THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 9, pp. 6364–6371, March 2, 2007

Glutathione Transferases Sequester Toxic Dinitrosyl-Iron Complexes in Cells

*A PROTECTION MECHANISM AGAINST EXCESS NITRIC OXIDE**

Received for publication, October 23, 2006, and in revised form, December 20, 2006 Published, JBC Papers in Press, December 30, 2006, DOI 10.1074/jbc.M609905200

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It is now well established that exposure of cells and tissues to nitric oxide leads to the formation of a dinitrosyl-iron complex bound to intracellular proteins, but little is known about how the complex is formed, the identity of the proteins, and the physiological role of this process. By using EPR spectroscopy and enzyme activity measurements to study the mechanism in hepatocytes, we here identify the complex as a dinitrosyl-diglutathionyl-iron complex (DNDGIC) bound to Alpha class glutathione *S*-transferases (GSTs) with extraordinary high affinity (K_D = 10^{-10} M). This complex is formed spontaneously through NO-mediated extraction of iron from ferritin and transferrin, in a reaction that requires only glutathione. In hepatocytes, DNDGIC may reach concentrations of 0.19 mM, apparently entirely bound to Alpha class GSTs, present in the cytosol at a concentration of about 0.3 mM. Surprisingly, about 20% of the dinitrosyl-glutathionyl-iron complex-GST is found to be associated with subcellular components, mainly the nucleus, as demonstrated in the accompanying paper (Stella, L., Pallottini, V., Moreno, S., Leoni, S., De Maria, F., Turella, P., Federici, G., Fabrini, R., Dawood, K. F., Lo Bello, M., Pedersen, J. Z., and Ricci, G. (2007) *J. Biol. Chem.* 282, 6372–6379). DNDGIC is a potent irreversible inhibitor of glutathione reductase, but the strong complex-GST interaction ensures full protection of glutathione reductase activity in the cells, and *in vitro* experiments show that damage to the reductase only occurs when the DNDGIC concentration exceeds the binding capacity of the intracellular GST pool. Because Pi class GSTs may exert a similar role in other cell types, we suggest that specific sequestering of DNDGIC by GSTs is a physiological protective mechanism operating in conditions of excessive levels of nitric oxide.

More than 30 years ago it was discovered that paramagnetic dinitrosyl iron complexes (DNICs)[2] can be formed in biological systems (1). These compounds can be observed in isolated cells or tissues incubated or perfused with NO or NO-generating systems (1–5), but traces are also present in tissues under physiological conditions (4). Such complexes, in which ferrous ion coordinates two nitric oxide molecules together with two other ligands, with characteristic EPR spectra centered at about g = 2.03 made possible their discovery in cells or tissues. Actually this is the only way in which NO can be observed directly in living systems. Although the natural occurrence of DNICs has been demonstrated unequivocally, their chemical identity *in vivo* is still ambiguous. In fact, even if DNICs exist as free low molecular mass complexes of the general formula $(NO)_2(RS)_2Fe$, *e.g.* the dinitrosyl-diglutathionyl iron complex (DNDGIC) and dinitrosyl-dicysteinyl iron complex, the existence of such free complexes *in vivo* has never been demonstrated; they always appear bound to unknown proteins (1). The binding to proteins is possible after replacing one thiol ligand of the free complex with a protein serine, tyrosine, or cysteine to complete the coordination shell of the iron. All these paramagnetic species show very similar EPR spectra centered around g = 2.03, and this technique is unable to define their precise chemical composition (6). Also the physiological role of DNICs is controversial; it has been suggested that they function as more stable natural NO carriers, but they are also known to have toxic effects in biological systems (1). In particular, DNDGIC at micromolar concentrations is a potent and irreversible inhibitor of glutathione reductase (7, 8).

