The modifier subunit of drosophila glutamate-cysteine ligase regulates catalytic activity by covalent and noncovalent interactions and influences glutathione Homeostasis in vivo
The Modifier Subunit of Drosophila Glutamate-Cysteine Ligase Regulates Catalytic Activity by Covalent and Noncovalent Interactions and Influences Glutathione Homeostasis in Vivo*

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Glutamate-cysteine ligase (GCL) has a key influence on glutathione homeostasis. It has been proposed that mammalian GCL is regulated by the redox environment, and we show here that cysteine residues in the Drosophila melanogaster GCL modifier subunit (DmGCLM) can form covalent interactions with the catalytic subunit (DmGCLC) and modify its activity. Candidate components of intersubunit disulfides (Cys213, Cys214, and Cys667) were identified using matrix-assisted laser desorption ionization time-of-flight spectroscopy of iodoacetamide-modified DmGCLM as well as examination of the evolutionary conservation of cysteines. Mutation of the 3 cysteine residues allowed DmGCLC to associate with DmGCLC, but inhibited the formation of intersubunit disulfides. This caused a 2-fold reduction in the catalytic efficiency of DmGCLC, although activity remained significantly higher than the catalytic subunit alone. The cysteine mutant was also more sensitive to inhibition by glutathione than the unmodified holoenzyme. Notably, human GCLM could substitute for DmGCLM in modification of DmGCLC activity. The role of DmGCLM in vivo was examined by analysis of a Drosophila mutant (I(3)L5800) containing a P-element insertion in Gclm. We found that the P-element is not responsible for the lethal phenotype and separated the recessive lethal mutation from the P-element by recombination. This yielded two fully viable and fertile recombinants bearing the P-element insertion, which Western and Northern blotting indicated is a severely hypomorphic allele of Gclm. Glutathione levels were 2-fold lower in the Gclm<sup>−/−</sup> mutants than in control strains, demonstrating the importance of DmGCLM in the regulation of glutathione homeostasis in vivo.

The intracellular redox environment is of critical importance in cell physiology. It has a major influence on signaling pathways and cell fate in response to stress (1). Glutathione is one of the key influences on the redox state of the cell; and accordingly, intracellular glutathione levels are subject to multilevel regulatory mechanisms (2, 3).

A major player in the regulation of glutathione homeostasis is glutamate-cysteine ligase (GCL),<sup>1</sup> which catalyzes the first and rate-limiting step in de novo synthesis of glutathione from precursor amino acids (4). GCL is located in the cytoplasm, and its activity appears to be regulated at several levels by the antioxidant status of the cell. In addition to being subject to transcriptional activation by pro-oxidants (5–7), evidence suggests that the catalytic activity is subject to redox regulation by the reversible formation of disulfide bridges between its two subunits (8, 9). Furthermore, GCL is subject to feedback inhibition by glutathione at concentrations that are physiologically relevant (10). The complex regulation of GCL activity highlights its pivotal role in controlling cellular glutathione synthesis.

GCL is a heterodimer composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM). The presence of GCLM modulates the catalytic properties of GCLC by lowering its sensitivity to inhibition by glutathione and by increasing its affinity for glutamate (8, 9). The sensitivity of GCLC to inhibition by glutathione is such that it has been proposed that GCLC would function poorly in vivo without the presence of GCLM. The pioneering biochemical studies of Meister and coworkers (9) in the 1990s provided evidence to suggest that GCLM could further enhance the function of GCLC by the formation of intersubunit disulfide bonds. This proposition was based on the observations that treatment of the GCL holoenzyme with dithiothreitol (DTT) lowered its affinity for glutamate and increased its sensitivity to inhibition by the glutathione analog ophthalmic acid. The effects of DTT were dependent on the presence of GCLM. The results prompted the hypothesis that intracellular GCL activity could be increased under conditions that deplete glutathione, where the oxidizing environment within the cell would promote disulfide bond formation within GCL.

More recently, the role of intermolecular disulfide linkages in modifying GCL activity was investigated by mutagenesis of cysteine residues in the catalytic subunit (11). 8 of the 14 cysteine residues in human GCLC were singly altered to glycine, and the effects on activity and ability to form disulfide linkages were examined. One of the 8 cysteines (Cys<sup>555</sup>) was shown to be involved in influencing the ability of GCLM to increase the activity of GCLC. The mutant holoenzyme was, however, still able to form a 114-kDa complex when analyzed by SDS-PAGE under non-reducing conditions. This suggests

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<sup>1</sup> The abbreviations used are: GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; DmA, Drosophila melanogaster; Hs, Homo sapiens; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
that covalent interactions could still occur between the mutated catalytic subunit and GCLM, indicating that Cys\textsuperscript{132} may not be the only cysteine involved in GCLC association with GCLM. It is possible that more than one disulfide linkage may form between the subunits.

