxynitrite, cytotoxic products formed by the reaction of nitric oxide (NO) with O_2 and superoxide, respectively [5–7]. In reactions catalyzed by the several isoforms of GSH peroxidase, GSH also participates in the reductive detoxification of hydrogen peroxide and lipid peroxides [8]. Each of these reactions leads directly or indirectly to the formation of glutathione disulfide (GSSG), a species that is reduced intracellularly to GSH by GSSG reductase in a NADPH-dependent reaction [9].

At normal levels of oxidative and nitrosative stress, GSSG reductase activity and NADPH availability are sufficient to maintain [GSH]/[GSSG] > 100 [10]. Under these circumstances there is essentially no net loss of GSH through oxidation. However, if stress levels increase sufficiently or if other factors limit the GSSG reductase reaction (e.g., glucose-6-phosphate dehydrogenase deficiency may limit NADPH synthesis), then GSSG may accumulate. This has two important conse-

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Dr. Owen W. Griffith earned his undergraduate degree in biochemistry from the University of California, Berkeley, and completed his graduate work at the Rockefeller University in New York City working on carnitine acetyltransferase with Dr. Leonard Spector. His work on γ-glutamylcysteine synthetase (γ-GCS) began in 1975 when he joined Dr. Alton Meister's group in the Department of Biochemistry at Cornell University Medical College. Dr. Griffith joined the faculty of that Department in 1980 and continued his work on the enzymes of glutathione metabolism and on carnitine-dependent enzymes. Among his contributions are the discovery of L-buthionine-S-sulfoximine as a highly selective, physiologically active γ-GCS inhibitor and numerous studies using that inhibitor to elucidate and pharmacologically control glutathione turnover. Other current interests include nitric oxide biology and microbial defenses against oxidative and nitrosative stress. Dr. Griffith is currently Professor and Chairman of Biochemistry at the Medical College of Wisconsin, a position he accepted in 1992.

quences: (i) the thiol redox status of the cell will shift, activating certain oxidant response transcriptional elements [11,12], and (ii) GSSG may be preferentially secreted from the cell [13]. Because GSSG is not taken up intact by cells, but is rather degraded extracellularly, loss of GSSG from cells under conditions of oxidative or nitrosative stress increases cellular requirements for de novo GSH synthesis.

Although GSH reacts spontaneously with some electrophiles [14], most such reactions require catalysis by an extended family of enzymes known as GSH S-transferases [15]. The initial products are chemically stable sulfides of GSH, but further metabolism removes the L-glutamate and glycine residues, forming S-substituted L-cysteines. Acetylation of the cysteinyl amino group can then form a mercapturic acid, which is easily excreted in the urine. Note that such metabolism results in the irreversible loss of the L-cysteine residue of GSH, a consequence of some importance because that amino acid is often limiting for GSH synthesis. In extreme cases such as acetaminophen intoxication (Tylenol; McNeil Consumer Products, Fort Washington, PA, USA), it is necessary to administer an L-cysteine precursor, typically N-acetyl-L-cysteine, in order to maintain survivable cellular GSH levels [16]. The present review summarizes our current understanding of the mechanisms controlling GSH synthesis and discusses recent advances in the pharmacologic control of GSH levels. These and related aspects of GSH synthesis and its regulation have also been addressed in several excellent earlier reviews [1–3,17–21].

The enzymes of synthesis

GSH is synthesized from its constituent amino acids by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (Reactions 1 and 2); the γ-GCS reaction is rate limiting [1,3]. Both enzymes are cytosolic, but there is as yet no evidence that they are physically linked in a "metabolon." The pathway intermediate, L-γ-glutamyl-L-cysteine, can, in fact, be acted on either by GSH synthetase, forming GSH, or by γ-glutamylcyclotransferase, forming 5-oxo-L-proline and L-cysteine. Partitioning of γ-glutamylcysteine between GSH synthesis and cyclotransferase-mediated degradation is apparently controlled only by the kinetics of the system. Thus, because the K_m of γ-glutamylcysteine is ~12-fold higher for γ-glutamylcyclotransferase than for GSH synthetase and the total activity of the cyclotransferase in cells is much lower, GSH synthesis is favored [22]. In fact, at the low levels of γ-glutamylcysteine normally present, it is estimated that >95% of the γ-GCS product is converted to GSH. As expected, significant diversion of γ-glutamylcysteine into 5-oxoproline and cysteine is seen in patients with inherited GSH synthetase deficiency [23].

Reaction 1:

$$\text{L-Glutamate} + \text{L-Cysteine} + \text{MgATP} \rightarrow \text{L-}\gamma\text{-Glutamyl-L-cysteine} + \text{MgADP} + \text{Pi}$$

Reaction 2:

$$\text{L-}\gamma\text{-Glutamyl-L-cysteine} + \text{Glycine} + \text{MgATP} \rightarrow \text{Glutathione} + \text{MgADP} + \text{Pi}$$

Mammalian γ-GCS is a heterodimer comprised of a catalytically active heavy subunit (γ-GCS_H, M_r ~73,000) that includes all substrate binding sites and a light subunit (γ-GCS_L, M_r ~31,000) that modulates the affinity of the heavy subunit for substrates and inhibitors. Both subunits have been cloned, sequenced and expressed [24–27], and their chromosomal location is known in mouse and man [28]. Although the rat γ-GCS_H subunit is represented by a single transcript [26], hybridization experiments show two mRNAs coding for the human γ-GCS_H subunit in most tissues, and two transcripts are consistently seen for γ-GCS_L in both rat and human [27]. It is not yet known if all mRNAs are translated.

As isolated, 30–70% of rat or human γ-GCS is stabilized by an intersubunit disulfide bond [29–31]. Because γ-GCS dimers persist even in the presence of thiols (e.g., GSH or dithiotheitol), it is clear that noncovalent forces also contribute importantly to dimer stability. Presence or absence of the disulfide bond is thought to modulate affinity for substrates and inhibitors ([31]; see also the next section on feedback inhibition).