We recently proposed that glutathione transferases (GSTs) could be involved in the DNIC binding, storage, and detoxification in living systems (9–11). GSTs represent a group of enzymes ubiquitously distributed in all organisms and devoted to the cell defense. The mammalian GSTs have been grouped in at least eight classes termed Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta, and Zeta (12–19). These enzymes catalyze the conjugation of GSH to the electrophilic center of many toxic compounds and also promote GSH-mediated reduction of organic hydroperoxides. In addition, GSTs may act as ligandins for xenobiotics (20) and also as antiapoptotic proteins through protein-protein interaction with Jun kinase (21). Recently, we demonstrated that Alpha, Pi, and Mu class GSTs, which represent 90–95% of all mammalian GSTs, bind the dinitrosyl-diglutathionyl iron complex with extraordinary high affinity, showing K_D values of 10^{-10}–10^{-9} M (9–11). The association of DNDGIC to GSTs has been thoroughly investigated, revealing

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[2] The abbreviations used are: DNIC, dinitrosyl-iron complex; DNDGIC, dinitrosyl-diglutathionyl-iron complex; DNGIC, dinitrosyl-glutathionyl-iron complex; GR, glutathione reductase; GST, glutathione *S*-transferase; GSNO, *S*-nitrosoglutathione.

that one of the glutathiones in the iron complex binds to the enzyme G-site, whereas the other GSH molecule is lost and is replaced by a tyrosine phenolate in the coordination of the ferrous ion (11). Thus, strictly speaking, the bound complex is a monoglutathionyl species (DNGIC). The x-ray crystallographic structure of DNGIC bound to GSTP1-1 has been solved recently, confirming the structure proposed on the basis of molecular modeling studies (22). Binding of DNGIC to the first subunit of the dimeric Alpha, Pi, and Mu GSTs also triggers a peculiar intersubunit communication, which lowers the affinity of the second subunit (11).

We found evidence that in crude liver homogenates one target of DNICs could be the pool of GSTs (10), which thus could represent a significant part of the "unknown" proteins that apparently bind DNICs. However, no previous studies have assessed the occurrence of such a complex-enzyme association in living cells nor has any physiological role of this phenomenon been defined. Furthermore, the intracellular iron source for DNIC formation has never been determined. This study demonstrates that DNDGIC is formed spontaneously in intact rat hepatocytes after exposure to GSNO; this complex is never detected as free species but always bound to GSTs. The preferential binding proteins in rat hepatocytes are the Alpha class GSTs, which stabilize the complex for many hours. Ferritin is the likely iron source for DNDGIC, but the amount of complex formed never exceeds the buffer capacity of the endogenous pool of GSTs. Evidence is also given that this highly specific interaction is essential to protect glutathione reductase against irreversible inactivation by DNDGIC.

EXPERIMENTAL PROCEDURES

Materials—DNDGIC was prepared essentially as described previously (9). Human GSTA1-1, GSTM2-2, GSTP1-1 were expressed in *Escherichia coli* and purified as described previously (23–25). MGST1, the microsomal GST, was a generous gift of Prof. R. Morgenstern. The enzyme concentrations reported in the text for all GSTs refer to the single subunit. Horse spleen ferritin (16% iron) was a Fluka product (Buchs, Switzerland). *S*-Nitrosoglutathione (GSNO) was prepared as described previously (9).

Preparation of Rat Liver Homogenate—Rat liver homogenate was prepared starting from 10 g of Sprague-Dawley male rat liver washed twice with 200 ml of phosphate-buffered saline. The tissue was homogenized in 100 ml of 0.25 M sucrose and centrifuged at 1000 × *g* to remove the nuclear fraction. The estimated concentration of the GST pool was 18 μM. Alternatively, the rat liver was homogenized in 30 ml of 0.25 M sucrose to obtain a more concentrated GST medium (56 μM).

Hepatocytes were isolated from male Wistar rats (2 months old, 100–120 g) as reported previously (26). Rats were anesthetized by pentobarbital (50 mg/kg body weight, injected intraperitoneally) before rapid killing by cervical dislocation and subsequent liver dissection. Experiments were carried out in accordance with the ethical guidelines for animal research (Italian Ministry of Health).