We reasoned that the most straightforward method of investigating the importance of intermolecular disulfide linkages in GCL catalysis would be to target the cysteine residues in the modifier subunit; GCLM has fewer cysteines, and interpreting the importance of intermolecular disulfide linkages in SgrAl (underlined sequence) and XhoI and used to replace the corresponding sites of pBluescript SK\textsuperscript{+}. The 254-bp cDNA fragment was subcloned into the EcoRI and XhoI sites of pET15b (Novagen) to generate pETDmGCLM plasmids. The mutated nucleotides are in lowercase letters.

### EXPERIMENTAL PROCEDURES

**Construction of pET20b DmGCLC**—To make recombinant DmGCLC with a histidine tag at the C terminus, the open reading frame of the Gclc gene was excised from pETDmgclc (13) and subcloned into the NdeI and XhoI sites of pET20b (Novagen), generating pET20b DmGCLC. A 254-bp fragment from the 3′-end of the open reading frame was amplified from pDmGCS4.3.3 (14) by PCR using upstream (5′-GCGGAAATTCGCGCGAGCTAATCACCACG-3′) and downstream (5′-CCGCTCGAGTTTCTCCTCGCAGCAGCC-3′) oligonucleotides designed to insert an EcoRI site into the 5′-end of the fragment and to remove the terminal stop codon. The 254-bp cDNA fragment was subcloned into the EcoRI and XhoI sites of pBluescript SK\textsuperscript{+} and sequenced before it was digested with SgrAI (underlined sequence) and XhoI and used to replace the corresponding SgrAI/XhoI fragment in pET20b DmGCLC.

**Site-directed Mutagenesis**—Site-directed mutagenesis was used to alter the cysteine residues within DmGCLM and was carried out using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were introduced via PCR-based site-directed mutagenesis using the DmGCLM open reading frame cloned into pBluescript SK\textsuperscript{+} (13) as a template and pairs of complementary oligonucleotides (Table I). Oligonucleotides were designed to introduce a single cysteine mutation resulting in an amino acid substitution from cysteine to serine. Mutations were introduced sequentially. Amplified plasmid DNA was isolated, and the presence of point mutation(s) was determined by sequencing before the mutated open reading frame was subcloned into the NdeI and XhoI sites of pET10b (Novagen) to generate pETDmGCLM plasmids, which were used to express mutant recombinant DmGCLM polypeptides.

**Expression and Purification of Recombinant Proteins**—pET20b DmGCLM and mutant forms of pETDmGCLM were expressed separately in Escherichia coli strain BL21 (DE3) and purified by nickel-agarose chromatography as described previously (13), except that LB broth was used in place of Terrific Broth for the culture medium. Recombinant human GCLM (HsGCLM) cloned into pET15b was expressed in BL21 (DE3) cells and purified as described previously (15). Preparations of the GCL holoenzyme were generated by mixing purified recombinant DmGCLM (with a C-termini nal histidine tag) and GCLM polypeptides (each tagged with histidine at the N terminus) and purifying the protein complexes by gel filtration chromato-
either
insertion (marked with \( \text{w} \)) respectively, similar to that of DmGCLM-FM (7). A DmGCLC/DmGCLM mixture was resolved, two peaks was subjected to gel filtration chromatography. When the wild-type DmGCLC/DmGCLM mixture was resolved, two peaks was estimated by Western blotting.

Regulation of mammalian GCLC activity by GCLM is thought to involve, at least in part, the transient formation of reversible covalent interactions promoting conformational change(s) around the active site of GCLC (9). To investigate the role of these interactions in DmGCL regulation, we attempted to generate a DmGCL holoenzyme using DmGCLC and DmGCLM to indicate its identity as a mutant allele of Gclm.

Revertants were obtained from the \( \text{w}^{+} \) stock by crossing to a stock bearing a third chromosome insertion of the P(\( \text{cy}^{+} \Delta 2 \text{-} 3 \))-element, which produces constitutively active P-transposase. Revertants were selected on the basis of loss of eye pigmentation, associated with loss of the \( \text{w}^{+} \)-bearing P-element. We confirmed the loss of the element by PCR. No apparent aberrations were seen. The level of DmGCLM in the revertant stock was estimated by Western blotting.

Multiple Sequence Alignment—Multiple sequence alignments were performed using the Genetics Computer Group Pileup software.

\textbf{RESULTS}

\textit{Mutation of All 5 Cysteines within DmGCLM Inhibits Interaction with DmGCLC}—DmGCLM contains 5 cysteine residues at positions 199, 213, 214, 224, and 269, which were named E, A, B, C, and D, respectively (Table II). To investigate the role of intersubunit disulfide linkages in the regulation of DmGCL activity, we performed site-directed mutagenesis on the modifier subunit to generate a polypeptide that entirely lacks cysteine (DmGCLM-FM). Purified recombinant DmGCLM-FM appeared as a single polypeptide with an apparent molecular mass of \( \sim 35 \) kDa when analyzed by SDS-PAGE (Fig. 1). Its mobility is slower than that of wild-type DmGCLM (Fig. 1), which has an apparent molecular mass of 31 kDa (13). The reason for the difference in electrophoretic motility is unknown, but it suggests that DmGCLM-FM may have a less compact structure than the wild-type protein. The secondary structures of the polypeptides were examined by CD spectroscopy over the absorbance range 260 to 320 nm. The CD analyses did not, however, reveal any significant differences between DmGCLM-FM and the unmodified DmGCLM polypeptide (data not shown). The predicted composition of \( \alpha \)-helices and \( \beta \)-sheets in DmGCLM is 6 \pm 1.3 and 61 \pm 1.45\% respectively, similar to that of DmGCLM-FM (7 \pm 1.5 and 62 \pm 1.6\%, respectively).

