Glutamate Cysteine Ligase Catalysis

DEPENDENCE ON ATP AND MODIFIER SUBUNIT FOR REGULATION OF TISSUE GLUTATHIONE LEVELS*

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Glutamate cysteine ligase (GCL), which synthesizes γ-glutamylcysteine (γ-GC), is the rate-limiting enzyme in GSH biosynthesis. γ-GC may be produced by the catalytic subunit GCLC or by the holoenzyme (GCLholo), which comprises GCLC and the modifier subunit GCLM. The Gclm(−/−) knock-out mouse shows tissue levels of GSH that are between 9 and 40% of the Gclm(+/+) wild-type mouse. In the present study, we used recombinant GCLC and GCLM and Gclm(−/−) mice to examine the role of GCLM on γ-GC synthesis by GCLholo. GCLM decreased the Km for ATP by ~6-fold and, similar to other species, decreased the Km for glutamate and increased the Kf for feedback inhibition by GSH. Furthermore, GCLM increased by 4.4-fold the Kνmax for γ-GC synthesis; this difference in catalytic efficiency of GCLholo versus GCLC allowed us to derive a mathematical relationship for γ-GC production and to determine the relative levels of GCLholo and GCLC; in homogenates of brain, liver, and lung, the ratio of GCLC to GCLholo was 7.0, 2.0, and 3.5, respectively. In kidney, however, the relationship between GCLC and GCLholo was complicated. Kidney contains GCLholo, free GCLC, and free GCLM, and free GCLC in kidney cannot interact with GCLM. Taken together, we conclude that, in most tissues, GCLM is limiting, suggesting that an increase in GCLM alone would increase γ-GC synthesis. On the other hand, our results from kidney suggest that γ-GC synthesis may be controlled post-translationally.

GSH is an abundant cellular antioxidant, being maintained at millimolar concentrations in most, if not all, cell types (1–3). Although the GSH concentration varies as much as 10-fold between different cell types, the concentration of GSH in a given cell type is quite consistent, unless modified by cellular redox reactions or perhaps external stimuli. Because of its elevated levels and high reduction potential, GSH together with GSSG are considered an important cellular redox couple. Because of its elevated levels and high reduction potential, GSH is a cofactor for many enzymes, including the glutathione transferases (5, 6). Thus, through enzyme reactions, GSH is both directly and indirectly involved in the elimination of reactive oxygen metabolites. GSH is also a signaling molecule. It is added to proteins as a post-translational modification (7). It modifies the activity of neurotransmitter receptors and may itself be active as a neurotransmitter (10). In conclusion, GSH is a multifunctional molecule with diverse and still emerging functions.

GSH is a tripeptide that is synthesized by two successive enzymatic reactions. The first, ligation of glutamate and cysteine, is catalyzed by glutamate cysteine ligase (GCL)(2) and forms γ-glutamylcysteine (γ-GC). The second, coupling of γ-GC with glycine, is catalyzed by glutathione synthetase to form GSH. The rate-limiting step in GSH biosynthesis is catalyzed by GCL (2). In its catalytically most active form, GCL is composed of a catalytic (GCLC) and a modifier (GCLM) subunit; these subunits are encoded by separate genes on different chromosomes. GCLC possesses the catalytic capacity for γ-GC formation. GCLM interacts with GCLC, making the holoenzyme (GCLholo) kinetically more efficient in γ-GC synthesis. Such roles for GCLC and GCLM have been suggested by experiments performed using knock-out mice (11). Disruption of the Gclc gene is embryolethal. Cells harvested from a Gclc(−/−) blastocyst inner cell mass continue to grow in culture, however, in the presence of GSH or N-acetylcysteine. When Gclc(−/−) cells are cultured in N-acetylcysteine, GSH is below the detection limit of about 1 μM, suggesting that GSH may not be essential for cell survival (12, 13). Disruption of the Gclm gene in mice, on the other hand, leads to no overt phenotype. The tissues from Gclm(−/−) mice, however, exhibit low GSH levels, and cells derived from these mice are very sensitive to oxidant exposure.

Studies examining the impact of GCLM on γ-GC synthesis have been conducted with the GCL enzyme, isolated or expressed from several species (11, 14–16). GCL uses three substrates: L-glutamate, L-cysteine, and ATP. For the rat, the GCLC Km for glutamate was determined to be 18.2 mM, 8.8-fold higher than the GCLholo and substantially higher than the cytoplasmic glutamate concentration in most tissues (14, 17–19). In contrast, the Km for glutamate has been demonstrated to be at or below the cellular glutamate concentration for partially purified or recombinant GCLholo from Drosophila, mice, or humans (11, 15, 16); for these species, GCLM did not modify the Km for cysteine. The impact of GCLM on the Km for ATP has not been reported. Studies on GCLC from each of these species also demonstrate competitive inhibition of glutamate binding by GSH; such inhibition occurs in the low millimolar concentration range and may be particularly important in cell types that accumulate high GSH levels, such as those in liver and kidney. Such inhibition is partially alleviated by GCLM binding (11, 14). Finally, under substrate saturation, there is conflicting evidence that GCLholo is catalytically more efficient than GCLC, possessing a higher Vmax (14–16).