Preparation of Subcellular Fractions—After perfusion with 0.25 M sucrose and heparin to remove blood, livers from male rats (about 10 g) were excised, minced, and homogenized in a Potter-Elvehjem in 0.25 M sucrose and 10 mM potassium phosphate buffer, pH 7.4 (50 ml per 5 g of liver). After a brief centrifugation to remove unbroken cells, the homogenate was incubated with 1 mM GSNO for 2 h and then centrifuged at 1000 × *g* for 10 min to isolate the nuclear fraction. The nuclear pellet was washed three times with 20 ml of 0.25 M sucrose and 10 mM potassium phosphate buffer, pH 7.4. The collected supernatants were centrifuged at 3,300 × *g* for 10 min to isolate the mitochondrial fraction. With similar procedures the lysosomal fraction (16,300 × *g* for 20 min) and the microsomal pellet (105,000 × *g* for 30 min) were isolated. Each fraction was washed three times with 10 volumes of 0.25 M sucrose in 10 mM potassium phosphate buffer, pH 7.4. Each fraction was tested for purity through measurement of the activities of several marker enzymes, typically located in separate cellular compartments as follows: glucose-6-phosphate dehydrogenase for the cytosol, cytochrome oxidase for mitochondria, acid lipase for lysosomes, and glucose-6-phosphatase for microsomes. In addition, the quality of isolated nuclei was examined using electron microscopy (not shown). Cross-contamination in each fraction was below 10%. The nuclear fraction showed less than 2% of cytosol contamination; the mitochondrial fraction contained less than 1% of nuclei as judged by DNA content.

GST Activity—GST activity was assayed in 0.1 M potassium phosphate buffer, pH 6.5, in the presence of 10 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene at 25 °C. The reaction was followed spectrophotometrically at 340 nm where the GSH-2,4-dinitrobenzene adduct absorbs ($\epsilon = 9{,}600\ \text{M}^{-1}\ \text{cm}^{-1}$).

Glutathione Reductase Activity—Glutathione reductase activity was assayed at 25 °C using a solution of 1 mM GSSG and 0.1 mM NADPH in 1 ml (final volume) of 0.1 M potassium phosphate buffer, pH 7.4. The activity was followed spectrophotometrically at 340 nm.

EPR Analysis—Samples for EPR experiments were usually prepared using hepatocytes in phosphate-buffered saline or rat liver homogenate in 0.25 M sucrose with DNDGIC added from a freshly made stock solution. EPR measurements were carried out at room temperature with a Bruker ESP300 X-band instrument (Bruker, Karlsruhe, Germany) equipped with a high sensitivity TM_{110}-mode cavity. To optimize instrument sensitivity, spectra were recorded using samples of 80 μl contained in flat glass capillaries (inner cross-section 5 × 0.3 mm) (27). Unless otherwise stated, spectra were measured over a 200-G range using 20 milliwatts power, 2.0 G modulation, and a scan time of 42 s; typically 4–40 single scans were accumulated to improve the signal to noise ratio. The EPR signal was quantified by comparison with standard samples containing known concentrations of DNDGIC and GST, as described previously (11). The limit of detection was ~2 μM, and the range was linear up to at least 50 μM DNGIC-GST.

Calculation of Intracellular DNIC Concentrations—DNDGIC and DNGIC-GST were determined on the basis of EPR spectra. Calculations of the cytosolic concentration of both DNGIC-GST and GSTs in rat hepatocytes and in rat liver homogenates were made assuming a hepatocyte volume of 8×10^{-12} liters and a cytosol volume corresponding to 56% of the cell volume. The volume of the cytosol is 0.28 ml per g of fresh liver (28). The concentration of the cytosolic GSTs was 0.7 mM.