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DmGCLM-FM was mixed with DmGCLC, and the mixture was subjected to gel filtration chromatography. When the wild-type DmGCLC/DmGCLM mixture was resolved, two peaks were identified corresponding to the holoenzyme complex (\( \sim 140 \) kDa) and uncomplexed DmGCLM (\( \sim 30 \) kDa) (Fig. 2). However, when the DmGCLC/DmGCLM-FM mixture was resolved, a major peak corresponding to the \( \sim 140\)-kDa complex was not observed, and the profile obtained was more akin to that of uncomplexed DmGCLC (\( \sim 80 \) kDa) and DmGCLM (\( \sim 30 \) kDa) (Fig. 2). This was confirmed by SDS-PAGE analysis of the peak fractions obtained from the DmGCLC/DmGCLM-FM profile, which showed that DmGCLM did not co-elute with DmGCLC in the \( \sim 80\)-kDa peak (data not shown). A small shoulder on the leading edge of the 80-kDa peak was observed and found to contain a trace of DmGCLM. Analyses of these fractions for GCL activity did not demonstrate an enhancement of activity compared with the catalytic subunit alone (data not shown). These findings suggest that mutation of all of the cysteine residues in DmGCLM substantially impairs its ability to interact with DmGCLC.

\textit{Redox Status of Cysteine Residues in DmGCLM}—The disulfide status of cysteine residues in DmGCLM was analyzed by peptide mass fingerprinting. Recombinant DmGCLM was treated with iodoacetamide under native, reducing, or reducing and denaturing conditions. Tryptic digests of iodoacetamide-treated DmGCLM were analyzed by MALDI-TOF mass spectrometry. The iodoacetamide-modified cysteine-containing polypeptide fragments are shown in Table II. In native DmGCLM, the fragment containing Cys\textsuperscript{213} (residues 137–147) and the fragment containing Cys\textsuperscript{214} and Cys\textsuperscript{224} (residues 177–260) were both singly modified by iodoacetamide (data not shown). In the reduced sample, a further iodoacetamide modification was identified in the fragment containing Cys\textsuperscript{213}, Cys\textsuperscript{214}, and Cys\textsuperscript{224}, whereas under denaturing and reducing

<table>
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<tr>
<th>Residues</th>
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<tr>
<td>137–147</td>
<td>PPFSEDSNVSR</td>
</tr>
<tr>
<td>177–260</td>
<td>QQITQLGIALDAAAAALELHSQAQVPITIAQVLSTCCVPPELQEFCTAHDIQLNTHSDPELLVPVEQFDGLVPGETIDWTLR</td>
</tr>
<tr>
<td>267–268</td>
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Stock Center. Recombinant progeny bearing the \( L0580 \) P-element insertion (marked with \( \text{w}^{+} \)) and either \( e^{3} \) or \( n^{1} \) were selected and tested for recessive lethality. Approximately 50% of the recombinants of each class were homozygous viable. We did not map the location of the lethal mutation. Viable stocks bearing the \( L0580 \) P-element and marked with either \( e^{3} \) or \( n^{1} \) were established. We have named the \( L0580 \) P-element \( Gclm\text{L0580}^{+} \) to indicate its identity as a mutant allele of Gclm.

Approximately 25% of the recombinants of each class were homozygous viable. We did not map the location of the lethal mutation. Viable stocks bearing the \( L0580 \) P-element and marked with either \( e^{3} \) or \( n^{1} \) were established. We have named the \( L0580 \) P-element \( Gclm\text{L0580}^{+} \) to indicate its identity as a mutant allele of Gclm.

\( 1.6\% \) respectively.

\( \sim 140 \) kDa and \( \sim 30 \) kDa (Fig. 2) (13). However, when the DmGCLC/DmGCLM-FM mixture was resolved, a major peak corresponding to the \( \sim 140\)-kDa complex was not observed, and the profile obtained was more akin to that of uncomplexed DmGCLC (\( \sim 80 \) kDa) and DmGCLM (\( \sim 30 \) kDa) (Fig. 2). This was confirmed by SDS-PAGE analysis of the peak fractions obtained from the DmGCLC/DmGCLM-FM profile, which showed that DmGCLM did not co-elute with DmGCLC in the \( \sim 80\)-kDa peak (data not shown). A small shoulder on the leading edge of the 80-kDa peak was observed and found to contain a trace of DmGCLM. Analyses of these fractions for GCL activity did not demonstrate an enhancement of activity compared with the catalytic subunit alone (data not shown). These findings suggest that mutation of all of the cysteine residues in DmGCLM substantially impairs its ability to interact with DmGCLC.