Results from mice lacking GCLM clearly demonstrate the importance of the modifier subunit in maintaining cellular GSH levels (11).

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2 The abbreviations used are: GCL, glutamate cysteine ligase; γ-GC, γ-glutamylcysteine; GCLholo, GCL holoenzyme; FPLC, fast liquid protein chromatography; GCLC, GCLC-bound GCLC; GCLM, free GCLM.
This finding, along with recent epidemiological and biochemical data demonstrating a dramatic impact of seemingly modest decreases in GCLM on the risk of myocardial infarction (20–23), underscore the need to understand more fully the mechanism(s) by which GCLM controls GSH levels. In the present study, we employ recombinant GCLC, GCLM, and Gclm(−/−) mice to investigate the mechanisms and extent to which GCLM regulates GSH accumulation.

MATERIALS AND METHODS

Chemicals—All chemicals were purchased from Sigma.

Gclm(−/−) Mice—The generation of Gclm(−/−) mice has previously been described (11). Gclm(+/+) and Gclm(−/−) were generated by crossing Gclm(+/+) mice. All animal experiments were conducted on littermates, and all studies were approved by the University of Cincinnati Medical Center Institutional Animal Care and Use Committee.

Construction, Expression, and Purification of GCLC and GCLM—cDNAs for mouse GCLC and GCLM were amplified (35 cycles) using Deep Vent Polymerase (New England Biolabs; Beverly, MA) from C57BL/6j hepatic RNA, following reverse transcription using oligo(dT) and Superscript (Invitrogen). The cDNAs were cloned into pSET vectors (Invitrogen) in frame with an N-terminal polyhistidine tag. Following transformation, the bacterium Escherichia coli BL21(DE3) PLYSS, containing either the GCLC or GCLM cDNA, was induced for 2 h with isopropyl-1-thio-β-D-galactopyranoside (final concentration 1 mM), and lysed (lysis buffer was 20 mM Tris-HCl, 500 mM KCl, 10 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, and 5 mM 2-mercaptoethanol, pH 7.9) by sonication. Lysates were cleared by centrifugation at 20,000 g for 1 h, and the supernatant was applied to nickel resin (Qiagen, Valencia, CA). Columns were washed with washing buffer (20 mM Tris-HCl, 150 mM KCl, 30 mM imidazole, 10% glycerol, and 5 mM 2-mercaptoethanol, pH 7.9) and eluted with elution buffer (20 mM Tris-HCl, 150 mM KCl, 300 mM imidazole, 10% glycerol, and 5 mM 2-mercaptoethanol, pH 7.9). Purified proteins, >95% homogeneous by Coomassie staining, were dialyzed overnight against dialysis buffer (10 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl₂, and 5 mM 2-mercaptoethanol, pH 8.4) and stored in 25% glycerol at −20 °C until use.

Tissue Homogenate Preparation—Tissue homogenates, as indicated, were prepared from freshly isolated tissue by homogenization in 9 volumes of homogenization buffer (154 mM KCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM MgCl₂, 0.1 M potassium phosphate, and 5 mM 2-mercaptoethanol, pH 6.8) using a Teflon homogenizer and clarified for Western immunoblot.

Gel Filtration Chromatography—Tissue GCLholo, GCLC, and GCLM were separated by fast liquid protein chromatography (FPLC) as previously described (11). Briefly, 1 ml of tissue cytosolic fractions was applied to a HiPrep 16/60 Sephacryl S-100 High Resolution column pre-equilibrated with homogenization buffer, and protein was separated with homogenization buffer. Elution fractions (0.5 ml) were collected and used for GCL activity assay immediately or stored in −80 °C for Western immunoblot.

PAGE and Western Immunoblot Analysis—Nondenaturing PAGE was conducted using a 7.5% separating gel cast using polyacrylamide and 1.5 M Tris-HCl (pH 8.8) and a 4% stacking gel cast using polyacrylamide and 0.5 M Tris-HCl (pH 6.8) as described (24); loading samples were performed in 0.125 M Tris-HCl (pH 6.8) and 7.5% glycerol. 12% denaturing SDS-PAGE was performed as previously described (25) to resolve tissue cytosolic or FPLC fractions. Western immunoblot analysis for the GCLC and GCLM proteins was conducted using rabbit polyclonal anti-GCLC (1:30,000) and anti-GCLM (1:25,000), respectively, as previously described (11).