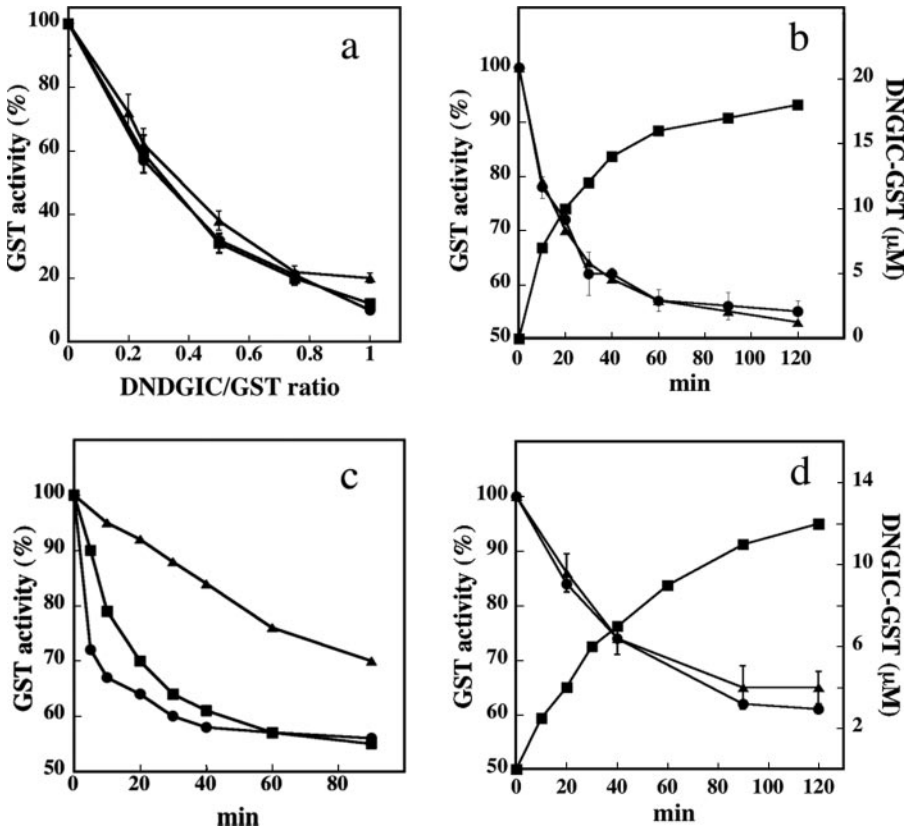


FIGURE 1. **DNDGIC-GST interaction in liver homogenate and hepatocytes.** *a,* inhibition of rat liver GSTs by substoichiometric DNDGIC. ▲, DNDGIC added to a rat liver homogenate (diluted 1:3 in 0.25 M sucrose). Final concentration of GSTs is 28 μM; ■, DNDGIC added to the purified pool of rat liver GSTs (28 μM final concentration); ●, theoretical inhibition curve for exclusive binding of DNDGIC to GSTs, calculated as reported under "Experimental Procedures." *b,* DNDGIC formation in rat liver homogenate. Rat liver homogenate (diluted 1:3 in 0.25 M sucrose) incubated with 1 mM GSNO at 25 °C; ■, DNGIC-GST measured by EPR. ●, GST activity; ▲, theoretical inhibition for an exclusive binding of DNDGIC to GSTs. *c,* dependence of DNGIC-GST formation on GSNO concentration. Variable amounts of GSNO were incubated at 25 °C with rat liver homogenate (diluted 1:3 in 0.25 M sucrose). ▲, 0.2 mM GSNO; ■, 1 mM GSNO; ●, 5 mM GSNO. *d,* DNGIC-GST formation in intact hepatocytes. Rat hepatocytes (6×10^7 of cells in Krebs-Henseleit buffer) were incubated with 1 mM GSNO at 25 °C. At variable times, aliquots of the cells were collected by centrifugation, sonicated, and centrifuged at 105,000 × *g*. The cytosolic fraction was then analyzed. ■, DNGIC-GST measured by EPR spectroscopy; ▲, GST activity; ●, theoretical inhibition for exclusive binding of DNDGIC to GSTs.

Theoretical Inhibition of the Cytosolic GSTs Because of DNDGIC Binding—An inhibition simulation algorithm has been developed based on the following assumptions. (*a*) In the male rat liver, Alpha and Mu GSTs are 43 and 56%, respectively (29, 30). These values were confirmed for our male rat liver preparations by means of high pressure liquid chromatography (31). (*b*) Specific activities of Alpha and Mu GSTs are 16 and 22 units/mg, respectively. These values are the weighted average of the specific activities of the three major Alpha isoenzymes, *i.e.* GSTA1-1 (18 units/mg), GSTA2-2 (18 units/mg), and GSTA3-3 (14 units/mg), and of the two major Mu isoenzymes, *i.e.* GSTM1- 1 (29 units/mg) and GSTM2-2 (15 units/mg) (32). (*c*) K_D values for the high and low affinity binding sites of Alpha and Mu GSTs were reported previously (11). (*d*) Half-site inhibition is operative for the Alpha GSTs, *i.e.* 95% inhibition when the enzyme is half-saturated (11).

