Molecular Mechanisms of Allosteric Inhibition of Brain Glycogen Phosphorylase by Neurotoxic Dithiocarbamate Chemicals*

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Dithiocarbamates (DTCs) are important industrial chemicals used extensively as pesticides and in a variety of therapeutic applications. However, they have also been associated with neurotoxic effects and in particular with the development of Parkinson-like neuropathy. Although different pathways and enzymes (such as ubiquitin ligases or the proteasome) have been identified as potential targets of DTCs in the brain, the molecular mechanisms underlying their neurotoxicity remain poorly understood. There is increasing evidence that alteration of glycogen metabolism in the brain contributes to neurodegenerative processes. Interestingly, recent studies with N,N-diethyldithiocarbamate suggest that brain glycogen phosphorylase (bGP) and glycogen metabolism could be altered by DTCs. Here, we provide molecular and mechanistic evidence that bGP is a target of DTCs. To examine this system, we first tested thiram, a DTC pesticide known to display neurotoxic effects, observing that it can react rapidly with bGP and readily inhibits its glycogenolytic activity ($k_{\text{inact}} = 1.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$). Using cysteine chemical labeling, mass spectrometry, and site-directed mutagenesis approaches, we show that thiram (and certain of its metabolites) alters the activity of bGP through the formation of an intramolecular disulfide bond (Cys318–Cys326), known to act as a redox switch that precludes the allosteric activation of bGP by AMP. Given the key role of glycogen metabolism in brain functions and neurodegeneration, impairment of the glycogenolytic activity of bGP by DTCs such as thiram may be a new mechanism by which certain DTCs exert their neurotoxic effects.

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§ The abbreviations used are: DTC, dithiocarbamate; DEDC, N,N-diethyldithiocarbamate; GP, glycogen phosphorylase; bGP, brain GP; S-1AF, S-iodoacetamide-fluorescein; DMDC, dimethylidithiocarbamate; NEM, N-ethylmaleimide; CAM, carboxymethyl.
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(bGP). These enzymes are regulated by both the binding of allosteric effectors (including AMP and ATP) and phosphorylation, in response to local or extracellular energy needs (22). Moreover, the activity of bGP can be redox-regulated through the formation of an intramolecular disulfide bond between Cys\(^{318}\) and Cys\(^{326}\), which controls the AMP-dependent activation of this GP isozyme. Together, these tight regulations of bGP enzyme may participate to control of glycogen metabolism in the brain (23).

Recent studies with \(N,N\)-diethyldithiocarbamate (DED) suggest that bGP and glycogen metabolism could also be altered by DTCs (7). To better understand the molecular mechanisms underlying the neurotoxic effects of DTCs, we investigated the potential molecular and functional effects of a neurotoxic DTC pesticide thiram, on bGP. We found that thiram (and certain of its metabolites), inhibited bGP through the formation of an intramolecular disulfide bond between Cys\(^{318}\) and Cys\(^{326}\), two residues involved in the redox regulation of bGP (23). Altogether, our results suggest that the modulation of bGP activity by DTCs may participate in the neurotoxic effects of these chemicals.

Results

bGP Is Inhibited in Brain Extracts Treated with Thiram—

DTCs (Fig. 1A) are chemicals known to directly modify proteins through the covalent addition of cysteine residues and/or the formation of disulfide bonds (Fig. 1B). In addition, DTCs can also be metabolized to certain reactive oxidative metabolites that irreversibly or reversibly modulate protein functions via the covalent modification of cysteine residues (1, 7, 24). Rat models and recent proteomic studies suggested that brain glycogen phosphorylase is a putative target of DTC chemicals in the brain (7). In addition, this isoenzyme has been described as presenting highly reactive cysteine residues (23, 25–27). To further determine the impact of DTCs on bGP activity and glycogen metabolism in the brain, we treated mice brain extracts with thiram (Fig. 1A), a well known neurotoxic DTC pesticide, using concentrations (0–20 \(\mu M\)) previously employed in toxicological \textit{in vitro} models (8, 9). As shown in Fig. 1C, thiram induced a dose-dependent inhibition of GP activity with full inhibition (with 15 ± 14% of control) obtained with 20 \(\mu M\).