\textbf{FIG. 1. SDS-PAGE analysis of DmGCLM-FM.} Recombinant DmGCLM polypeptides were purified from \( E. \) coli by nickel-agarose chromatography and analyzed by SDS-PAGE. Protein (0.75 \( \mu \)g) was loaded as follows: lane 1, DmGCLM; lane 2, DmGCLM-FM.

were identified corresponding to the holoenzyme complex (\( \sim 140 \) kDa) and uncomplexed DmGCLM (\( \sim 30 \) kDa) (Fig. 2) (13). However, when the DmGCLC/DmGCLM-FM mixture was resolved, a major peak corresponding to the \( \sim 140\)-kDa complex was not observed, and the profile obtained was more akin to that of uncomplexed DmGCLC (\( \sim 80 \) kDa) and DmGCLM (\( \sim 30 \) kDa) (Fig. 2). This was confirmed by SDS-PAGE analysis of the peak fractions obtained from the DmGCLC/DmGCLM-FM profile, which showed that DmGCLM did not co-elute with DmGCLC in the \( \sim 80\)-kDa peak (data not shown). A small shoulder on the leading edge of the 80-kDa peak was observed and found to contain a trace of DmGCLM. Analyses of these fractions for GCL activity did not demonstrate an enhancement of activity compared with the catalytic subunit alone (data not shown). These findings suggest that mutation of all of the cysteine residues in DmGCLM substantially impairs its ability to interact with DmGCLC.
conditions, all 3 cysteine residues within this fragment were modified. We were unable to identify the fragment containing Cys267 (residues 267–268) due to its being small and poorly retained on the high pressure liquid chromatography column. Nevertheless, MALDI-TOF analysis implicated Cys267 as being involved in disulfide formation, as peaks corresponding to mixed disulfides between Cys 267 and Cys139 and between Cys267 and Cys213, Cys214, or Cys224 were identified when the non-reduced iodoacetamide-treated samples were analyzed. These peaks were absent in samples treated with DTT.

It is important to note that, in the absence of DmGCLC and glutathione or DTT, purified recombinant DmGCLM was predominantly multimeric (data not shown). This suggests that some of the disulfide bonds that become reduced upon DTT treatment and subsequently modified by iodoacetamide are likely to be intermolecular disulfides rather than intramolecular disulfides. It is unclear whether this has biological significance, but it is possible that multimerization may occur as a result of the artificial environment of the *E. coli* expression system, in which reactive cysteines could readily form intermolecular disulfide bonds giving rise to homodimers or trimers. We reasoned that cysteine residues involved in forming inter-
molecular disulfide bonds in the absence of DmGCLC are potential candidates for forming disulfide bridges with DmGCLC in the holoenzyme.

Our findings from the proteomic analyses indicate that two cysteines on the surface of DmGCLM are predominantly in a reduced state and can be modified by iodoacetamide, two surface cysteines are involved in formation of disulfide bonds, and one cysteine is inaccessible to iodoacetamide without denaturation of the protein. The MALDI-TOF analyses suggest that Cys139 is principally (but not entirely) present as a free thiol on the surface of DmGCLM and that Cys267 can participate in disulfide bridge formation with other cysteines. Tryptic fragment 177–260 appears to contain one free thiol, one cysteine as part of a disulfide, and one cysteine that is buried within the native protein. A caveat to our interpretation is that the vicinal cysteines may not be amenable to simultaneous modification by iodoacetamide in the nondenatured protein. Disulfide shuffling may also occur. Due to the positions of the tryptic cleavage sites, we were unable to dissect this further, and we used a bioinformatics approach to gain additional insight into which of the cysteine residues in tryptic fragment 177–260 could be involved in disulfide formation with DmGCLC.

Comparison of the amino acid sequences of DmGCLM, Hs-GCLM, and hypothetical GCLM polypeptides from Caenorhabditis elegans (GenBankTM/EBI accession number NP_491305), Xenopus laevis (accession number AAH44107), and Danio rerio (accession number AAH44532) showed that both Cys213 and Cys214 are conserved in representatives from mammals, amphibians, fish, arthropods, and nematodes (Fig. 3). A partial sequence of an expressed sequence tag encoding a hypothetical GCLM mixture (data not shown), suggesting that mutation of Cys139 and Cys224 together has little or no impact on the ability of DmGCLM to associate with DmGCLC to form an 140-kDa complex. This was confirmed by SDS-PAGE analysis, which showed that the 140-kDa complex obtained following gel filtration chromatography of DmGCLM with a single mutation at Cys224 also exhibited retarded motility (data not shown). This was not observed in DmGCLM with a single mutation at Cys139, implicating Cys224 in this effect.

DmGCLM-ABD and DmGCLM-CE were each mixed separately with DmGCLC, and the mixtures were resolved by gel filtration chromatography. The profiles obtained following separation of DmGCLC/DmGCLM-ABD and DmGCLC/DmGCLM-CE were identical to that of the wild-type DmGCLM/DmGCLM mixture (data not shown), suggesting that mutation of Cys139 and Cys224 together or Cys267, Cys213, and Cys214 together has little or no impact on the ability of DmGCLM to associate with DmGCLC to form an 140-kDa complex. This was confirmed by SDS-PAGE analysis, which showed that the 140-kDa complex obtained following gel filtration contained both DmGCLC and the mutant forms of DmGCLM (data not shown).