The kinetic and chemical reaction mechanism of rat γ-GCS as well as the several partial and alternative reactions catalyzed by γ-GCS have been recently reviewed [1]. Steady state kinetics indicate the reaction normally proceeds through a quarternary complex in which all substrates are bound before any products are formed [32,33]. Nevertheless, the chemical mechanism involves two discreet steps in which L-glutamate and ATP first react to form tightly bound γ-glutamylphosphate, and that intermediate then reacts with L-cysteine to form the final products. Both steps occur within the kinetic quarternary complex, thus accounting for the steady state kinetic results [1].

Mammalian GSH synthetase is a homodimer with each subunit (M_r ~52,000) containing about 2% carbohydrate [34–36]. The rat enzyme has been cloned and sequenced [35]. In mouse a single gene produces six distinct mRNAs, three of which code for the same native enzyme; the other mRNAs encode truncated proteins that

have not yet been established to have activity [36]. Dimer stability is apparently dependent only on noncovalent forces as no intersubunit disulfide bonds have been detected. The GSH synthetase kinetic and chemical mechanisms are apparently similar to those of γ-GCS; L-γ-glutamyl-L-cysteine and ATP first react to form tightly bound L-γ-glutamyl-L-cysteinylphosphate, and that intermediate then reacts with glycine to form GSH [37].

Modulation of cellular GSH levels—Overview

Cellular GSH levels reflect a steady state balance between synthesis and loss. As noted, synthesis includes de novo synthesis from the constituent amino acids (Reactions 1 and 2) as well as GSSG reductase-mediated regeneration of GSH from GSSG. Although the latter reaction is quantitatively important in that flux through the GSSG reaction is generally high relative to de novo synthesis, GSSG reductase normally maintains the total glutathione pool in a predominantly reduced state [9,10]; thus, redox cycling between GSSG and GSH does not usually have a major influence on cellular GSH levels. On the other hand, extreme levels of oxidative or nitrosative stress can quickly and substantially diminish GSH levels in favor of GSSG, particularly if GSSG reductase activity is low due to inherited deficiency [38] or administration of an inhibitor (e.g., the cancer chemotherapeutic agent BCNU [39]). As noted, at least some cell types efflux GSSG rapidly, increasing the demand for de novo GSH synthesis [13].

De novo GSH synthesis is regulated by at least three factors: (i) the level of γ-GCS present in the cell; (ii) the availability of its substrates, particularly L-cysteine; and (iii) feedback inhibition of GSH on γ-GCS. In addition, there is recent evidence that γ-GCS activity can also be modulated by phosphorylation and nitrosation. Each of these factors is individually moderately well understood, as is discussed later in this article. On the other hand, our understanding of how these control mechanisms integrate to establish a tissue- or cell-specific rate of GSH synthesis is limited. One can confidently predict that increased γ-GCS expression, greater availability of L-cysteine, and decreased intracellular levels of GSH will each increase the rate of GSH synthesis, but it is not possible at present to predict the magnitude of change following any of those perturbations. Moreover, our understanding of the inter-relationship between the control mechanisms governing GSH synthesis and the less understood mechanisms governing GSH and GSSG utilization is poor. The steady state level of GSH can not yet be predicted for any cell type or tissue from information on levels of substrates, enzymes, and transporters.

Reactions contributing to the net loss of intracellular GSH are limited to spontaneous or catalyzed addition of GSH to various electrophiles, most of which are formed endogenously by the cytochrome P450 system [14–16]. In the absence of intoxication with cytochrome P450 substrates, these reactions consume relatively little GSH, and, under normal conditions, the net loss of GSH from cells is due almost entirely to the transport of GSH and, to a smaller degree, GSSG across the plasma membrane and out of the cell [40,41]. Extracellular degradation of GSH and GSSG, initiated by γ-glutamyltranspeptidase, is both rapid and irreversible. With the exception of bile, which may contain 10-mM GSH [40], extracellular levels of GSH and GSSG are low. Plasma GSH + GSSG levels are, for example, typically 5–50 μM, depending on vascular bed and species examined. Note that the extreme concentration gradient between intracellular GSH (1–8 mM) and extracellular GSH (a few μM) assures that transport of intact GSH or GSSG back into cells is thermodynamically unfavorable even for transporters shown to be reversible in vitro. As expected, there is substantial current interest in characterizing the plasma membrane GSH and GSSG transporter(s) and their regulation, and reviews of this work are available [13,40,41]. For our purpose it is sufficient to re-emphasize that the rate of GSH transport, which is subject to regulation [42–44], is a major factor controlling intracellular GSH levels.

Control of GSH synthesis by regulation of γ-GCS expression

The γ-GCS_H and γ-GCS_L genes are located on different chromosomes, and their transcription does not appear to be tightly coordinated. Gipp et al. [27] surveyed normal human tissues to determine mRNA levels for the heavy and light γ-GCS subunits and found that both absolute amounts and ratios varied widely. Some tissues such as lung showed γ-GCS_H mRNA levels that exceeded those for γ-GCS_L, whereas other tissues such as skeletal muscle, testes, and colon exhibited γ-GCS_L mRNA levels that exceeded those for γ-GCS_H. Furthermore, the relative abundance of heavy and light chain mRNA species in human tissues differed from those seen in rat [26,27]. The significance of these tissue- and species-specific differences is not yet clear. It is also noted that the general assumption that γ-GCS holoenzyme accounts for essentially all γ-GCS activity in normal tissues is supported by little, if any, direct data. It may be that imbalances normally occur between the γ-GCS subunits, thus reflecting the discrepant mRNA levels. It is known that in many tumors γ-GCS_H is overexpressed, often without corresponding overexpression of γ-GCS_L

Table 1. Factors Affecting γ-GCS Activity or Transcription[a]