The specific reactivity of DTCs toward cysteine residues suggest that reducing agents, including the reducing agent DTT, at least partially, restore protein functions. To investigate the reversibility of endogenous GP inhibition by thiram, we further incubated brain extracts treated with thiram and DTT. As shown in Fig. 1C, DTT partially restored endogenous GP activity (57 ± 13% of control), suggesting the formation of both reversible and irreversible modifications of GP enzymes in the brain by thiram treatment.

bGP Is Reversibly Inhibited by Thiram through the Modification of Cysteine Residues and Intramolecular Disulfide Bonds—

In the brain, bGP is both expressed in astrocytes and in neurons. To investigated the molecular mechanism of GP inhibition by DTCs, we first exposed the recombinant human bGP to increasing concentrations of thiram (0.5–10 \(\mu M\)) (Fig. 1D). The bGP was inhibited in a dose-dependent manner with an \(IC_{50}\) value of 1 \(\mu M\), similar to what we observed with mice brain homogenates. We also investigated the impact of other DTCs on human bGP (Fig. 1A). As shown in Table 1, other DTCs such as disulfiram, as well as metal-complexed DTCs such as maneb and mancozeb, also lead to the inhibition of the recombinant enzyme (with residual activities comprised between 15 and 35% of the positive control), confirming the high sensitivity of bGP to DTCs.

To ascertain whether bGP was inhibited by thiram through the covalent modification of the enzyme, thiram-inhibited bGP was buffer-exchanged using a PD10 column. In agreement with DTC reactivity (covalent modification of cysteines) (1, 4), thiram-exposed bGP demonstrated a residual activity of 25% before and after buffer exchange, suggesting that the enzyme is covalently inhibited by thiram (Fig. 2B). In addition, we carried out second order kinetic analysis and determined the second order rate constant (\(k_{\text{inact}}\)) of bGP inhibition by thiram. We obtained a \(k_{\text{inact}}\) of 1.4 \(10^{5}\) \(M^{-1}\) \(s^{-1}\) (Fig. 2C), suggesting a fast inhibition of the enzyme within seconds, in agreement with previously reported for other enzymatic systems (28–30).
As we stated above, DTCs are known to interact with proteins through the formation of mixed disulfide (adducts) with cysteine residues and/or to induce the formation of disulfide bonds (1, 4) (Fig. 1B). 5-Iodoacetamide-fluorescein (5-IAF), an alkylating agent that specifically and covalently modifies reduced thiol groups, was used in chemical labeling experiments to investigate the modification of βGP cysteines by thiram. The results presented in Fig. 2d show that treatment with thiram leads to a loss of 5-IAF fluorescence signal, indicating a reduction of cysteine alkylation by 5-IAF after treatment. These results thus confirmed the modification of cysteine residues of βGP upon thiram exposure. Furthermore, SDS-PAGE of treated and untreated βGP showed that inhibited βGP migrated faster than the positive control, indicating the formation of intramolecular disulfide bonds upon treatment with thiram (Fig. 2a, lower panel).

Because disulfide bonds are oxidative modifications of proteins that can be reduced by reducing agents, we carried out reactivation experiments using DTT. As shown in Fig. 2e, DTT fully reactivated βGP and restored the migration profile βGP on SDS-PAGE similarly to the control enzyme (non-treated βGP). Together, these findings indicated that βGP inhibition by thiram occurred through the reversible modification of cysteine residues with the concomitant formation of intramolecular disulfide bonds.