Enzyme Activity and Kinetic Analysis—Reactions to form γ-GC from l-glutamate and l-cysteine were conducted at 37 °C in the standard assay buffer (100 mM Tris-HCl, 20 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 3 mM dithiothreitol, 10 mM l-glutamate, 3 mM l-cysteine, and 10 mM ATP, pH 7.2). Such concentrations for individual substrates were selected to reach saturation. For Kᵣ and Kᵢ studies, concentration series of individual substrates were used, with other components unchanged in the standard assay buffer, as described under “Results.” A fluorescent derivative of γ-GC was generated using o-phthalaldehyde, and the derivative was separated from GSH and quantified, using high pressure liquid chromatography as previously described (11). Kinetic constants were calculated using the Sigma Plot Enzyme Kinetics Module (SPSS Inc., Chicago, IL).

Protein Quantification—Protein concentration was determined using the Bradford method (Bio-Rad).

Statistical Analysis—Statistical analyses were performed using SigmaStat Statistical Analysis software (SPSS). All data are reported as the means ± S.E.

RESULTS

In Vitro Formation of Holo-GCL—Mouse GCLC and GCLM proteins, containing polyhistidine tags, were expressed in E. coli, and each was purified to near homogeneity by affinity chromatography. GCLC is catalytically active in the formation of γ-GC, whereas GCLM is not. Interaction between GCLC and GCLM forms a heterodimeric complex, which hereafter is referred to as GCLholo. To compare the catalytic characteristics of GCLC and GCLholo, we first must demonstrate the efficient formation of the heterodimeric GCLholo complex. For this purpose, we added increasing amounts of GCLM to GCLC and followed the formation of the complex by nondenaturing polyacrylamide gel electrophoresis. Proteins were identified by Coomassie Blue staining (Fig. 1a). GCLM (30.5 kDa; isoelectric point 5.19) migrated most rapidly and was clearly separated from GCLC (72.6 kDa; isoelectric point 7.9). Upon the addition of GCLM to GCLC, a more slowly migrating complex was formed, probably representing GCLholo; antibodies to either GCLM or GCLC recognized this complex and confirmed its identity (Fig. 1b).

Immunoblots for GCLC demonstrated that increasing GCLM
Regulation of Glutamate Cysteine Ligase

TABLE ONE

| Enzyme    | \(V_{\text{max}}\) | \(K_{\text{cat}}\) | \(K_{\text{m}}\) | \(K_{\text{cat}}/K_{\text{m}}\) |
|-----------|---------------------|---------------------|---------------------|---------------------|
|           | \(\mu\text{mol/min/mg}\) | \(s^{-1}\) | \(s^{-1} \text{mM}^{-1}\) | \(s^{-1} \text{mM}^{-1}\) |
| GCLholo   | \(6.8 \pm 0.1\) | \(8.2 \pm 0.1\) | \(0.22 \pm 0.3\) | \(0.48 \pm 0.1\) |
| GCLC      | \(1.54 \pm 0.1\) | \(1.9 \pm 0.1\) | \(0.27 \pm 0.1\) | \(1.6 \pm 0.3\) |

TABLE TWO

Determination of \(K_i\) of GSH

| Enzyme | \(K_{\text{GSH}}\) | \(K_{S}\) | \(K_{I}\) | \(K_{S}\) | \(K_{II}\) |
|--------|---------------------|----------|----------|----------|----------|
| GCLholo | \(0.8 \pm 0.2\) | \(3.1 \pm 0.5\) | \(6.5 \pm 1.4\) | \(3.9 \pm 1.1\) |
| GCLC   | \(0.3 \pm 0.1\) | \(0.8 \pm 0.2\) | \(1.3 \pm 0.2\) | \(0.4 \pm 0.1\) |

GCL activity was only 2–8% that of GCLholo. Thus, compared with GCLholo, GCLC is very inefficient in \(\gamma\)-GC synthesis.

Catalytic Efficiency of GCLC Versus GCLholo—GCLC exerts a marked effect on GCLholo by increasing the maximal rate at which \(\gamma\)-GC is produced. At a fixed GCLC amount upon the addition of GCLM, the production of \(\gamma\)-GC, under substrate saturation, increased to a maximal \(4.4 \pm 0.1\)-fold (Fig. 2a); this increase in product formation was reflected as an increase in the maximal velocity for GCLholo, relative to GCLC (TABLE ONE). \(K_{\text{cat}}/K_{\text{m}}\), a measure of the catalytic efficiency of an enzyme, is about 14-fold higher with respect to glutamate and 24-fold higher with respect to ATP for GCLholo than GCLC (TABLE ONE).