Condition	Response	References
Oxidant stress—various sources[b]	Increased activity/transcription	54,55,57,62–64
Oxidant stress—lipopolysaccharide	Decreased activity/transcription	59
Nitrosative stress	Increased activity/transcription	65,66
Inflammatory cytokines (IL-1β, TNF-α)	Increased activity/transcription	56
TGF-β; dexamethasone; erythropoietin	Decreased activity/transcription	63,67,72
Cancer/cancer chemotherapy	Increased activity/transcription	46,68,107
Ionizing radiation	Increased activity/transcription	69–71
Heat shock	Increased activity/transcription	72
Inherited genetic changes	Increased or decreased activity/transcription	23,73
Inhibition of γ-GCS activity	Increased transcription	55,74
GSH depletion/conjugation	Increased activity/transcription	57,75
Prostaglandin A2	Increased activity/transcription	76,77
Insulin, glucocorticoid	Required for normal activity/transcription	78,79
High glucose levels	Decreased activity/transcription	56,79
Metals (heavy metals, CH_3-Hg, Se)	Increased activity/transcription	80–83
Antioxidants	Increased activity/transcription	84,85

[a] Adapted from S.C. Lu [20].
[b] Oxidative stress undoubtably contributes to the induction of γ-GCS in several of the other conditions listed below including exposure to inflammatory cytokines, ionizing radiation, and some of the heavy metals and antioxidants.

[45,46]. Other tumors show coordinated overexpression of both subunits [47].

The 5′-flanking region of the human γ-GCS_H gene contains putative AP-1, AP-2, nuclear factor kappa B (NF-κB), antioxidant and electrophile response elements (ARE/EpRE), and Sp-1 binding sites within 1500 bp of the transcription start site [48,49]. Several studies implicate binding of Jun family transcription factors to an AP-1 site in this proximal region as critical to upregulation of γ-GCS_H transcription in response to a variety of oxidant stress agents [45,50–53]. Among the oxidants and oxidant-related species reportedly acting through this mechanism are H_2O_2, redox cycling drugs, cigarette smoke, and tumor necrosis factor-α (TNF-α) [49,52,54–57]. Although the ARE element in this proximal promoter region is apparently not important for the response to at least some oxidants [49,52], Mulcahy et al. [48] report that a distal ARE/EpRE site located between −3802 and −2752 is required both for constitutive expression and for induced expression in response to β-naphthoflavone, an agent that increases expression of phase II detoxifying enzymes. It is known that oxidant stress induces NF-κB binding activity in addition to AP-1 activity [11,12], and several studies have implicated NF-κB in the upregulation of γ-GCS_H expression in response to inflammatory cytokines (e.g., interleukin-1β and TNF-α) [56], redox cycling drugs [57], and buthionine sulfoximine-mediated GSH depletion [57]. Other studies in different cell types suggest, however, that the NF-κB site is not involved in the response to TNF-α [58]. Cisplatin-mediated induction of γ-GCS_H in a lung cancer cell line is attributed to distinct regions of the proximal promoter [46].

It should be noted that responses in vivo may not match those seen in vitro. In contrast to the results cited above, Buetler [59] reports that γ-GCS_H mRNA and GSH levels are decreased in livers of rats administered lipopolysaccharide, a bacterial cell wall component that increases oxidant stress as well as interleukin-1β and TNF-α expression. Similarly, Maher et al. [60] find that injury stimulates γ-GCS transcription in liver stellate cells in culture but not in vivo. An additional complication is that in some cases increased mRNA levels are due mainly to increased mRNA stability, a possibility that has not always been examined [61]. Thus, although there is currently much known about what stimuli increase or decrease γ-GCS activity or γ-GCS_H mRNA levels in specific systems (Table 1), the generality of the response is not always clear, and the detailed mechanisms responsible for those changes are incompletely understood.

Factors regulating transcription of the γ-GCS_L gene have received less attention than those affecting the catalytic, heavy subunit, but Moinova and Mulcahy [86] recently reported that constitutive γ-GCS_L transcription is controlled in part by an AP-1 site located 334–340 bp upstream of the transcription start site. Although an unidentified element located further upstream also affects constitutive expression, it is notable that sites binding Jun family proteins have major importance for both subunits. Transcription of the γ-GCS_L gene, like the γ-GCS_H gene, is stimulated by the oxidant stress resulting from exposure to redox cycling quinones or BSO-mediated GSH depletion [55]. Addressing the mechanisms involved, it was recently shown that induction of γ-GCS_L gene transcription by β-naphthoflavone depends mainly on an EpRE site 291–301 bp upstream of the start site [86].

Table 2. Kinetic Constants Reported for γ-GCS Holoenzyme and Heavy Subunit

Source of enzyme [ref.]	K_m^{GLU} (mM)	K_m^{CYS} (mM)	$K_m^{\alpha\text{-ABA}}$ (mM)	K_i^{GSH} (mM)
Rat kidney holoenzyme [91]	n.d.	0.35	3	2.3
Rat kidney holoenzyme [92,93]	1.4	0.2	1.2	8.2
Rat kidney heavy subunit [92,93]	18.2	0.2	0.8	1.8
Human holoenzyme [30]	1.9	0.1	1.3	3.3
Human heavy subunit[a]	3.2	0.13	2.7	1.0
His-tagged human holoenzyme [94][b]	0.7	0.8	3.4	2.2
His-tagged human heavy subunit [94][b]	3.5	0.5	1.7	25.5

[a] I. Misra and O.W. Griffith, unpublished.

[b] Whereas rat and human γ-GCS and $\gamma\text{-GCS}_H$ have specific activities of ~1500 μmol/hr per mg protein, the His-tagged human γ-GCS and $\gamma\text{-GCS}_H$ had specific activities of only 370 and 69 μmol/hr per mg protein, respectively. Activity of the His-tagged γ-GCS reportedly increased 40% when the tag was proteolytically removed, but the resulting specific activity, presumably 518 μmol/hr/mg, is still only about 1/3 that seen with human γ-GCS expressed in *E. coli*. In addition. GSH inhibition of His-tagged γ-GCS and $\gamma\text{-GCS}_H$ was non-competitive in contrast to the competitive inhibition seen with all other mammalian γ-GCS preparations and most other $\gamma\text{-GCS}_H$ preparations.