**βGP Is Inhibited by Parent Thiram and Certain Oxidative Metabolites**—DTCs, such as thiram, can be metabolized to different reactive metabolites that lead to the reversible and/or irreversible modification of cysteine residues (1). Indeed, DTCs such as thiram are readily reduced in blood and can undergo intracellular re-oxidation (31). In addition, they can be decomposed leading to the formation of isothiocyanate of carbon disulfide intermediates. Additionally, reduced DTCs might also be oxidized to sulfoxide and sulfone derivatives (Fig. 3a) (7, 24, 32, 33). Interestingly, all are able to interact with cysteine lateral side chain (albeit with different reactivities) and to impact protein functions (4, 7). We thus investigated the impact of DTCs, such as thiram, on βGP.
metabolites on bGP activity. Because the metabolites of thiram (DMDC-sulfoxide, DMDC-sulfone, and methylisothiocyanate) are not commercially available, we used the metabolites of disulfiram (DEDC-sulfoxide, DEDC-sulfone, and ethylisothiocyanate), which only differ from thiram metabolites by one methyl group (4, 7). As shown in Fig. 3b, DEDC-sulfoxide metabolite was found to be a potent inhibitor of bGP similar to what we found for thiram. However, we did not observe inhibition of bGP activity following incubation with DEDC-sulfone, isothiocyanate, or carbon disulfide (Fig. 3b). These results suggest that thiram-but also certain metabolites might be involved in the inhibition of bGP. We further investigated the inhibition of bGP by DEDC-sulfoxide by treating the recombinant bGP with increasing concentrations of this metabolite. Similarly to what we found with thiram, bGP was inhibited in a dose-dependent manner (Fig. 3, c and d) and was concomitant with a loss of 5-IAF fluorescence signal (Fig. 3e), suggesting that inhibition occurred through the modification of essential sulfhydryl groups of cysteine residues. However, DEDC-sulfoxide exposure of bGP did not lead to a shift in bGP migration on SDS-PAGE (Fig. 3, b and c). Sulfoxide metabolites of DTCs are known to induce bonds (34), as well as adducts on cysteines (24). Recently, bGP has been shown to be regulated through the modification of essential sulfhydryl groups (4, 7). As shown in Fig. 3a, the metabolism of DTCs. In the organism, DTCs are found as an equilibrium between their reduced and oxidized form. In addition, reduced DTCs can be decomposed in carbon disulfide (CS₂) and isothiocyanate. In addition, DTCs can undergo oxidation and form sulfoxide and sulfone metabolites. bGP was independently incubated with 10 μM of thiram metabolites for 30 min at 37 °C prior to activity measurement and SDS-PAGE analysis. c, recombinant bGP was incubated with different concentrations of DEDC-sulfoxide (0–10 μM) for 30 min at 37 °C. Residual activity was assayed, and aliquots were also subjected to SDS-PAGE analysis under non-reducing conditions. The results are expressed as percentages of the control. The data represent mean values of three independent experiments ± S.D. ***, p < 0.001 compared with positive control. d, recombinant bGP was first inhibited by DEDC-sulfoxide and subsequently incubated with 5 mM DTT prior to activity measurement and SDS-PAGE analysis. e, to confirm the modification of cysteine residues upon exposure to DEDC-sulfoxide, recombinant bGP was inhibited by thiram, and free cysteine residues were specifically labeled using the fluorescent probe 5-IAF. The samples were run on SDS-PAGE in the presence of 2-mercaptoethanol and blotted onto nitrocellulose membrane. 5-IAF fluorescence was measured (λexc = 492 nm, and λem = 520 nm), and bGP was revealed by Ponceau red coloration. The untreated bGP is used as a positive control.

Cys³¹⁸ and Cys³²⁶ Are Involved in bGP Inhibition by Thiram—bGP has 12 cysteine residues in its primary sequence. Using the crystal structure of the human bGP (35), we previously identified two potential intramolecular disulfide bonds: between Cys³¹⁸ and Cys³²⁶ and between Cys³⁷³ and Cys⁴⁴⁵ (Fig. 4, a and b), the first one being specific for the brain isoform of GP (Cys³²⁶ is not present in muscle and liver GPs) and involved in the redox regulation of bGP activity (23). To test whether this disulfide bond was involved in bGP inhibition by thiram, we performed site-directed mutagenesis experiments, substituting Cys³¹⁸ and Cys³²⁶ with serine residues, and treated the mutated enzymes with thiram. As shown in Fig. 4c, mutation of Cys³¹⁸ or Cys³²⁶, as well as the simultaneous mutation of the two cysteines into serine residues protected bGP from its inhibition by thiram, indicating that the modification of these cysteine residues (likely through disulfide bonds) was involved in bGP inhibition by thiram. Similar protection was observed after exposing WT and mutants bGP to DEDC-sulfoxide (Fig. 4d), indicating that this metabolite likely inhibits bGP through the modification of these cysteine residues and disulfide bond formation. Moreover, despite the bGP mutants being fully active following exposure to thiram, non-reducing SDS-PAGE analysis showed that the enzymes still presented a shift in their migration profile, suggesting that disulfide bonds still occurred after treatment by thiram without obvious effect on the enzyme activity (Fig. 4c). Because Cys³⁷³ and Cys⁴⁴⁵ can be involved in a disulfide bond as well, it is likely that this bond still forms after exposure to thiram but has no impact on the activity. The mod-
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**FIGURE 4.** Thiram induced the formation of the Cys\(^{318}\)–Cys\(^{326}\) disulfide bond, which impairs bGP activity. 