Statistics—Results are shown as the mean ± S.D. of at least three experiments.

RESULTS

DNDGIC-GST Interaction in Rat Liver Homogenate—In a first approach, we verified that in rat liver GST represents almost exclusively the binding protein for DNDGIC among all the cytosolic protein components present. Kinetics and EPR experiments were used for this purpose. Incubation of variable amounts of DNDGIC in a liver homogenate (56 μM total GSTs) caused instantaneous and concentration-dependent loss of GST activity. By considering the relative levels of Alpha and Mu GSTs, their different affinities for the complex ($K_D = 10^{-10}$ and 10^{-9} M for Alpha and Mu GSTs, respectively (11)), and their different specific activities (see "Experimental Procedures"), it is possible to calculate the extent of this inhibition in case DNDGIC binds stoichiometrically and exclusively to GSTs, assuming that the isoenzyme with higher affinity (Alpha GST) is involved first. As shown in Fig. 1*a*, the inhibition calculated corresponds well to that found experimentally. The inhibition pattern of the purified pool of liver GSTs is also very similar (Fig. 1*a*). Somewhat less inhibition can be observed using more diluted samples (5 μM GSTs; data not shown), probably because of incomplete saturation of the low affinity sites of GSTs and to the instability of free DNDGIC at very low concentrations (10). The inhibition pattern observed using Alpha class specific co-substrates, like cumene hydroperoxide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, gave further indication that Alpha GST but not Mu GST is primarily involved in DNDGIC interaction (data not shown). As expected, the EPR analysis of the homogenate after reaction with substoichiometric DNDGIC confirmed that all complex is bound to protein (Fig. 2). It should be remembered that in rat liver homogenate the GST-DNGIC signal is stable for many hours, whereas DNDGIC in a GST-depleted homogenate appears as a free species and is highly unstable, with a $t_{1/2}$ of 10 min (10).

GSNO Forms DNDGIC in Rat Liver Homogenate—Incubation of 1 mM GSNO in rat liver homogenate (56 μM GSTs) depleted only of the nuclear fraction induces a time-dependent accumulation of DNIC that reaches an apparent plateau of ~18 μM after about 2 h of incubation (Fig. 1*b*). This is followed by a second phase with a very slow increase that ends only after 14–16 h, at a concentration of ~26 μM DNIC (not shown). The EPR spectra showed that the iron complex does not exist as a free species but is entirely bound to proteins (Fig. 3), and the spectrum is identical to that obtained after addition of authentic DNDGIC to the homogenate. The identity of DNGIC-GST