Control of glutathione synthesis by substrate availability

Intracellular levels of amino acids vary with species, tissue, and nutritional status, but L-cysteine levels are consistently and substantially lower than levels of L-glutamate and glycine. For example, Tateishi et al. [87] report that liver concentrations of L-glutamate, L-cysteine, and glycine are 1.8, 0.15, and 2.2 mM, respectively, in rats starved 24 h and are 2–4, 0.18–0.25, and 1.5–1.8 mM, respectively, in refed rats. From these data and additional feeding studies Tateishi et al. [87] concluded that L-cysteine availability was generally limiting for GSH synthesis. Subsequent studies support this view [88–90]. Note that L-cysteine limits GSH synthesis even though all of the amino acid substrates for γ-GCS and GSH synthetase have affinities that are in the physiologic range (i.e., for γ-GCS, K_m^{GLU} ~1.7 mM and K_m^{CYS} ~0.15 mM (Table 2); for GSH synthetase, K_m^{GLY} ~0.8 mM [34]). L-Cysteine thus limits GSH synthesis not because it is present at levels far below its K_m but because only a small pool of L-cysteine is available to sustain a much larger and often metabolically active pool of GSH. For example, rat liver contains ~4.5 mM GSH and releases 20–50% of that pool into bile and plasma in 60 min [95,96]. Maintenance of the hepatic GSH pool thus consumes an amount of L-cysteine equivalent to its entire intracellular pool every 4–15 min.

In liver, the new L-cysteine needed to sustain GSH synthesis is derived by transsulfuration of L-homocysteine, a metabolite of L-methionine, and by uptake from the portal circulation of L-cyst(e)ine originating from dietary protein or from recapture in the gut of L-cyst(e)ine derived from biliary GSH and GSSG.[1] To the extent that the liver takes up and degrades proteins synthesized in extrahepatic tissues, protein degradation also supplies L-cysteine to the liver. For other tissues, degradation of plasma GSH and GSSG and uptake of the resulting L-cyst(e)ine constitutes a major source of new L-cysteine for GSH and protein synthesis (L-cystine is rapidly reduced intracellularly by GSH). Because plasma GSH originates mainly from the liver, hepatic GSH synthesis begins a process that efficiently delivers L-cysteine to extrahepatic tissues to sustain both cellular GSH levels and protein synthesis [41,97,98].

Appreciation of the fact that GSH synthesis is often limited by L-cysteine availability has stimulated investigation of the relationship between diet and tissue GSH levels [99,100], the development of pharmacologically useful L-cysteine prodrugs [19,101,102] and the study of L-cyst(e)ine transport [89]. As expected, factors that stimulate L-cysteine or L-cystine uptake typically increase cell or tissue GSH levels. Nerve growth factor, for example, reportedly increases GSH levels in pheochromocytoma cells in part by stimulating L-cyst(e)ine uptake [103]. Although not affecting L-cysteine transport directly, the fact that some tumors express relatively high levels of γ-glutamyltranspeptidase allows them to efficiently degrade plasma GSH and GSSG, providing abundant L-cyst(e)ine for uptake and synthesis of intracellular GSH [104,105]. Maintenance of high GSH levels can, as noted, confer resistance to therapy [106–108].

Although L-glutamate and glycine levels are rarely, if ever, limiting for GSH synthesis [89], the fact that L-glutamate and L-cystine share the System χ_c^- amino acid transporter means that L-glutamate can affect L-cystine uptake and thus its availability for GSH synthesis. System χ_c^- is a Na^+-independent transporter that normally exchanges intracellular L-glutamate for extracellular L-cystine, thus facilitating GSH synthesis from the L-cysteine formed by intracellular reduction of L-cystine [89]. On the other hand, if extracellular levels of L-glutamate are high, L-cystine uptake by System χ_c^- is competitively inhibited, decreasing intracellular L-cysteine levels and limiting GSH synthesis. Plasma L-glutamate levels are high in patients with advanced cancer [109] and HIV infection [110], potentially limiting their ability to maintain GSH levels in tissues relying on L-cystine uptake.

As noted, some tissues take up L-cysteine rather than

[1] Cysteine and cystine are transported by distinct carriers, and tissues typically transport one more efficiently than the other. Although liver has little or no capacity for cystine transport, effluxing GSH reduces cystine at the cell surface, and the resulting cysteine is readily taken up. Other cell types take up L-cystine readily and reduce it intracellularly to L-cysteine in a GSH-mediated reaction (i.e., L-cystine + 2 GSH ⇆ 2 L-cysteine + GSSG).

L-cystine, and, for them, L-glutamate-mediated inhibition of System χ_c^- cystine transport is, of course, not relevant. Interestingly, in that circumstance high intracellular L-glutamate levels can stimulate GSH synthesis by overcoming nonallosteric feedback inhibition by GSH (next section) rather than by increasing substrate availability per se. This effect, which appears to be rare, is seen in dog erythrocytes, cells that have a genetically determined high uptake rate for L-glutamate and L-glutamine, a L-glutamate precursor. Such cells show abnormally high GSH levels [111].

In addition to its constituent amino acids, GSH synthesis requires ATP, and that substrate can also be limiting. Suzuki and Kurata [112], for example, found that GSH synthesis in erythrocytes depended on the level of ATP. Similarly, Papadopouos et al. [113] report that glucose deprivation injury in astrocytes is correlated with low GSH levels and suggest that ATP may limit synthesis when energy metabolism is disrupted. As the latter authors point out, glucose deprivation under normoxic conditions can occur in diabetes, and the resulting ATP and GSH depletion may sensitize tissues to oxidative damage. ATP may also limit GSH synthesis during ischemic injuries such as stroke.