Using GCLM Complementation to Determine GCLC and GCLholo Concentrations—The sizeable difference in \(K_{\text{cat}}\) between GCLC and GCLholo may allow determination of the relative proportion of GCLC and GCLholo, in a given tissue or cell type, by confirming the following relationship. By definition, the GCL activity (GCLA) under substrate saturation is the sum of the activities of GCLC and GCLholo, or as follows.

\[
\text{GCLA} = \text{GCLC} + 4.4[\text{GCLCb}]
\]

For purposes of clarity, GCLC can be viewed as existing in two discrete pools: free GCLC (GCLCf) and GCLM-bound GCLC (GCLCb). In this case, the activity represented by GCLholo is equal to 4.4[GCLCb], representing the activity increase of GCLholo, where the constant 4.4 is equal to the -fold increase in \(\gamma\)-GC synthetic capacity observed when GCLM binds GCLC. Thus, the following is true.

\[
\text{GCLA} = \text{GCLCf} + 4.4[\text{GCLCb}]
\]

Combining Equations 3 and 4, we have the following.

\[
\text{GCLtot} = 4.4[\text{GCLCf}] + \text{GCLA} - \text{GCLCf}
\]
and the activity attributed to GCLCf is as follows.

\[
\text{GCLCf} = \frac{(\text{GCLtot} - \text{GCLa})}{3.4} \quad \text{(Eq. 6)}
\]

Furthermore, the activity contributed by GCLCb from Equation 3 is as follows.

\[
\text{GCLCb} = \frac{(\text{GCLa} - \text{GCLCf})}{4.4} \quad \text{(Eq. 7)}
\]

Therefore, in theory, by determining the activity of an enzyme preparation before and after complementation with GCLM, one may derive the relative proportions of GCLC and GCLholo. To test the validity of this relationship, we compared the activity of recombinant GCLC/GCLholo mixtures using recombinant enzymes (Fig. 2a) with the theoretical activity calculated using the molar ratio of GCLM added and the anticipated increase in activity using the equations above (Fig. 2b). The observed values were highly correlated \((r^2 = 0.9638)\) with the theoretical values.

**Complementation of Tissue Cytosols from Gclm\((-/-)\) Mice Using GCLM**—The use of the mathematical relationship described above to determine the relative levels of GCLC and GCLholo in cells or tissues depends on the assumption that complementation of tissue extracts with GCLM results in activity increases that approach a 4.4-fold maximum, compared with extracts devoid of GCLM. Because we previously had generated the \(\text{Gclm}\!(/-/-)\) knock-out mouse (11), we are in a position to test this possibility. In these analyses, we used tissue cytosolic fractions isolated from \(\text{Gclm}\!(/-/-)\) mice and measured \(\gamma\)-GC synthesis, following titration with GCLM (Fig. 3a). Of the tissues selected, liver and kidney have the highest levels of GSH and GCL among tissues surveyed, and lung and brain have relatively low levels (Fig. 3b). In all tissues, the addition of GCLM led to a dose-dependent increase in GCL activity, which was saturable. Also, as expected, the amount of GCLM needed for maximal GCL activity was considerably less with lung or brain cytosol, compared with liver or kidney cytosol. Liver, lung, and brain cytosol, when saturated with GCLM, showed 4.3-, 4.6-, and 4.3-fold increases, respectively; these values are not significantly different from that found with recombinant GCLC (Fig. 2a; TABLE ONE). On the other hand, saturation of GCLC from kidney cytosol resulted in only a 3.6-fold increase in activity. Thus, in many but not all tissues, GCLC shows an increase in catalytic efficiency similar to that with recombinant GCLC.

**GCLM Is Limiting in Liver, Lung, and Brain but Apparently Not in Kidney**—The difference in the maximal velocity of GCLC and GCLholo may be exploited to determine the relative amount of free GCLC in a given tissue, by comparing the maximal rate of \(\gamma\)-GC production of a tissue homogenate, before and after complementation with recombinant GCLM. In these analyses, cytosolic extracts were prepared from tissues derived from wild-type mice and assayed for \(\gamma\)-GC formation at saturating substrate concentrations. Parallel assays were complemented with GCLM and similarly assayed. The results in Fig. 3c show that \(\gamma\)-GC production increased significantly with GCLM complementation in all tissue cytosols assayed except kidney. The most dramatic increase in \(\gamma\)-GC production was observed in brain cytosol, which increased \(~3.1\)-fold following GCLM complementation. Using Equations 6 and 7 above, \(\text{GCLCf}/\text{GCLCb} = \text{GCLC}/\text{GCLholo} \) in brain was calculated to be \(3.0\). GCLC was also in excess in liver and lung, and in these tissues the \(\text{GCLC}/\text{GCLholo} \) ratio was 2.0 and 3.5, respectively. In kidney cytosol, GCLM was unable to increase \(\gamma\)-GC synthesis significantly, suggesting the absence of GCLCf in kidney extracts. As shown in Fig. 3a, however, the GCLC in kidney cytosol from \(\text{Gclm}\!(/-/-)\) mice failed to be fully complemented, upon the addition of GCLM. This intriguing finding suggests a difference in GCLC in kidney from that in the other tissues, which we explore below.