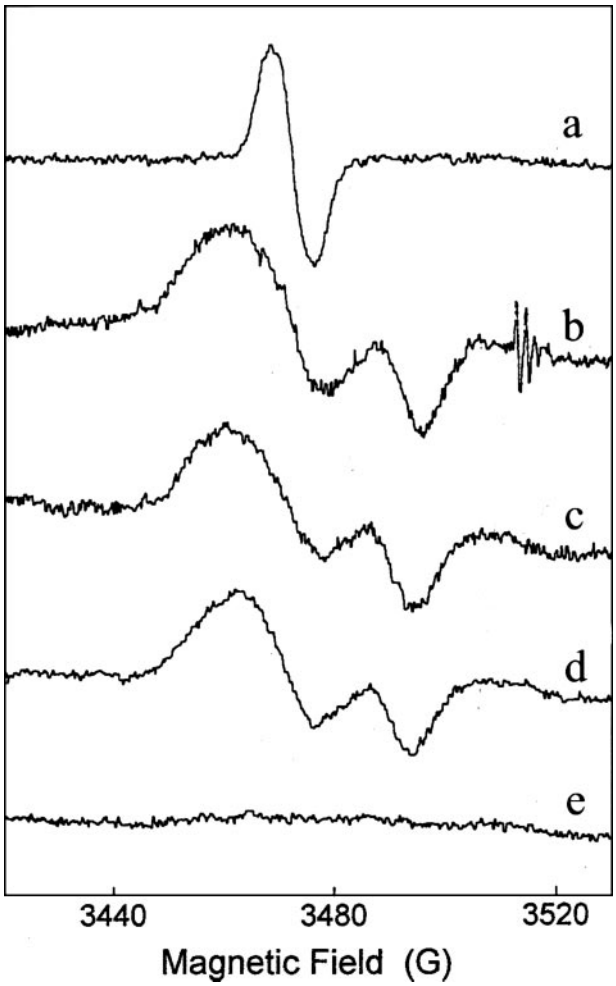


FIGURE 2. **EPR spectra of DNDGIC and DNGIC-GST.** *Spectrum a,* authentic DNDGIC (5 μM) in 0.1 M potassium phosphate buffer, pH 7.4. *Spectrum b,* DNDGIC (9 μM) added to a rat liver homogenate containing 18 μM GSTs. *Spectrum c,* DNDGIC (9 μM) added to the purified pool of rat liver GSTs (18 μM) at pH 7.4. *Spectrum d,* DNDGIC (10 μM) added to purified GSTA1-1 (20 μM). *Spectrum e,* rat liver homogenate as a control.

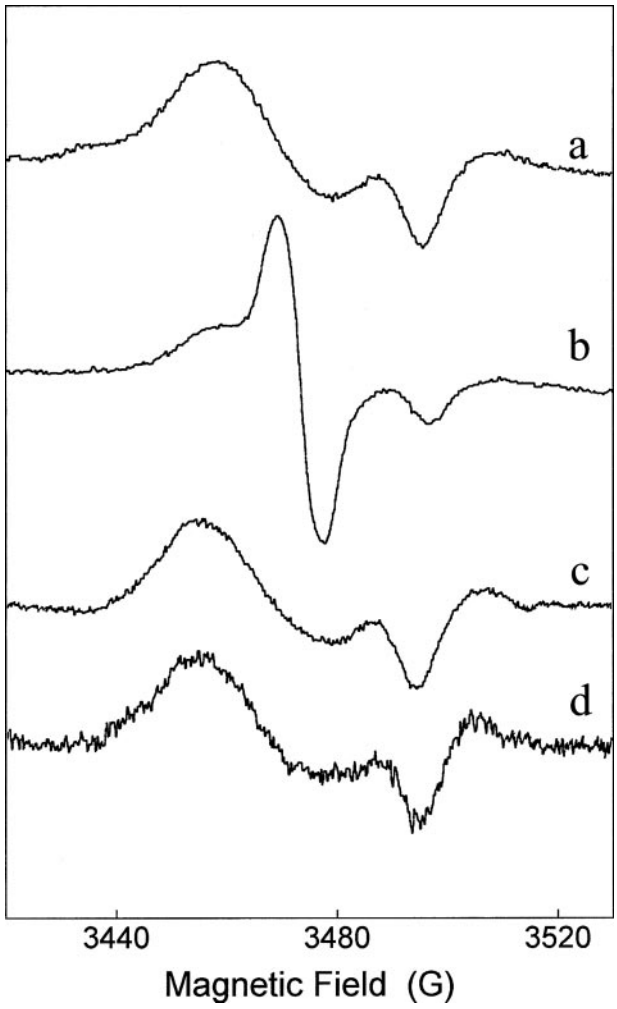


FIGURE 3. **EPR spectra of DNGIC-GST formed by GSNO.** *Spectrum a,* homogenate (56 μM GSTs) after 1 h of incubation with 1 mM GSNO. *Spectrum b,* as in *a* with 50 μM Fe(II) added before incubation; spectrum is shown at half the actual size. *Spectrum c,* hepatocytes (4×10^7 cells) after 1 h of incubation with 1 mM GSNO. *Spectrum d,* membrane fraction isolated from sample *c*; spectrum was amplified twice.

is confirmed by the GST inhibition pattern that is close to that expected assuming GSTs to be the sole target of this complex (Fig. 1*b*). Increasing the final concentration of GSH in the homogenate up to the physiological levels in rat hepatocytes (10 mM) results in faster kinetics of the first phase for DNDGIC formation, but the final amount of complex formed is the same (not shown). The kinetics of DNDGIC formation also depends on GSNO concentration (in the range from 0.2 to 5 mM), but the final concentration of DNGIC-GST does not change appreciably (Fig. 1*c*). Thus it appears that iron availability is the limiting factor for the final level of the complex. In our experimental conditions, DNDGIC never exceeds the amount of the endogenous GST pool, which is 56 μM. Only by adding 50 μM of exogenous ferrous ions to the homogenate can the typical EPR signal of unbound DNICs be seen, superimposed on a large GST-DNGIC signal (Fig. 3). In that case, the GST activity almost disappears, and the amount of the bound DNIC corresponds to the concentration of the entire pool of cytosolic GSTs.