Feedback inhibition of γ-glutamylcysteine synthetase

Richman and Meister reported in 1974 [91] that GSH is a nonallosteric feedback inhibitor of rat kidney γ-GCS. Glutathione binding was found to be competitive with L-glutamate ($K_i^{GSH} = 2.3$ mM) and dependent on the cysteinyl thiol group. Inhibition by L-γ-glutamyl-L-cysteine was comparable to that seen with GSH, but S-methyl-GSH, GSSG, ophthalmic acid (L-γ-glutamyl-L-α-aminobutyrylglycine) and L-γ-glutamyl-L-α-aminobutyrate were much weaker inhibitors [91]. Observation of competitive inhibition strongly suggested that the glutamyl residue of the peptides occupies the L-glutamate-binding site. Because L-cysteine binds more tightly than L-α-aminobutyrate ($K_m^{CYS} \ll K_m^{\alpha\text{-ABA}}$; Table 2), the relative strength of inhibition by GSH and ophthalmic acid suggested further that the cysteinyl or α-aminobutyryl residue of the inhibitory peptides might occupy the L-cysteine binding site of γ-GCS. Supporting this model, S-methyl-L-cysteine and L-α-aminobutyrate bind with comparable affinity as γ-GCS substrates, and S-methyl-GSH and ophthalmic acid are comparably effective as inhibitors. Other observations are less easily rationalized. L-cysteinylglycine is neither a substrate nor an inhibitor of γ-GCS, and it is thus difficult to postulate a binding site to be occupied by the glycyl moiety of inhibitory tripeptides. Similarly, inhibition by GSSG is not easily rationalized by the proposed binding model because L-cystine is not a substrate; inhibition by GSSG would seemingly require that the L-cysteine binding site accommodate the mixed disulfide of L-cysteine and GSH, a structure even larger than L-cystine.

In more recent studies, Huang et al. [92,93] established that thiols and γ-GCS_L significantly modulate peptide binding and feedback inhibition. Thus, whereas ophthalmic acid and dithiothreitol are very weak inhibitors of γ-GCS holoenzyme when added separately, ophthalmic acid in the presence of dithiothreitol inhibits moderately well with a K_i of 11.4 mM. In contrast, addition of thiols has little effect on inhibition of γ-GCS_H by ophthalmic acid ($K_i = \sim$12.5 mM with or without dithiothreitol) [92]. These observations led to the suggestion that GSH and other thiols reductively alter γ-GCS in a manner that decreases the specificity of the substrate binding sites and, thus, increases the ability of di- and tri-peptide inhibitors to bind to the holoenzyme [92]. Based on an original suggestion by Huang et al. [92], Figure 1 shows a plausible model that attributes the critical reductive alteration of γ-GCS to cleavage of an intersubunit disulfide bond. Although alternative or additional thiol-mediated changes might occur, the model illustrated accounts conceptually for the results reported to date.

As shown in Table 2, Huang et al. [92,93] report that GSH is a much less effective inhibitor of rat kidney γ-GCS holoenzyme than of γ-GCS_H. Conversely, the holoenzyme has better affinity for L-glutamate than does γ-GCS_H. These differences led to the suggestion that γ-GCS_H would be an inefficient catalyst at physiologic concentrations of GSH (1–8 mM [95]) and L-glutamate (1–4 mM [87]). For example, at 2 mM L-glutamate and 4 mM GSH, the kinetic constants reported by Huang et al. [92,93] predict that γ-GCS_H would have <7% of the activity of the holoenzyme. Because some tumors overexpress only γ-GCS_H [45,46], the issue is of potential clinical importance; if γ-GCS_H exhibits little activity in vivo, such tumors may not exhibit the GSH-dependent resistance to therapy characteristic of tumors overexpressing γ-GCS holoenzyme. Indeed, Mulcahy et al. [114] expressed human γ-GCS and γ-GCS_H in COS cells, which have low endogenous γ-GCS activity, and found that the holoenzyme is more effective than γ-GCS_H in increasing cellular GSH levels. However, unless COS cells maintain unprecedentedly high L-glutamate levels, the modest GSH differences seen by Mulcahy et al. [114] are much less than predicted by the kinetic constants reported by Huang et al. [92,93] for rat holoenzyme and γ-GCS_H.

Addressing this discrepancy, we have recently expressed and purified human γ-GCS and γ-GCS_H, and find that the differences in K_m^{GLU} and K_i^{GSH} for human γ-GCS and γ-GCS_H are much smaller than reported for rat (Table 2) ([30]; I. Misra and O. W. Griffith, unpublished). The results with the expressed human proteins

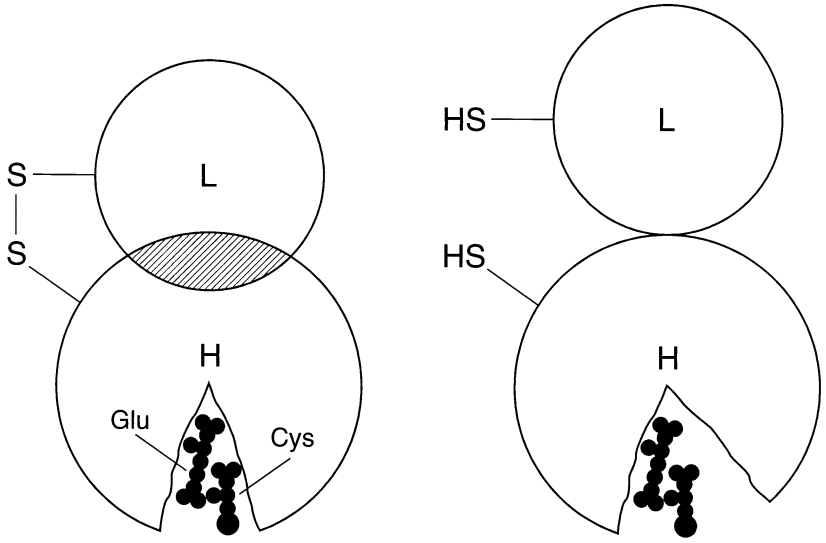

Fig. 1. Model of how reductants affect γ-GCS. The left panel shows the heavy (H) and light (L) subunits of γ-GCS connected by a disulfide bond; the substrate binding site is relatively tight, making it difficult for GSH and other tripeptides to bind. The right panel shows the subunits after reduction of the intersubunit disulfide bond. A conformational change has relaxed the substrate binding site such that tripeptides would be bind and inhibit with greater affinity. Based on a drawing by Huang et al. [92].