To explore the role of cysteine residues within DmGCLM in disulfide interactions, we investigated the ability of mutant DmGCLM to form the 140-kDa complex with DmGCLC under non-reducing conditions. The peak fractions obtained from gel filtration were subjected to SDS-PAGE and Western blotting under non-reducing conditions (Fig. 5). In the control DmGCLC/DmGCLM sample, a complex with an approximate molecular mass of 140 kDa was apparent when the sample was probed with antiserum raised against recombinant DmGCLC.
formation within DmGCL. Conversely, the 140-kDa band was not detectable in the lane containing DmGCL/DmGCLM-ABD, suggesting that mutation of these residues inhibits disulfide bond formation between the DmGCL subunits under non-reducing conditions. Taken together with the results from the peptide fingerprinting experiments, these findings implicate Cys^{206} and either Cys^{213} or Cys^{214} (or both) as the cysteine residues within DmGCLM responsible for intermolecular disulfide linkages.

Cysteine Residues in DmGCLM Impact on GCL Activity—To determine whether inhibition of intermolecular disulfide bond formation affects the catalytic characteristics of DmGCL, we compared the catalytic activities of the dialyzed DmGCL and DmGCLC/DmGCLM-ABD samples. Kinetic analyses of DmGCL activity were carried out on at least four separate occasions on different preparations of DmGCL. We found that, unlike the $K_m$ values, which were fairly consistent between experiments, the specific activity and maximal velocity of DmGCLC varied between preparations by as much as 2-fold. Despite this interbatch variation, the magnitude of changes in $V_{\text{max}}$ and $K_m$ between the unmodified and mutant DmGCL polypeptides and the catalytic subunit alone was consistent between preparations.

The $K_m$ of DmGCL for L-glutamate is 0.91 mM, whereas the $K_m$ of DmGCLC/DmGCLM-ABD is 0.97 mM (Table III). This observation implies that loss of disulfide bond formation has little impact on the affinity of DmGCL for L-glutamate. In contrast, the $V_{\text{max}}$ of DmGCL was found to be 12.1 μmol/min/mg, approximately twice that of DmGCLC/DmGCLM-ABD. This suggests that intermolecular disulfide bridge formation has a significant impact on the catalytic efficiency of the DmGCL holoenzyme. The $V_{\text{max}}$ of DmGCLC is substantially lower than that of either DmGCLC/DmGCLM-ABD (−4-fold) or unmodified DmGCL (−8-fold) (Table III), emphasizing the importance of non-covalent subunit interactions in regulating DmGCL activity. In contrast, the $K_m$ obtained for DmGCLC is not significantly different from that of DmGCL or DmGCLC/DmGCLM-ABD.

Absence of Intermolecular Disulfide Linkages between DmGCLM and DmGCLC Enhances Sensitivity to Feedback Inhibition by Glutathione—The DmGCL holoenzyme is subject to feedback inhibition by glutathione (13). Inhibition is mixed, and reduction of intermolecular disulfide linkages by glutathione may facilitate its access to the active site to inhibit activity.

We hypothesized that a DmGCL mutant unable to form disulfide linkages would be more susceptible to inhibition by glutathione.

Wild-type DmGCL activity was lowered in the presence of glutathione by a maximum of ~40% (Fig. 6). By contrast, DmGCLC/DmGCLM-ABD activity was more susceptible to inhibition by glutathione; activity was decreased by ~60% at the highest concentration of GSH (16 mM). The difference in the extent of inhibition between DmGCL and DmGCLC/DmGCLM-ABD was particularly evident at lower concentrations of glutathione. These findings highlight the potential importance of intermolecular disulfide bridges within DmGCL in regulating the mechanism of feedback inhibition by glutathione in the holoenzyme. The susceptibility of DmGCLC/DmGCLM-ABD to inhibition by glutathione was not, however, as marked as that of DmGCLC, where activity was almost completely abolished at higher concentrations of GSH (Fig. 6). Inhibition of the catalytic subunit by glutathione is competitive (13), and the difference in the extent of inhibition between DmGCLC and DmGCLC/DmGCLM-ABD underscores the impact that non-covalent intersubunit interactions have upon the susceptibility of DmGCLC to glutathione inhibition.

HsGCLM Is Able to Interact with DmGCLC in Vitro via Covalent Interactions—To gain a better understanding about some of the regions on GCLM that may be involved in subunit interactions, we investigated whether we could create a hybrid GCL holoenzyme using HsGCLM and DmGCLC. Purified re-
pared with that of the DmGCLC/HsGCLM hybrid. The lytic activity of the wild-type DmGCL holoenzyme was estimated to be 1.01 mM, which is consistent with the profile obtained following resolution of the DmGCLC/DmGCLM mixture. This indicates that, despite having only 27% sequence identity to DmGCL, HsGCLM is capable of forming disulfide linkages with DmGCLC. As only 2 cysteine residues (Cys213 and Cys214) are conserved between the Drosophila DmGCLC and human GCLC, it seems likely that either one or both residues are involved in intermolecular disulfide bond formation.