a. bGP sequence representation with relative positions of the cysteine residues. Four cysteine residues are specific from the brain isoform (C326*, C436*, C757*, and C808*). Cys\(^{318}\) and Cys\(^{326}\), which have been previously identified as forming an intramolecular disulfide bond are represented in red. Cys\(^{373}\) and Cys\(^{445}\), which can also form an intramolecular disulfide bond are presented in green. Sequences of muscle (PYGM), liver (PYGL), and brain (PYGB) glycogen phosphorylase from rat, mouse, and human containing these four cysteines have been aligned.

b. ribbon representation of C\(\alpha\)/H9251 trace of the dimer of bGP. The AMP-binding site is marked by the allosteric effector AMP (surface representation). Cys\(^{318}\), Cys\(^{326}\), Cys\(^{373}\), and Cys\(^{445}\) are represented as red sticks and balls. The distance separating the C\(\alpha\) of Cys\(^{318}\) and Cys\(^{326}\) (7.9 Å), as well as Cys\(^{373}\) and Cys\(^{445}\) (8.4 Å) is shown.

c. single mutation and double mutations of Cys\(^{318}\) and Cys\(^{326}\) were performed and tested for thiram inhibition resistance as described above. The results are expressed as percentages of the control. The data represent the mean values of three independent experiments ± S.D. ***, \(p < 0.001\) compared with control.

d. to determine whether Cys\(^{318}\) and Cys\(^{326}\) are involved in the inhibition of bGP by DEDC-sulfoxide, WT, and mutants (C318S, C326S, C318/326S) were incubated with DEDC-sulfoxide for 30 min at 37 °C prior to activity measurement. The data represent mean values of three independent experiments ± S.D. ***, \(p < 0.001\) compared with positive control.

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AMP-dependent Activation of bGP Is Altered by Thiram but Not Phosphorylation-dependent Activation of bGP—bGPs are allosteric enzymes found in at least two states: an inactive state (or T-state) and an active state (or R-state). GP activation is under the control of the binding of AMP in the AMP binding site and/or the phosphorylation of Ser\(^{14}\). Cys\(^{318}\) and Cys\(^{326}\) are located in the adenine loop of bGP, which belongs to the AMP binding site. We previously reported that the formation of an intramolecular disulfide bond between Cys\(^{318}\) and Cys\(^{326}\) by
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**Discussion**

DTCs are highly reactive compounds widely used as pesticides, in the industry and in several therapeutic applications (1). These chemicals are highly reactive and covalently modify proteins through the adduction of cysteine residues and/or the subsequent formation of disulfide bonds (1, 4). The wide use of DTCs has raised many concerns about their impact on the environment and human health. Many studies have thus associated DTCs with the development of several pathologies including the Parkinson’s disease-like syndromes (2, 3). However, the molecular basis of DTC toxicity in the brain is poorly understood. Here, we report that bGP activity is impaired by DTCs such as thiram, as well as certain DTC oxidative metabolites. Using human recombinant bGP enzyme, we demonstrated that thiram and certain metabolites (sulfoxide form) inhibited bGP activity through the modification of cysteine residues and the subsequent formation of intramolecular disulfide bonds (Figs. 1B and 2). One of the disulfide bonds involved Cys318 and Cys326, two cysteines located in the AMP binding site that participated to the activation of bGP by the allosteric activator AMP (Fig. 4) (23). GPs are allosteric enzymes tightly regulated by the binding of allosteric effectors, including AMP, and by phosphorylation, in response to extracellular signals (such as neurotransmitters). We found that thiram induces the formation of a disulfide bond between Cys318 and Cys326, impairing the AMP-dependent activation but poorly affecting the phosphorylation-dependent activation of the enzyme.