suggest that at 2-mM L-glutamate and 4-mM GSH, γ-GCS_H should thus have about 35% the activity of the holoenzyme. Although the L-glutamate concentration in COS cells is not known, our kinetic results are in general agreement with the observation by Mulcahy et al. [114] that γ-GCS_H transfection increases GSH levels ~46% as much as does transfection with γ-GCS holoenzyme. In contrast, the transfected COS cell results are not consistent with kinetic constants reported for His-tagged human γ-GCS and γ-GCS_H (Table 2) [94]. Those kinetic data, which imply that γ-GCS_H would be essentially inactive at any plausible intracellular L-glutamate and GSH levels, were obtained with enzyme preparations having very low specific activity [94]; we conclude that the His-tagged γ-GCS proteins do not reflect the kinetic properties of the native species.

Regulation of γ-GCS by post-translational modification

Following an early suggestion by Estrela et al. [115], Lu et al. reported in 1991 [116] that cholera toxin, dibutyryl cAMP, glucagon, phenylephrine, vasopressin, and phorbol ester decreased GSH synthesis in cultured rat hepatocytes. Based on inhibitor studies, the authors attributed decreased synthesis to phosphorylation and consequent inhibition of γ-GCS by either cAMP-dependent protein kinase (PKA) or protein kinase C (PKC). Studies with isolated γ-GCS established that PKA, PKC, and Ca^{2+}/calmodulin-dependent protein kinase II (CMK) can all phosphorylate the γ-GCS heavy subunit on serine and threonine residues. Phosphorylation was Mg^{2+}-concentration dependent with ~20% inhibition seen at 1 mM Mg^{2+}, a physiologic concentration. At higher Mg^{2+}-concentrations, incorporation of phosphate ranged from 0.6- to 1.17-mol/mol γ-GCS, and in all cases phosphorylation decreased V_{max} without affecting K_m^{GLU} or K_m^{CYS} or causing subunit dissociation [117]. As the authors point out, even 20% inhibition of γ-GCS can have a biologically significant effect on GSH levels, particularly in liver where activation of PKA and PKC also stimulates GSH efflux [118]. Injury-induced elevation of intracellular-free Ca^{2+} would, for example, be expected to increase γ-GCS phosphorylation and thereby diminish the effectiveness of GSH-dependent protective mechanisms [20,117]. Interestingly, Goss et al. [119] report that hepatocytes from protein-energy malnourished rats are not susceptible to cAMP-mediated inhibition of GSH synthesis. This adaptation has the advantageous consequence of allowing feeding to rapidly restore GSH levels in starved animals, but the mechanism accounting for loss of inhibition is not yet established.

Most eukaryotic γ-GCS are potently inhibited by cystamine and other agents able to modify a specific cysteine residue in the L-glutamate binding site of the heavy subunit [1]. Because there is now abundant evidence that many protein thiols can be S-nitrosylated under conditions of nitrosative stress, we examined the effect of a variety of NO donors on rat γ-GCS activity. S-Nitroso-L-cysteine, S-nitroso-L-cysteinylglycine, and other small S-nitrosothiols were potent inhibitors, but S-nitroso-glutathione caused little inhibition. S-Nitrosothiol-mediated inhibition was prevented by prior reaction of γ-GCS with

cystamine. The data are consistent with direct transnitrosation between S-nitrosothiol and the active-site thiol. Other NO donors such as various NONOates also inactivated γ-GCS, but the reaction was less efficient. Exposure of mouse peritoneal macrophages to cytokines that induce expression of nitric oxide synthase caused increased NO synthesis with consequent γ-GCS inhibition and GSH depletion. Inhibitors of nitric oxide synthase prevented those effects [119,120]; J. Han, H. Li, J.S. Stamler, and O.W. Griffith, unpublished). The findings suggest that GSH synthesis will be acutely compromised at sites of rapid nitric oxide synthesis.

Perhaps compensating for NO-mediated inhibition, Kuo et al. [66] report that interleukin-1-mediated induction of γ-GCS in hepatocytes is NO-dependent, and Moellering et al. [65] report that vascular smooth muscle cells exposed for several hours to donors releasing 7–14 nM NO/sec, a physiologically relevant rate, exhibit initial GSH depletion followed by enhanced expression of γ-GCS. The mechanism(s) accounting for γ-GCS induction in these systems is (are) not yet established although the authors provide evidence and argument against simple oxidant stress-mediated induction and cGMP-dependent or protein kinase G Iα-dependent mechanisms. Although additional studies are necessary, it appears that transcriptional activation of γ-GCS expression may result in increased γ-GCS activity at low to moderate levels of NO production. The net effect on activity at sites experiencing higher rates of NO synthesis (e.g., sites of inflammation) are harder to predict. If direct post-translational inhibition of γ-GCS by NO is more important than increased expression, tissue damage could be augmented by decreased antioxidant defenses.

Pharmacologic control of γ-GCS

Because GSH has a critical role in the detoxification of electrophiles and oxidative and nitrosative stress agents, strategies for pharmacologically maintaining or increasing tissue GSH levels have received considerable attention. Most commonly, L-cysteine precursors are administered to increase the availability of this limiting amino acid and to replace L-cysteine irreversibly lost through formation of GSH S-transferase products. Because L-cysteine itself auto-oxidizes to insoluble L-cystine and is reportedly toxic to cultured cells and newborn mice [19], most studies and trials are carried out using N-acetyl-L-cysteine, a species that can be given orally or intravenously and that is readily deacetylated intracellularly. A wide range of additional L-cysteine precursors have been developed and studied at least in experimental animals. Most are made by reaction of a physiologically acceptable aldehyde with L-cysteine to form a 2-substituted-4-carboxythiazolidine. These derivatives protect the L-cysteine thiol from oxidation but spontaneously dissociate in vivo to reform L-cysteine and the original aldehyde. Among the aldehydes used in this way are acetaldehyde and ribose [121].