**Kidney GCLCf Fails to Interact with GCLM**—Based on their difference in size, GCLholo, GCLC, and GCLM in tissue cytosols are separable by gel filtration; this separation was performed on liver, lung, brain, and kidney cytosols, and GCLC and GCLM were detected by Western immunoblotting (data shown for liver and kidney; Fig. 4). In cytosolic proteins separated by gel filtration, GCLholo may be implied in fractions eluting early, as fractions that have both GCLC and GCLM on the Western immunoblot. Free GCLM was identified in fractions without associated GCLM. Based on these analyses, both liver and kidney contain GCLholo and GCLC. Although the precise ratio of GCLC and GCLholo (GCLCb) cannot be determined based on these analysis, principally because these peaks overlap, the ratio of GCLC to GCLholo, determined to be 2.0 in liver (see above), is consistent with this analysis. Free GCLM, eluted after GCLholo and GCLC, was not detected in liver (Fig. 4); this was also true in cytosolic fractions from lung and brain (data not shown) and is consistent with the notion (see above) that GCLC is in excess in these tissues.

In contrast, kidney cytosol contains free GCLM. Because liver cytosol from wild-type mice was complemented by the addition of recombinant GCLM, whereas kidney cytosol was not, we examined GCLM-mediated increases in \(\gamma\)-GC synthesis for partially purified fractions of GCLC from liver and kidney (Fig. 5a). For this analysis, we pooled gel filtration fractions devoid of GCLM, as assessed by Western immunoblot. GCLC
FIGURE 3. Complementation of tissue cytosolic fractions, using recombinant GCLM. a. Tissue cytosols (1 mg of protein) from Gclm(−/−) mice were mixed with increasing amounts of recombinant GCLM and incubated at 37 °C for 15 min and assayed for γ-GC formation at saturating substrate concentrations. The data depict the fold increase in γ-GC formation, compared with that in tissue cytosol alone. b, Western immunoblot for GCLC and GCLM in tissue cytosolic fractions (as indicated, 30 μg/lane) from Gclm(+/+) and Gclm(−/−) mice. c, tissue cytosols from Gclm(+/+) mice were assayed, or were mixed with saturating recombinant GCLM and then assayed, for γ-GC formation at saturating substrate concentrations. The fold increases after GCLM complementation, compared with tissue cytosol alone, are indicated. Data represent the means ± S.E. of triplicate determinations.

FIGURE 4. Separation of GCLholo, GCLC, and GCLM in liver and kidney cytosol by gel filtration chromatography. Kidney or liver cytosol, as indicated, isolated from wild-type mice, was separated by FPLC, and the fractions were analyzed for GCLC and GCLM by Western immunoblot analysis. Fractions corresponding to GCLholo, free GCLC, and free GCLM are indicated.
from liver was complemented by GCLM addition, and the increase in GCL activity (4.5-fold) is consistent with the increase noted above for recombinant GCLC for liver, brain, or lung tissue cytosols from Gclm(−/−) mice, was incubated with or without saturating recombinant GCLM at 37 °C for 15 min, followed by assay for γ-GC formation at saturating substrate concentrations. b, Western immunoblot analysis of GCLC in the eluate.

It should be noted that these analyses were performed under conditions of substrate saturation, and the kinetic properties of kidney GCLC were not further evaluated. We also evaluated the interaction of GCLM with partially purified GCLC from liver and kidney. GCLM modified the elution profile of partially purified liver GCLC, consistent with the formation of GCLholo, but did not affect the elution profile of kidney GCLC (data not shown), suggesting, as expected, that partially purified liver GCLC, but not kidney GCLC, interacts with GCLM.

DISCUSSION

GCL is the rate-limiting enzyme in GSH biosynthesis (28). Although bacteria apparently possess only a single gene encoding GCL, higher eukaryotes possess both GCLM and GCLC. Indeed, sequence analysis suggests that GCLM is present to complement GCLC, even in primitive eukaryotic organisms, but formal proof is still awaited. GCLC possesses all GCL catalytic activity and is therefore sufficient to fuel GSH biosynthesis. Why has nature diversified the control of γ-GC formation and, ultimately, GSH biosynthesis, to two unlinked gene products? It seems reasonable to speculate that the increased complexity of this two-gene system is intrinsic to the need of the cell to exert careful control over the synthesis of GSH and that such homeostasis may be more fully understood by understanding the catalytic differences between GCLC and GCLholo and the relative levels of these enzymes.