Finally, we used the fluorescent AMP analog mant-AMP to investigate the ability of the treated enzyme to bind AMP. When it binds to proteins, mant-AMP emits a fluorescence signal that decreases following the addition of AMP (Fig. 5c). We found similar behavior in the treated and non-treated enzymes toward mant-AMP binding, suggesting that the thiram-induced Cys318–Cys326 disulfide bond did not modify the binding of AMP in the AMP binding site but likely altered the transmission of the allosteric activation signal (Fig. 5c).

**FIGURE 5.** Thiram induced Cys318/Cys326 disulfide bond alter the AMP-dependent regulation of bGP by blocking the transmission of the allosteric signal. a, bGP was incubated with 10 μM thiram for 30 min at 37 °C. The inhibited enzyme was then incubated with AMP or phosphorylase kinase prior to activity measurement. The results are expressed as percentages of the control. The data represent mean values of three independent experiments ± S.D. ***, p < 0.001 compared with control; ###, p < 0.001 when two groups are compared. b, to assess the inhibition of active R state bGP by thiram, bGP was preincubated with AMP or phosphorylase kinase prior to exposure to 10 μM thiram. The mixture was then assayed for activity measurement. The results are expressed as percentages of the control. The data represent mean values of three independent experiments ± S.D. ***, p < 0.001 compared with control; ###, p < 0.001 when two groups are compared. c, analysis of the binding of AMP to reduced and oxidized bGP was performed by incubating the treated or untreated enzyme with 5 μM of fluorescent mant-AMP. To ascertain that the fluorescence was due to binding of mant-AMP to the enzyme, increasing concentrations of AMP were added. The addition of increasing concentration of AMP resulted in a loss of fluorescence.
the different reactivity of these chemicals. Indeed, thiram is highly reactive and can form adduct on cysteine residues (1, 4). In addition, contrary to \( \text{H}_2\text{O}_2 \), we observed that bGP exposure to thiram also leads to the formation of a second intramolecular disulfide bond between Cys\(^{373}\) and Cys\(^{445}\). These two cysteine residues, found in the three GP isozymes, are located next to the glycogen storage site of GP, a site involved in the binding of glycogen. Although this disulfide bond does not impact bGP activation and activity (Fig. 4), it might affect the enzyme behavior toward its substrate, such as substrate affinity. Moreover, because of the conservation of these two residues among GP isoenzymes, the Cys\(^{373}\)–Cys\(^{445}\) disulfide bond might also impact glycogenolysis in muscle and liver. Further studies are needed to ascertain this point.

Proteomic studies revealed highly reactive cysteine residues in bGP (Cys\(^{109}\), Cys\(^{326}\), and Cys\(^{406}\)) (25–27), which can constitute potent targets of electrophilic compounds such as DTCs. Consequently, DTCs may directly modulate glycogenolysis through the modification of key cysteine residues of bGP, known to redox regulate the enzyme activity. Moreover, considering that DTCs induce oxidative stress in cells, particularly through the depletion of the GSH pool and the inhibition of antioxidant enzymes (36–38), bGP may also constitute an indirect target a DTC through the oxidation of highly reactive cysteine residues. GP inhibition in brain extracts was not fully rescued by the subsequent addition of high concentrations of reducing agents (DTT) (Fig. 1C). It is thus likely that thiram (and other DTCs) may be metabolized to reactive metabolites (or may induce strong oxidative stress), leading to the irreversible inhibition of brain GP enzymes and subsequent alteration of glycogen metabolism. In addition, the brain displays a limited content in reducing power, which could thus emphasize the inhibition of glycogenolysis in the brain and the toxicity of DTCs (39).

The relationship between brain glycogen accumulation, and brain disease is now well established. Accumulation of glycogen in neurons is a direct cause of neurodegeneration (21) and is observed in several brain diseases, including Lafora disease, Alzheimer’s disease, and amyotrophic lateral sclerosis, and in aging (21, 40–42). DTCs are known to induce several neurological effects including ataxia, convulsion, behavioral abnormalities (43), and Parkinson’s disease-like syndrome (2, 3). Our results suggest that brain glycogen metabolism could be involved in the brain toxicity of DTCs, particularly through the impairment of brain energy metabolism and the toxic accumulation of glycogen in neurons and in astrocytes. In addition, the isozyme-specific inhibition of bGP by thiram suggests that DTCs constitute a basis for the development of drugs targeting glycogen metabolism.