2-Oxothiazolidine-4-carboxylate (OTC) represents an alternative approach to protecting the L-cysteine thiol in a biologically reversible way. This agent is an alternative substrate for 5-oxo-L-prolinase, a widely distributed intracellular enzyme; enzymatic ring opening forms S-carboxy-L-cysteine, which spontaneously loses CO_2 to form L-cysteine [19,101–103,122]. Recently Chen et al. [123] have shown that at least some tumors are relatively 5-oxo-L-prolinase deficient, suggesting that OTC may be useful in selectively protecting normal tissues from cytotoxic cancer chemotherapy agents.

Because GSH itself is taken up poorly or not at all by cells and is rapidly degraded in the gut and circulation, oral or intravenous administration of GSH serves only to provide cells with its constituent amino acids [1–3]. GSH is a good (albeit expensive) precursor of L-cysteine, and in this sense oral or intravenous administration of GSH mimics the physiologic release of GSH by liver into the bile or blood, respectively. Esters of GSH, on the other hand, are readily taken up by cells and are de-esterified intracellularly to release GSH [19,101,102]. Because administration of GSH esters bypasses the regulated steps in GSH synthesis, cellular levels well above physiologic can be attained. Most commonly, ethanol is used as the esterifying alcohol, but other physiologically tolerated small alcohols appear to serve just as well. Both monoesters (esterified on the glycyl carboxylate) and diesters of GSH are effective. Recent studies show that the diester is more effective because it is rapidly transported into cells and converted to the monoester, which does not efflux. Slower intracellular hydrolysis of the monoester yields GSH [124]. It should be noted that in several early studies significant cytotoxicity was attributed to GSH esters, particularly monoesters. More recent work has established that toxicity is due to heavy metal contaminants in the preparations; metal-free preparations reportedly show little, if any, toxicity [19,101,124].

While strategies for increasing GSH levels were developed to increase cellular defenses and resist toxicity, strategies for depleting GSH were developed to increase the sensitivity of tumors and certain parasites to radiation, drugs or endogenous killing mechanisms [1,106–108,125]. Two basic approaches to GSH depletion have been used, administration of agents that oxidize or derivatize GSH and administration of γ-GCS inhibitors. Because it is difficult to selectively oxidize or react with GSH, the former approach is potentially problematic and finds little application in systems more complex than cultured cells [126]. Nonetheless, GSH depletions effected by thiol-reactive oxidants and electrophiles are

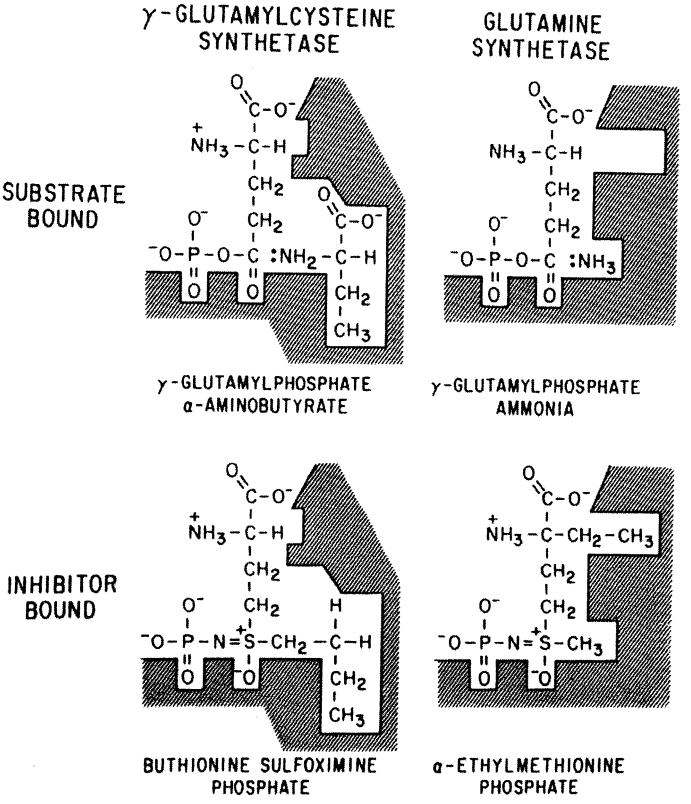

Fig. 2. Binding of substrates and sulfoximine inhibitors to γ-GCS. The left panels show the binding of L-γ-glutamylphosphate and L-α-aminobutyrate to γ-GCS (upper panel) and the isosteric binding of L-S-BSO-P to the same enzyme (lower panel). The right panels show similar interactions for glutamine synthetase. The upper panel shows L-γ-glutamylphosphate and ammonia, and the lower panel shows isosteric binding of γ-ethyl-L-methionine sulfoximine phosphate. Note that the selectivity of BSO for γ-GCS is controlled by the larger S-alkyl group, which is not accommodated by glutamine synthetase. Adapted from Griffith and Mulcahy [1].

more rapid and often more extensive than can be obtained with γ-GCS inhibitors. Commonly used oxidants include diamide [diazenedicarboxylic acid bis(N,N-dimethylamide] and various organic hydroperoxides; e.g., tert-butyl hydroperoxide. Commonly used electrophiles are diethyl maleate, other α,β-unsaturated carbonyl compounds, and various cytochrome P450 substrates [126].