In this report as in others (29), it was demonstrated that the formation of GCLholo is efficient in vitro; this finding is important not only in assessing the catalytic properties of GCLC and GCLholo but also because it suggests that there is no barrier intrinsic to either subunit for the assembly of GCLholo in vivo. Thus, it is likely that newly synthesized GCLC or GCLM could associate with its previous heterodimerization partner within the cell (i.e. interaction does not depend on co-translation). Formal proof of this notion will require careful evaluation of γ-GC and GSH biosynthesis in cells containing ectopically regulated GCLC and GCLM genes in the absence of the endogenous genes. Such analyses should be possible as a consequence of the development of null mutants for both Gclc and Gclm (11, 12).

Impact of the Glutamate Concentration on γ-GC Biosynthesis—The catalytic characteristics of GCLC and GCLholo were first determined for rat GCL. In these studies, the $K_m$(Glu) values for purified and recombinant GCLC were determined to be 12.4 and 18.2 μM, respectively, whereas the $K_m$(Glu) for purified and recombinant GCLholo were determined to be 1.4 and 2.8 μM, respectively (14, 29). Based on these data, GCLC was predicted to function inefficiently at normal cellular concentrations of glutamate. Subsequent studies utilizing recombinant human (16), recombinant Drosophila (15), purified mouse (11), and recombinant mouse (the present study) have shown that the $K_m$(Glu) for GCLC ranges between 1.7 and 3.5 μM, and the $K_m$(Glu) for GCLholo ranges between 0.5 and 1.8. Thus, in all studies, the $K_m$(Glu) for GCLC is substantially higher than that for GCLholo.

The intracellular concentration of glutamate has been determined in several studies to be between 4 and 30 μM, depending on the cell or tissue type (14, 17–19). Thus, in many cell types, the cellular concentration of glutamate is greater than or equal to the $K_m$(Glu) for GCLC. Hence, although GCLC is less efficient compared with GCLholo with regard to glutamate, the effect of glutamate on GSH biosynthesis by GCLC versus GCLholo may be modest in many cell types. This conclusion may not be true in rats, in which the $K_m$(Glu) for GCLC is substantially higher than that reported for other species.

Impact of the ATP Concentration on γ-GC Biosynthesis—The $K_{m(ATP)}$ (TABLE ONE) was found to differ appreciably (6-fold) between mouse GCLC and GCLholo and, like $K_m$(Glu), was lower for GCLholo. The $K_{m(ATP)}$ comparing GCLC and GCLholo for other species, is not reported in the literature. The recombinant GCLC and GCLholo purified for these analyses contain an N-terminal polyhistidine tag. Out of concern that this tag might influence the determination of $K_{m(ATP)}$, we also determined the $K_{m(ATP)}$ of GCLC and GCLholo, partially purified from liver. The $K_{m(ATP)}$ values for GCLC and GCLholo from liver were 6.3 and 0.41 μM, respectively; these values agree well with those reported in TABLE ONE for the recombinant proteins.

The cellular ATP concentration in a variety of cell and tissue types ranges from 1 to 7 mM (30–33). Based on these values, it may be concluded that GCLC produces γ-GC at only approximately half-maximal velocity, even in cell or tissue types containing the highest levels of ATP. In contrast, the $K_{m(ATP)}$ for GCLholo is below the cellular concentration of ATP, even in cell or tissue types having relatively low levels of ATP. Based on the $K_{m(ATP)}$ values and ATP concentrations, the obvious conclusion is that ATP concentrations in the normal cellular range may have a profound impact on the velocity of γ-GC production. In the absence of experimental proof, however, caution should be exercised before adopting this view. Nevertheless, the difference in $K_{m(ATP)}$ between GCLC and GCLholo is intriguing, and further study regarding the impact of cellular ATP on GSH biosynthesis seems warranted.

Impact of GSH Concentration on γ-GC Biosynthesis—Determinations using human, rat, mouse, and Drosophila GCLC and GCLholo.
much lower constants, GSH tends to compete greatly with the glutamate binding site on the noncompetitive inhibitor. Based on the determined inhibition constant, GSH favors to bind to ATP-bound enzyme, indicated by a much lower $K_{i}$ (ATP) than $K_{i}$ (Glu); on the other hand, GSH favors to bind to ATP-bound enzyme, indicated by a much lower $K_{i}$ (ATP) than $K_{i}$ (Glu). In both cases, GCLM bound to GCLC decreases dramatically the sensitivity of the holoenzyme to GSH inhibition, presumably by changing the conformation of the enzyme (Fig. 6). It should be noted that the pathways shown in Fig. 6 are kinetic, not mechanistic.