**Experimental Procedures**

**Materials**

Thiram, Disulfiram, Maneb, Mancozeb, DMDC, isothiocyanate, carbon disulfide, protease mixture inhibitors, rabbit muscle phosphorylase kinase, phosphoglucomutase, AMP, ATP, glycogen, glucose-1,6-diphosphate, DTT, 5-IAF, and BSA were purchased from Sigma-Aldrich. Anti-bGP antibody was provided by Santa Cruz (sc-81751). DEDC-sulfoxide and DEDC-sulfonylamine were provided by Tokyo Research Chemical Inc. Glucose-6-phosphate dehydrogenase was purchased from Roche. Mant-AMP was purchased from Jena Bioscience. NADP\(^{+}\) was purchased from Apollo Scientific.

**Methods**

**Cloning and Site-directed Mutagenesis**—The human bGP cDNA was obtained in the eukaryotic pCMV6-XL4 vector (provided by OriGene Technologies, Inc.) and subcloned into the pET28a vector for expression and purification of recombinant bGPs. The resulting construct encoded for His\(_6\)-tagged fusion recombinant proteins (His-bGP). *Escherichia coli* C41(DE3)/pGro7 (encoding the GroEL/GroES chaperonin protein complex) strains were transformed with plasmid pET28acarrying His-bGP and used to express and purify the recombinant enzymes.

For site-directed mutagenesis of bGP, cysteine residues 318 and 326 were mutated into serine residues using the Agilent QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. Briefly, the mutagenesis of each cysteine residue was performed by amplification of the whole plasmids (pCMV6 and pET28a) carrying bGP, using 5’ and 3’ primers containing the single point mutation to induce. The sequence of each oligonucleotides pair used is presented in supplementary Table S2.

**Expression and Purification of Recombinant bGP**—Recombinant human bGP was expressed using C41 *E. coli* bacteria containing pGro7 plasmid responsible for the expression of the chaperonin protein complex GroEL/GroES and cultured at 37 °C. Expression of the GroEL/GroES chaperonin protein complex was first induced by the addition of 1 mM l-arabinose to culture. Expression of recombinant His-bGP was then induced by the addition of 500 \( \mu \text{M} \) of isopropyl-1-thio-\( \beta \)-D-galactopyranoside to culture. The bacteria were further cultured at 16 °C overnight. The bacteria were then pelleted by centrifugation (4,000 \( \times \) g, 10 min), washed with cold PBS, harvested by centrifugation (4,000 \( \times \) g, 10 min), and stored at \(-80^\circ\text{C}\) until required.

The bacteria were suspended in 35 ml of lysis buffer (PBS, pH 8, 300 mM NaCl, 0.5% Triton X-100, 1 mg/ml lysozyme, protease inhibitor mixture) and incubated for 1 h at 4 °C. The lysate was sonicated on ice (8 s pulses for up to 7 min) and centrifuged (17,000 \( \times \) g, 30 min, 4 °C). The supernatant was collected and incubated with 1 ml of nickel-nitrilotriacetic acid Superflow resin in the presence of 10 mM imidazole (final concentration) for 2 h at 4 °C. The resin was then poured into a column and washed successively with washing buffer (PBS, pH 8, 300 mM NaCl) containing 0.1% Triton X-100 and a stepwise gradient of imidazole in washing buffer until a concentration of 20 mM imidazole (final concentration) was achieved. His-tagged proteins were eluted with washing buffer containing 300 mM imidazole. Purified proteins were incubated for 10 min with 10 mM DTT and protease inhibitor mixture and then exchanged against PBS, pH 7.1, using a PD 10 desalting column. The protein concentration was measured using the standard Bradford assay with BSA as standard and by absorbance measurement at 280 nm, using a theoretical \( \varepsilon_{280} \) 115,170 \( \text{M}^{-1} \cdot \text{cm}^{-1} \). The purity of the protein was assessed by SDS-PAGE analysis.
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GP Activity Assay—GP activity was measured in the direction of glycogen breakdown as previously described (44). Briefly, the formation of glucose-1-phosphate was determined using a coupled assay system composed of phosphoglucomutase, glucose-6-phosphate dehydrogenase and NADP, by measuring (NADPH) formation at 340 nm. The phosphorylase activity assay was carried out at 37 °C in PBS, pH 6.9. The mixture consisted of GP (final concentration, 0.1 mM) with or without 1 mM AMP, 0.25% glycogen, 2 mM EDTA, 0.8 mM NADP, 10 mM magnesium acetate, 5 μM glucose-1,6-diphosphate, 5 units of glucose-6-phosphate dehydrogenase, and 5 units of phosphoglucomutase in a final volume of 250 μl. Each measurement was performed in triplicate. GP activity was expressed as the percentage of the control (enzyme in absence of thiram).