DNDGIC Formation in Intact Hepatocytes—Exposure of rat hepatocytes to GSNO causes a time-dependent intracellular accumulation of a paramagnetic species with an EPR spectrum centered at $g = 2.03$, very similar to that obtained in the crude homogenate after incubation with GSNO and reasonably because of a DNGIC-GST complex (Fig. 3). Also in this case, the kinetics of DNIC formation is proportional to the GSNO concentration (within 0.5 and 2 mM), whereas the final level of the complex is almost independent (data not shown). After 2 h of incubation with 1 mM GSNO, DNGIC-GST reaches a plateau of 12 μM in the sample, corresponding to an intracellular concentration of about 0.19 mM (Fig. 1*d*). As in the homogenate, the EPR signal was stable for several hours; this stability might be due to a steady-state equilibrium between decomposition and re-synthesis of the complex in the presence of an excess of GSNO. However, after repeated washing of the cells, the EPR signal was still stable for hours, thus suggesting that true stabilization occurs in the cell. At fixed times, hepatocytes were sonicated and centrifuged at 105,000 $\times$ *g*. The cytosolic DNGIC-GST was measured by EPR spectroscopy and compared with the degree of GST inhibition. As observed in the homogenate, the inhibition pattern parallels DNDGIC formation, and it also

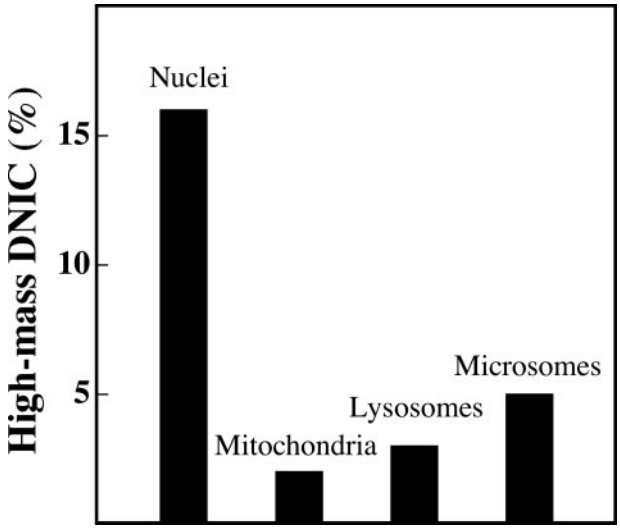


FIGURE 4. **Subcellular association of high mass DNICs.** Rat liver homogenate (18 μM GSTs) was incubated with 1 mM GSNO for 60 min at 25 °C. After incubation, nuclei, mitochondria, lysosomes, and microsomes were isolated by differential centrifugation, washed three times with 10 volumes of 0.25 M sucrose in 10 mM potassium phosphate buffer, pH 7.4, and analyzed by EPR spectroscopy. Values represent the percentage of DNIC compared with the total high mass DNIC found in the cytosol.

approaches the inhibition curve calculated for exclusive binding of DNDGIC to the endogenous GSTs (Fig. 1*d*). Interestingly, DNGIC-GST never exceeds the concentration of the intracellular GSTs pool; it actually becomes similar to the concentration of the high affinity binding sites of Alpha GST (0.15 mM).