HsGCLM Can Enhance the Activity of DmGCLC—The catalytic activity of the wild-type DmGCL holoenzyme was compared with that of the DmGCLC/HsGCLM hybrid. The kinetic constants for the DmGCLC/HsGCLM hybrid enzyme are 1.01 mM, which is similar to the value of 0.89 mM obtained for the Drosophila GCL holoenzyme. The Vmax for the DmGCLC/HsGCLM hybrid enzyme was found to be 8.39 μmol/min/mg, similar to the Vmax determined for the DmGCL holoenzyme (8.45 μmol/min/mg) generated from the same preparation of DmGCLC. These results indicate that HsGCLM is capable of enhancing the activity of DmGCLC in a similar way to DmGCLM.

Effect of DmGCLM Ablation on Glutathione Homeostasis in Vivo—The gene encoding DmGCLM (Gclm) maps to 94C on the third chromosome. We obtained the P-element-induced recessive lethal mutant stock I(3)3L0580, which contains a P{lacW} element inserted in the S′-noncoding region of the Gclm gene. The P-element in this stock was responsible for the lethality, as judged from failure to recover dysgenically induced revertants, from failure to rescue lethality with a transgene, and by deficiency mapping (data not shown). Accordingly, we separated the recessive lethal mutation in this stock from the P-element by recombination with an e4 w1 ro1 chromosome. This yielded two viable recombinants bearing the P-element insertion, e4 GelmL0580 and GelmL0580 ro1. We have named this P-element allele GelmL0580 and refer here to the two marked recombinant derivatives as e GelmL0580 and GelmL0580 ro1. Levels of DmGCLM in homozygous e GelmL0580 and GelmL0580 ro1 flies were examined by Western blotting. Fig. 9A shows that DmGCLM was substantially diminished in e GelmL0580 or GelmL0580 ro1 fly lysates. Fig. 9B shows that the reduction in DmGCLM was not accompanied by any change in DmGCLC protein levels. Northern blotting showed a dramatic reduction in Gclm transcription in adult GelmL0580 homozygous flies (data not shown). Collectively, these data indicate that GelmL0580 is a severely hypomorphic allele. GelmL0580 homozygous flies are viable and fertile under normal laboratory conditions and have no obvious phenotype. We obtained a revertant (e GelmL0580 ro1) by mobilizing the L0580 element from the e GelmL0580 chromosome. The e GelmL0580 ro1 homozygotes have wild-type levels of DmGCLM, as judged by Western blotting (Fig. 9). As DmGCLM enhances the catalytic efficiency of DmGCLC and reduces its sensitivity to feedback inhibition by glutathione, we hypothesized that e GelmL0580 and GelmL0580 ro1 flies may have an impaired capacity to synthesize glutathione. We analyzed the whole body glutathione contents of e GelmL0580 and GelmL0580 ro1 flies and compared them with those of Canton S and e GelmL0580 ro1 flies, both of which are wild-type for Gclm. The e GelmL0580 ro1 flies contained slightly less glutathione than the wild-type strains.
Glutamate-cysteine ligase has a profound influence on intracellular redox status. This is due to the complex regulatory mechanisms that modify GCL activity to control glutathione homeostasis. In this study, we have examined the role of intermolecular disulfide linkages in regulating Drosophila GCL activity and showed that aberration of the ability to form disulfide bridges between the catalytic and modifier subunits has a significant impact on the catalytic efficiency of the holoenzyme as well as sensitivity to feedback inhibition by glutathione. The proposed importance of DmGCLM in regulating glutathione homeostasis in vivo was substantiated by the observation that Drosophila strains with a mutation in Gclm have approximately half as much glutathione as wild-type strains.

Using a combination of MALDI-TOF mass spectroscopy and examination of evolutionary conservation of cysteines, we identified Cys213, Cys214, and Cys207 in DmGCLM as candidate disulfide-forming cysteines important for the interactions with DmGCLC. A mutant form of DmGCLM that lacks these 3 cysteine residues (DmGCLM-ABD) could form a stable holoenzyme complex with DmGCLC, but was unable to form intermolecular disulfide bridges under non-reducing conditions. Our kinetic analyses showed that the mutant DmGCLC/DmGCLM-ABD holoenzyme was less active than the unmodified holoenzyme complex with DmGCLC that we had used previously (13). We were surprised, however, to find that there was no significant difference in the Km values for glutamate between DmGCLC and either the mutant or wild-type DmGCLC holoenzyme. In our earlier study (using cysteine as substrate and DmGCLC with an N-terminal tag), we found that the Km value for glutamate was nearly six times higher when the modifier subunit was absent. This difference in Km values is highly reproducible. We are uncertain why the more active C-terminally tagged DmGCLC does not exhibit this DmGCLM-dependent difference in Km for glutamate. It is possible that the cysteine residue that has been proposed to be at or near the active site (12, 22) is more susceptible to oxidation by the cysteine substrate in uncomplexed DmGCLC due to allow access of glutathione and competition with glutamate. Interestingly, the degree of inhibition observed for DmGCLC/DmGCLM-ABD in the presence of very high concentrations of glutathione was still greater than that observed for wild-type DmGCL. It would be expected that, under such conditions, the disulfide linkages between DmGCLC and DmGCLM would be fully reduced, and the sensitivity of wild-type DmGCL to glutathione inhibition would be identical to that of the DmGCLC/DmGCLM-ABD mutant. It is possible, however, that a proportion of the recombinant holoenzyme contains glutathione-resistant disulfides. Pertinent, it was shown previously that a proportion of native rat kidney GCL remained as undissociated holoenzyme when subjected to SDS-PAGE after incubation with the physiologically attainable concentration of 10 μM glutathione (9).