SDS-PAGE and Western Blotting—Proteins were loaded onto 7.5% polyacrylamide gels, and electrophoretic protein separation was carried out at 110 V (constant voltage) under non-reducing conditions. For SDS-PAGE, the presence of protein was revealed using R-250 Coomassie Blue. After SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane at a constant current of 200 μA at 4 °C for 1 h. Membranes were incubated at 4 °C overnight with appropriately diluted primary antibody. After washing, the membranes were incubated for 2 h at room temperature with peroxidase-coupled secondary antibody. The proteins were visualized by chemiluminescence detection using ECL substrate and LAS 4000 (Fujifilm). The images were analyzed using Gimp 2 software.

Phosphorylation of Ser14 of bGP—bGP was phosphorylated using phosphorylase kinase from rabbit muscle (1 unit/mg bGP), activated by preincubation for 1 h in phosphorylation buffer 20 mM Tris-HCl buffer, pH 7.7, 0.22 mM ATP, 3.3 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM NaF for activation. Phosphorylation was performed by the addition of activated phosphorylase kinase to the bGP solution, in phosphorylation buffer, and incubation for 2 h at room temperature. The resulting activity was assessed with or without AMP. Phosphorylation of rabbit muscle GP was used as a control. Phosphorylated bGP was then subjected to buffer exchange against PBS buffer, pH 6.9, using a PD Minitrap G25 (GE Healthcare) desalting column.

Effect of DTCs on Recombinant bGP Activity—To test the effect of DTCs (thiram, DMDC, disulfiram, maneb, and mancozeb) and metabolites (DEDC-sulfoxide, DEDC-sulfone, isothiocyanate, and carbon disulfide) on recombinant bGP, 1 μM recombinant human bGP was incubated with DTCs or DTC metabolites for 30 min at 37 °C in a final volume of 25 μl. The reaction was stopped by the addition of the activity assay mixture (which results in a 10 times dilution). GP activity in absence of DTCs was considered as 100% activity. Each measurement was performed in triplicate.

To determine whether thiram-dependent inhibition of bGP is irreversible, bGP exposed to thiram was exchanged against PBS, pH 7.1, using PD 10 desalting column (GE Healthcare) prior to activity assay. To test whether the reducing agent could reactivate the enzyme, recombinant bGP treated with thiram was incubated with 5 mM DTT for 10 min at 37 °C prior to activity measurement. The results are expressed as percentages of the control (enzyme in absence of thiram).

5-IAF Labeling—To test whether thiram or DEDC-sulfoxide-dependent inhibition was concomitant with the modification of cysteine residues, we performed cysteine labeling experiments using 5-IAF. To this end, 1 μM bGP was incubated with thiram as described above. The mixture was then incubated with 20 μM 5-IAF for 10 min at 37 °C and then run on SDS-PAGE under reducing condition. The proteins were then transferred on nitrocellulose membrane, and fluorescence was measured (λ_exc was 492 nm, and λ_em was 520 nm) using the ImageQuant LAS-4000 system (Fujifilm-GE Healthcare). bGP was also revealed using Ponceau red staining.

Mass Spectrometry Analysis—bGP was fully reduced and precipitated using TCA to stabilize cysteine residues. Proteins were then centrifuged, and pellets were successively washed using 1) acetone containing 5 mM HCl and 2) acetone. We employed mass spectrometry with differential cysteine labeling to identify the modified cysteines in the enzyme. With that aim, 1 μM of precipitated bGP was resuspended in PBS buffer containing 250 mM IAA or incubated with 2 mM thiram for 10 min at room temperature. Proteins were precipitated again using TCA and washed as previously described. Pellets were finally resuspended in loading buffer containing 250 mM IAA to alkylate any unreacted cysteine residues. The samples were first purified on a 7.5% SDS-polyacrylamide gel stained with Coomassie blue R250. After in-gel reduction (final concentration, 5 mM DTT; 30 min, 56 °C) and alkylation (200 mM NEM for 20 min at room temperature in the dark), gels slices are recovered by trypsin (Roche) at 12.5 ng/μl in 25 mM ammonium bicarbonate, 0.05% CaCl₂, and incubated overnight at 37 °C. Then the reaction was stopped with 100 μl of 5% formic acid. Peptides were extracted from gel and incubated twice in 5% formic acid prior to sonication and incubation acetonitrile. Then supernatants from all the fractions of the same sample were pooled and dried using a SpeedVac.