**Impact of GCLM on GCL Catalysis**—Studies using rat purified and recombinant GCLC and GCLholo suggested little difference in the maximal velocity of these enzymes under substrate saturation (14). In contrast, although the specific activity of recombinant Drosophila GCLC and GCLholo is comparatively low, a 3-fold increase in the $V_{\text{max}}$ of GCLholo compared with GCLC was reported (15). Similarly, using recombinant human GCLC and GCLholo, a 5-fold increase in the $V_{\text{max}}$ of GCLholo compared with GCLC was reported (16). Herein we report a 4.4-fold increase in mouse GCLholo compared with GCLC. Furthermore, $\gamma$-GC synthesis was found to increase in a linear fashion, following complementation of recombinant GCLC with increasing amounts of GCLM. Such properties of these enzymes allowed derivation of a mathematical relationship for theoretical complementation of GCLC with GCLM, which agreed well with experimental values. Using tissue extracts from Gclm(−/−) mice, we confirmed that, at least for liver, lung, and brain, GCLC is able to be complemented by the addition of GCLM, to achieve the theoretical maximal increase in $\gamma$-GC synthetic capacity. These data suggest that, at least in these tissues, the relative level of $\gamma$-GC synthesis probably reflects the kinetic constants determined by our in vitro analysis using recombinant proteins. The establishment of this mathematical relationship, in conjunction with complementation of wild-type tissue extracts, further allowed the calculation of the relative levels of free GCLC and GCLholo in tissues.

**GCLM Is Limiting in Most Tissues**—We conducted detailed analyses of liver, lung, and brain to ascertain the relative levels of GCLC and GCLM; in these tissues, GCLC was found to be in excess by 2.0-, 3.5-, and 7-fold, respectively. We believe these determinations to be reliable for three reasons: (a) extracts from these tissues, prepared from Gclm(−/−) mice, became fully complemented upon the addition of GCLM (this suggests that no factors present in the extract, nor alterations as a result of extract preparation, negatively affect the interaction between GCLC and GCLM); (b) chromatography of cytosolic fractions, from these tissues isolated from wild-type mice, detected no free GCLM (this suggests that dissociation of the GCLC and GCLM occurs as the result of extract preparation); and (c) the relative levels of GCLholo, GCLC, and GCLM, as detected in Western immunoblots following gel filtration, qualitatively agree with our results of complementation. We also conducted complementation on several other tissues, and all studies demonstrated an increase in $\gamma$-GC synthetic capacity. The results of these analyses show that, in cytosol from heart, spleen, pancreas, and skeletal muscle, the GCLC/GCLM ratio was 1.5, 1.5, 2.0, and 4.5, respectively. It must be noted that neither experiments to complement these tissues from Gclm(−/−) mice nor gel filtration followed by Western immunoblotting were conducted using these tissues. Nevertheless, the ability to complement these tissues from wild-type mice does suggest an excess of GCLC, which is competent and able to interact with GCLM.

Similar conclusions, regarding a tissue excess of GCLC, have been recently reported using Western immunoblot analysis (34). In that study, cytosols from mouse heart and liver were analyzed, and the GCLC/GCLM ratio was determined to be 1.0 and 10, respectively; in heart, the GCLC/GCLM ratio was similar to that found in our present study. In liver, the ratio was considerably higher than what we have determined, 10 compared with 2.0; the reason for this is unclear. Nevertheless, it is intriguing to speculate that the ratio of GCLC/GCLM is dynamic and may fluctuate, depending on the metabolic state of the cells being measured.

Overall, these data suggest that, in most tissues, the GCLC/GCLM ratio is >1.0. As a consequence, it may be that many cell types stand poised to increase their $\gamma$-GC synthesis by increasing GCLM. In many studies, the GCLC and GCLM genes have been demonstrated to be transcriptionally responsive to extracellular stimuli, including reactive oxygen species (35). In many instances, this regulation may be coordinated for both genes (36–38); in other cases, induction of one gene is favored over the other (39–41). As the regulation of these genes becomes better defined, it will be interesting to correlate the signal transduction mechanisms responsible for their disparate regulation with the ability and need to synthesize GSH.