At the outset of this study, we noticed that replacing the N-terminal histidine tag with one at the C terminus caused GCL activity to increase by ~10-fold. This implies that the N-terminal tag may interfere with the conformation of the protein. Although we have not analyzed DmGCLC activity without a histidine tag, previous studies have shown that an N-terminal histidine tag has a modest inhibitory effect on HsGCLC activity (11). The low activity of N-terminally tagged DmGCLC made it less amenable to study than the more active C-terminally tagged protein, and therefore, we carried out all of our subunit interaction studies using the latter DmGCLC form. Furthermore, to circumvent potential problems due to the rapid oxidation of the cysteine substrate in vitro, we performed our enzyme assays with α-aminobutyrate rather than cysteine, which we had used previously (13). We were surprised, however, to find that there was no significant difference in the Km values for glutamate between DmGCLC and either the mutant or wild-type DmGCLC holoenzyme. In our earlier study (using cysteine as substrate and DmGCLC with an N-terminal tag), we found that the Km value for glutamate was nearly six times higher when the modifier subunit was absent. This difference in Km values is highly reproducible. We are uncertain why the more active C-terminally tagged DmGCLC does not exhibit this DmGCLM-dependent difference in Km for glutamate. It is possible that the cysteine residue that has been proposed to be at or near the active site (12, 22) is more susceptible to oxidation by the cysteine substrate in uncomplexed DmGCLC due to

(258.52 ± 22 pmol of glutathione/fly). The concentrations of glutathione in the e Gclm−/− and Gclm−/− stock were significantly different from each other, but did differ from the levels in the other three genotypes (p < 0.001); the levels in the other Gclm−/− stock were significantly different from those in each of the other genotypes in the analysis.

**FIG. 10. Effect of mutation of Gclm on glutathione levels in vivo.** Glutathione levels in whole fly homogenates were determined as described under “Experimental Procedures.” Glutathione concentrations are expressed as the mean ± S.D. of three experiments. Glutathione levels differed significantly by genotype (analysis of variance, F14,40 = 68.42, p < 0.001). Post hoc comparisons indicated that the levels in Canton S and w1118 flies did not differ significantly from each other, but did differ from the levels in the other three genotypes (p < 0.001); the levels in the two Gclm−/− stocks did not differ from each other, but did differ from the levels in the other three genotypes (p < 0.001); and the levels of glutathione in the Gclm−/− stock were significantly different from those in each of the other genotypes in the analysis.

**DISCUSSION**

Glutamate-cysteine ligase has a profound influence on intracellular redox status. This is due to the complex regulatory mechanisms that modify GCL activity to control glutathione homeostasis. In this study, we have examined the role of intermolecular disulfide linkages in regulating Drosophila GCL activity and showed that aberration of the ability to form disulfide bridges between the catalytic and modifier subunits has a significant impact on the catalytic efficiency of the holoenzyme as well as sensitivity to feedback inhibition by glutathione. The proposed importance of DmGCLM in regulating glutathione homeostasis in vivo was substantiated by the observation that Drosophila strains with a mutation in Gclm have approximately half as much glutathione as wild-type strains.

Using a combination of MALDI-TOF mass spectroscopy and examination of evolutionary conservation of cysteines, we identified Cys213, Cys214, and Cys207 in DmGCLM as candidate disulfide-forming cysteines important for the interactions with DmGCLC. A mutant form of DmGCLM that lacks these 3 cysteine residues (DmGCLM-ABD) could form a stable holoenzyme complex with DmGCLC, but was unable to form intermolecular disulfide bridges under non-reducing conditions. Our kinetic analyses showed that the mutant DmGCLM/DmGCLM-ABD holoenzyme was less active than the unmodified holoenzyme, but significantly more active than DmGCLC, supporting the notion that both covalent and non-covalent interactions are important in regulating DmGCL activity. We also found that the DmGCLM/DmGCLM-ABD mutant was more sensitive to inhibition by glutathione than the wild-type holoenzyme. This observation is in keeping with the hypothesis that disulfide linkages generate a conformational change that causes the active site to be less susceptible to competitive inhibition by glutathione (9); in the absence of disulfide bridges (or where intermolecular disulfides are reduced by glutathione), the active site would adopt a more open conformation, which would allow access of glutathione and competition with glutamate. Interestingly, the degree of inhibition observed for DmGCLC/DmGCLM-ABD in the presence of very high concentrations of glutathione was still greater than that observed for wild-type DmGCL. It would be expected that, under such conditions, the disulfide linkages between DmGCLC and DmGCLM would be fully reduced, and the sensitivity of wild-type DmGCL to glutathione inhibition would be identical to that of the DmGCLC/DmGCLM-ABD mutant. It is possible, however, that a proportion of the recombinant holoenzyme contains glutathione-resistant disulfides. Pertinent, it was shown previously that a proportion of native rat kidney GCL remained as undissociated holoenzyme when subjected to SDS-PAGE after incubation with the physiologically attainable concentration of 10 μM glutathione (9).