Samples were purified by Zip-Tip (Millipore) before the LC-MS/MS analysis by eluting in a solution containing 60% acetonitrile, 0.1% formic acid. Desalted samples were then diluted 10 times prior to fractionation on a capillary reverse phase column (nano C18 Dionex Acclaim PepMap100, 75 μm inner diameter × 50 cm) at constant flow rate of 220 nl/min, with a gradient of 2% to 40% of buffer B containing 90% acetonitrile, 10% water, 0.1% formic acid (buffer A: 98% water, 2% acetonitrile, 0.1% formic acid). LC was directly coupled to a Qq Orbitrap mass
spectrometer (Q Exactive, ThermoFisher Scientific). MS experiments consisted of a survey MS scan (400–2,000 m/z; resolution 70,000) followed by an MS/MS analysis of the most 10 intense precursors, with a dynamic exclusion of 30 s of the previously fragmented precursors.

After processing raw files with the in-house developed software MaxQuant 1.5.3.8, (45) data were searched against the PYGB sequence with Andromeda (46). Carbamidomethylated cysteines and NEM cysteines were set as variable as modifications like oxidation of methionine, and N-terminal acetylation. Mass deviation of 0.5 Da was set as maximum allowed for MS/MS peaks, and a maximum of two missed cleavages were allowed. Maximum false discovery rates were set to 0.01 both on peptide and protein levels. The minimum required peptide length was 7 amino acids.

Perseus v.1.5.1.6 was used for data analysis and processing. We use the evidence.txt file to compare the abundance for each peptide modified with carbamidomethyl (CAM) or NEM. The results are expressed as the ratios of NEM/CAM labeling. A decrease in the NEM/CAM ratio suggests that the cysteine residue is modified upon addition of thiram.

**AMP Binding**—AMP binding experiments were carried out using fluorescent mant-AMP, an analog of AMP that fluoroescences when bound to proteins. bGP (0.25 μM, either reduced or treated) was incubated with mant-AMP (5 μM) and increasing concentrations of AMP (0–50 μM) in Tris-HCl buffer, pH 7.5, for 30 min at 25 °C. Fluorescence of bound mant-AMP was determined by spectrofluorometry using λ_{exc} = 355 nm, λ_{em} = 448 nm (Flex Station 3; Molecular Devices).

**Total Brain Extraction and Exposure to DTCs**—Three-month-old male Swiss mice brains were crushed in PBS buffer, pH 7.1, and protease inhibitors mixture prior to short month-old male Swiss mice brains were crushed in PBS buffer, pH 7.1, 0.5% Triton, protease inhibitors mixture prior to short. Total brain extracts from 30 min at 37 °C were centrifuged during 30 min at 15,000 × g, and the protein concentration was determined using the standard Bradford assay with BSA as standard. 0.125 mg/ml of brain extract were then exposed to the indicated concentrations of thiram for 30 min at 37 °C prior to activity measurement, as described above. Reactivation of GP activity by reducing agents was investigated by further incubating the sample with 10 mM DTT for 10 min at 37 °C, prior to activity assay.

**Statistical Analysis**—The results are presented as means ± S.D. of three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Bonferroni’s post hoc test, using OriginPro 8 software. If only two groups were compared, a mean comparison t test was used. A p < 0.05 was considered to be significant. p < 0.05, p < 0.01, and p < 0.001 are indicated by one, two, and three asterisks.

**Author Contributions**—C. M., F. R.-L. designed experiments, managed the project, and wrote the manuscript. C. M., L.-C. B., E. P., I. H., O. A., J. V., J.-M. D., and F. R.-L. contributed reagents, performed experiments, and/or analyzed the data. All the authors discussed the results and commented on the manuscript.

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