**Kidney Has Free GCLC That Fails to Interact with GCLM**—Of the tissues that we tested, only kidney GCLC failed to be complemented upon the addition of GCLM (Fig. 3c). Analysis of kidney cytosol by...
FPLC revealed GCLholo, GCLC, and GCLM (Fig. 4). GCLC from kidney, following partial purification, failed to complement or bind with GCLM. Upon making this observation, we were quite concerned that this result represented artificial dissociation of GCLholo and modification of GCLC during the isolation process. There are several lines of evidence that suggest this is not the case. First, this was not observed for cytosols isolated from liver, brain, or lung. Gel filtration analysis on cytosols from these tissues (liver shown in Fig. 4) demonstrated the absence of free GCLM, and free GCLC from these tissues was complemented, upon the addition of recombinant GCLM. Thus, if modification of GCLholo occurred, it was specific to kidney, which seems unlikely. Furthermore, the relative amounts of GCLholo, GCLC, and GCLM were quite consistent from several kidney cytosol preparations, with free GCLC representing far more than half of the GCLC present in the cytosolic fraction. Second, GCLC from Gclm(−/−) kidney cytosol was able to complement, in large part, the recombinant GCLM. If modification of GCLholo occurred, it was specific to kidney, which seems unlikely. Third, a modest but consistent portion of GCLC (approximately one-fourth, based on complementation analysis) from kidney cytosol, isolated from Gclm(−/−) mice, failed to complement. Although this proportion of recalcitrant GCLC is considerably less than that detected as free GCLC in gel filtration analysis from wild-type kidney cytosol, it does suggest that a modification exists for GCLC, even in the Gclm(−/−) mouse, which has low levels of GSH. This result also suggests that the relative amount of GCLC that is not competent to interact with GCLM may be dynamic and might change upon alteration of GSH levels or other cellular cues. We are currently trying to purify free GCLC from kidney, in an attempt to discover potential post-translational modifications. It should be noted that a previous report has shown that GCLC may be phosphorylated in vivo (42); phosphorylation, which altered some catalytic characteristics of GCL, however, did not affect its capacity to interact with GCLM.

Conclusions—By taking advantage of catalytic differences between GCL and GCLholo, we have determined that GCL is limiting in all tissues, except in kidney, where post-translational regulation of GCLC might uniquely be occurring. The GCLC/GCLM ratio ranged from 7.0 in brain to 1.5 in heart and spleen; despite this excess of GCLC, the role of GCLM in maintaining GSH levels should not be underestimated. GSH levels in Gclm(−/−) mice are between 9 and 40% of wild-type levels in a battery of tissues, with depletion being most severe in red blood cells and least severe in brain (11). It should be noted that the change in tissue GSH levels would not be expected to reflect merely the relative ratio of GCLC/GCLM. For example, in liver, loss of GCLM leads to a compensatory increase in GCLC (11) (present study). In addition, tissue GSH levels reflect not only its accumulation in cells but also its eflux out of cells. In liver, it has been estimated that 90% of GSH is exported into the plasma and bile (43). Indeed, in the Gclm(−/−) mouse, plasma GSH levels decrease 84% (11). Hence, the GSH level, upon loss of GCLM, depends on multiple factors, some probably unappreciated.

It should also be noted that the measurements of GCLC/GCLM ratios presented herein represent an average contribution of GCLC and GCLM present in a given tissue. Thus, in brain, where the ratio of GCLC/GCLM is 7.0, it is reasonable to speculate that some cell types have an even higher ratio and some have a lower ratio. Cell types with a very high GCLC/GCLM ratio would be expected to be more dependent on GCLC and the kinetic constants associated with GCLC; this may be particularly important when considering diseases associated with these cell types. In this regard, it is interesting that decreases in GSH levels seem to be intimately associated with pathologies in the central nervous system, most notably Parkinson disease (44–46). It is worth noting that mitochondrial dysfunction is a critical feature of this disease, and mitochondrial dysfunction leads to the generation of reactive oxygen species and a decline in cellular ATP (47). In light of the dramatic difference in the KmATP between GCLholo and GCLC, it will be important to determine the relative ratio of GCLC/GCLM in dopaminergic neurons and to assess the role of ATP in maintaining GSH levels.

Summary—Studies in Gclm(−/−) mice reveal the essential role for the GCLM subunit in controlling GSH levels. Such studies have not previously been possible in animal model or cell culture. Assessment of the catalytic characteristics of GCLholo, compared with that of GCLC, reveals that GCLholo is much more active in γ-GC synthesis in the presence of elevated GSH and under conditions of low glutamate and especially low ATP. Nevertheless, GCLM is limiting in most tissues. Our current understanding of the function of GSH suggests that GSH is highly beneficial; however, the complex mechanism for the regulation of GSH levels suggests stringent controls on the overaccumulation of GSH. With an understanding of the kinetic parameters that govern GSH biosynthesis, we should be able to gain a better perception of cellular conditions that limit or exacerbate GSH accumulation.

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