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DmGCLM-induced conformational alterations. Indeed, we have shown previously that DmGCLC is more susceptible to inactivation by cystamine than the DmGCL holoenzyme (13). Alternatively, it is possible that oxidation of cysteine to cystine in the assay mixture could cause differential inhibition, which might be reflected in apparent differences in $K_m$ values. One other possibility is that the C-terminal histidine tag on DmGCLC actually generates a conformational change in the catalytic subunit to increase activity and to decrease the $K_m$ for glutamate. Despite this caveat, we have demonstrated convincingly that interaction with DmGCLM increases the catalytic efficiency of DmGCLC and that the potential to form intersubunit disulfide linkages can further modulate activity and susceptibility to inhibition by glutathione.

We found that HsGCLM was able to functionally substitute for DmGCLM in the regulation of DmGCLC activity. The modifying effect of HsGCLM was very similar to that of unmodified DmGCLM, suggesting that intersubunit disulfide bridges may form between HsGCLM and DmGCLC to enhance activity. Supporting this notion, HsGCLM was able to form disulfide bonds with DmGCLC. These data strongly implicate Cys$^{213}$ and/or Cys$^{214}$ as the principal modulator of GCLC activity, as Cys$^{213}$ and Cys$^{214}$ are the only cysteines that are conserved between HsGCLM and DmGCLM. We should note, however, that mutant DmGCLM polypeptides in which only Cys$^{213}$ and Cys$^{214}$ were changed to serine could still form coherent linkages with DmGCLC when analyzed by SDS-PAGE under non-reducing conditions (data not shown). In this study, it was therefore important to perform the biochemical analyses on GCL protein complexes in which the formation of disulfide linkages in vitro was not apparent.

In view of the number of hypothetical GCLM proteins identified from representatives of a variety of eukaryotic classes, it seems likely that many eukaryotes utilize a modifier subunit to regulate glutathione synthesis. Nevertheless, it is probable that this mechanism is not common to all eukaryotes. Previous work has shown that GCL from Trypanosoma brucei lacks the conserved cysteine residue (Cys$^{253}$ in HsGCLC) that has been proposed to be involved in disulfide interactions between HsGCLC and HsGCLM (11). When we examined GCLC sequences from organisms in which we found a hypothetical GCL subunit (D. rerio, X. laevis, G. gallus, C. elegans, Anopheles gambiae, S. pombe, and Neurospora crassa), as well as the characterized mammalian forms, all were found to contain the conserved Cys$^{253}$, supporting the hypothesis that this cysteine residue plays an important role in regulating GCL activity by participating in intermolecular disulfide bridge formation. It is of interest to note that, unlike S. pombe GCLC, Saccharomyces cerevisiae GCLC does not contain the conserved Cys$^{253}$, and we were unable to find a GCLM ortholog in the genome sequence.

Despite the wide distribution of GCLM, Gclm$^{L058K}$ flies are fully viable and fertile. The P-element insertion in the 5'-untranslated region of Gclm almost entirely ablates expression, and the mutants have $\sim$50% less glutathione than control strains. However, the lack of phenotype under standard laboratory conditions is perhaps not surprising in light of a recent study in which targeted disruption of mouse Gclm caused no overt phenotype, despite causing significant decreases in glutathione levels in all tissues examined (24). Gclm$^{L058K}$ mouse fetal fibroblasts were, however, substantially more susceptible to $H_2O_2$ toxicity than those with the Gclm$^{+/+}$ or Gclm$^{L058K}$ genotype.

The genetic experiments with fruit flies and mice have highlighted the importance of GCLM in glutathione homeostasis in vivo. It is likely that an impaired capacity to synthesize glutathione would sensitize mutant Gclm animals to cellular oxidative damage. As oxidative stress has been proposed to play a prominent role in aging (25), it will be interesting to study the relationship between glutathione homeostasis, resistance to various stresses, and aging in mutant Gclm flies. The use of Drosophila as a model system will allow us to address the importance of reversible disulfide bridges as a mechanism for regulating glutathione synthesis in vivo. We should be able to use gene replacement strategies to generate flies that express the DmGCLM-ABD mutant instead of the wild-type protein, furthering our understanding of the fundamental mechanisms of regulation and the biological relevance of this essential thiol antioxidant.

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