Glutathione can also be depleted by inhibiting γ-GCS and allowing the ongoing reactions of GSH utilization, mainly efflux, to deplete cellular stores. With this strategy the rate of GSH depletion depends on the rate of GSH utilization and varies from tissue to tissue. In mice administered buthionine sulfoximine (BSO), the most commonly used inhibitor, kidney, liver, and pancreas deplete quickly ($t_{1/2}$ 30–60 min), most other tissues deplete significantly within a several hours, and a few cell types such a red blood cells ($t_{1/2}$ ~4 d [127]) deplete too slowly for the loss to be easily measured [95]. Often depletion plateaus at GSH levels 10–20% of control; the residual pool is sequestered in the mitochondria, from which it effluxes slowly [128,129]. In contrast, most cells in culture deplete almost completely (>95%) within 24–48 h, usually with negligible toxicity if the cells are not otherwise stressed. A few tissues or cells apparently resist GSH depletion because the γ-GCS inhibitor used is not taken up well; failure to efficiently cross the blood-brain barrier may account in part for the slow GSH depletion seen in the brain after parenteral administration of BSO [95].

Over a dozen γ-GCS inhibitors have been described and recently reviewed [1]. Although many of those inhibitors served to elucidate the chemical mechanism or substrate binding site geometry of γ-GCS, the only inhibitors that are currently used with cultured cells or in vivo are the various S-alkyl-L-homocysteine sulfoximines. Although the parent compound in this series, L-methionine sulfoximine, inhibits glutamine synthetase more effectively than γ-GCS and is therefore toxic, derivatives with S-alkyl substituents of n-propyl or larger are γ-GCS selective and highly effective inhibitors in vitro and in vivo. The most widely used inhibitor is L-buthionine-SR-sulfoximine (L-SR-BSO), a commercially available mixture of two diastereomers [130].

Although both the L,S- and L,R-diastereomers of

BSO bind to γ-GCS as transition state analogs, only L-buthionine-S-sulfoximine (L-S-BSO) is a mechanism-based inhibitor and subject to ATP-dependent, enzyme-catalyzed phosphorylation on the sulfoximine nitrogen to form L-buthionine-S-sulfoximine phosphate (L-S-BSO-P) [131]. In the presence of MgATP, that product is bound very tightly, but noncovalently to the γ-GCS active site. Because cells contain MgATP, BSO is generally regarded as an irreversible inhibitor when used in vivo or with cells.

We have recently examined this assumption in more detail by measuring the rate at which L-S-BSO-P dissociates from purified human γ-GCS. Although the dissociation rate is extremely slow (1–3%/h initially [I. Misra and O.W. Griffith, unpublished]), it is not negligible in terms of γ-GCS reactivation following a pulsed exposure to BSO (i.e., cells exposed to BSO and then replated in BSO-free medium or animals given a bolus injection of BSO). Because reactivation of even a small fraction of cellular γ-GCS can rapidly replete a substantial amount of GSH when initial GSH levels are low, this slow dissociation of L-S-BSO-P may be pharmacologically important. Unfortunately, it is not possible from available data to determine if restoration of GSH synthesis after BSO washout is due to reactivation of γ-GCS or to de novo γ-GCS synthesis. De novo synthesis is likely to be at least contributory, however, because it is known that BSO exposure increases γ-GCS transcription (Table 1) [55,74].

Of the simple S-alkyl-L-homocysteine sulfoximines, BSO is the optimal mammalian γ-GCS inhibitor. Alkyl groups shorter than n-propyl allow significant inhibition of glutamine synthetase and alkyl groups larger than n-pentyl yield homologs that are toxic when administered to mice [130]. As shown in Fig. 2, the S-butyl group of BSO is thought to be bound in the site normally occupied by L-cysteine or the commonly used L-cysteine mimetic, L-α-aminobutyrate. Missing from the BSO structure, however, is anything to occupy the site normally occupied by the carboxylate of L-cysteine. Anderson and Meister [132] reported that S-((2-methyl)butyl)-L-homocysteine sulfoximine, in which the 2-methyl group would be located in the L-cysteine carboxylate site, has a Ki_{app} ~30-fold higher than that of L-SR-BSO, suggesting that substitution is not helpful. Very recently, Tokutake et al. [133] have synthesized S-((2-carboxy) butyl)-L-homocysteine sulfoximine and reported that it is a very potent inhibitor of *E. coli* γ-GCS, an enzyme only slowly inhibited by L-SR-BSO ([131]; M.A. Hayward and O.W. Griffith, unpublished). We have prepared compounds of similar structure and find that they are good inhibitors of human γ-GCS (B.R. Babu, I. Misra, and O.W. Griffith, unpublished).

Although Table 1 lists a few agents that decrease γ-GCS transcription, none is sufficiently selective to be pharmacologically useful for reducing cellular γ-GCS activity and GSH levels. Kijima et al. [134] recently reported, however, the construction of two hammerhead ribozymes against $\gamma\text{-GCS}_H$ and $\gamma\text{-GCS}_L$ mRNA, and showed that transfection of these species into a mouse pancreatic islet cell line resulted in decreased γ-GCS activity and reduced GSH levels. Additional studies with these ribozymes are eagerly awaited.

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ABBREVIATIONS

GSH—glutathione
GSSG—glutathione disulfide
NO—nitric oxide
γ-GCS—γ-glutamylcysteine synthetase
γ-GCS_H—γ-GCS heavy subunit
γ-GCS_L—γ-GCS light subunit
IL-1β—interleukin-1β
TNF-α—tumor necrosis factor-α
AP-1—activator protein-1
AP-2—activator protein-2
NF-κB—nuclear factor kappa B
ARE/EpRE—antioxidant and elctrophile response elements
Sp-1, PKA—cAMP-dependent protein kinase
PKC—protein kinase C
CMK—Ca^{2+}/calmodulin-dependent protein kinase II
OTC—2-oxothiazolidine-4-carboxylate
BSO—buthionine sulfoximine
L-SR-BSO—L-buthionine-S,R-sulfoximine
L-S-BSO—L-buthionine-S-sulfoximine
L-S-BSO-P—L-buthionine-S-sulfoximine phosphate