We noticed that the concentration of DNIC measured in the cytosol (0.16 mM) is about 20% lower than that observed in intact cells, suggesting that a not negligible amount is retained by intracellular organelles or cell membranes. In fact, the 105,000 × *g* pellet showed the presence of a bound DNIC with an EPR spectrum very similar to that of the DNGIC-GST complex (Fig. 3). Further details were obtained by isolating nuclear, mitochondrial, lysosomal, and microsomal fractions after 1 h of incubation of a rat liver homogenate with 1 mM GSNO. All subcellular fractions contain detectable amounts of the bound DNIC, but it is mainly localized in the nuclear fraction (Fig. 4). An identical distribution of bound DNICs was found by incubating separately each subcellular component with DNDGIC, indicating that the protein counterpart is constitutively bound to these fractions and not associated as a consequence of DNDGIC binding. As Alpha and Mu GSTs are considered cytosolic enzymes and the peculiar membrane-bound microsomal MGST1 is found to have scarce affinity for DNDGIC,[3] these results might indicate the presence of unknown proteins associated with subcellular organelles, able to bind DNDGIC but different from cytosolic GSTs. Unexpectedly, after treatment with 10 mM KCN to displace the complex (9), we find considerable GST activity associated with these components (not shown). The accompanying paper (31) demonstrates that the EPR signal is entirely because of the DNGIC-GST species and that significant amounts of GSTs, mainly GSTA1-1 and GSTA2-2, are associated with the nucleus.

Ferritin Is the Likely Iron Source for DNDGIC in Hepatocytes—The amount of GST-DNGIC generated both in intact hepatocytes and in liver homogenates after exposure to GSNO requires mobilization of iron from the intracellular iron storage proteins. The cytosolic free iron pool is only 5 μM (33), a concentration 2 orders of magnitude lower than that of the DNDGIC formed in the cell after GSNO treatment. It has been reported previously that iron can be mobilized from ferritin by NO-generating systems (34). We confirm here that, in the presence of GSNO and GSH, iron is readily extracted from purified horse ferritin to produce free DNDGIC (Fig. 5*a*), and similar results were obtained using transferrin as the iron source (data not shown). Interestingly, the kinetics of DNDGIC formation from ferritin and its final concentration are independent of the presence of GST (data not shown), indicating that GST is not a kinetic or thermodynamic drawing force for DNDGIC; the complex is formed at the same rate in the presence or absence of GST, the only difference being that in the first case the complex will bind immediately to the transferase. Although the kinetics of DNDGIC formation depends directly on GSNO concentration and on ferritin (Fig. 5, *b* and *c*), the final amount of DNDGIC is determined by the amount of ferritin available (Fig. 5*a*). Importantly, only a small fraction of the iron present in the ferritin protein can be mobilized by GSNO (about 0.3%). The mobilization of iron from horse ferritin also occurs in a complex milieu such as the crude homogenate. Addition of horse spleen ferritin to the rat liver homogenate in the presence of 1 mM GSNO and 10 mM GSH causes a net increase in the DNDGIC formed (Fig. 5*d*). This overproduction of DNDGIC corresponds to that calculated by assuming the homogenate does not alter the reaction observed with the purified system. Interestingly, the amount of iron extractable from the endogenous rat liver ferritin appears 10-fold higher than that coming from the purified horse spleen protein. In fact, the total ferritin iron present in our homogenate is about 1 mM, whereas the final concentration of DNDGIC is 28 μM (3%). A higher propensity for iron mobilization from rat ferritin compared with that of the horse protein has been observed previously, in the case of iron extraction by superoxide ions (35).

GSTs Protect Glutathione Reductase against Irreversible Inhibition by DNDGIC—It is known that DNDGIC irreversibly inactivates glutathione reductase (GR). This reaction was studied in detail by Boese *et al.* (7), and the x-ray crystal structure of the DNDGIC-inactivated enzyme has been solved by Karplus and co-workers (8). It has been clearly demonstrated *in vitro* that free DNDGIC at micromolar levels (IC_{50} = 3–4 μM) oxidizes irreversibly the essential thiol group of Cys-63 to sulfinic acid (8). Therefore, we tested whether the complex bound to GST was still able to inactivate GR. Exposure of rat hepatocytes to 1 mM GSNO did not cause any detectable inhibition of GR even after 120 min of incubation (not shown), although the estimated cytosolic concentration of GST-DNGIC reached 0.16 mM. To prove the involvement of GSTs with this protection and to evaluate the maximal defense capacity of the cell, we compared the effects of increasing amounts of DNDGIC added to rat liver homogenate. Inactivation of GR is observed only

[3] G. Ricci and J. Z. Pedersen, unpublished observations.

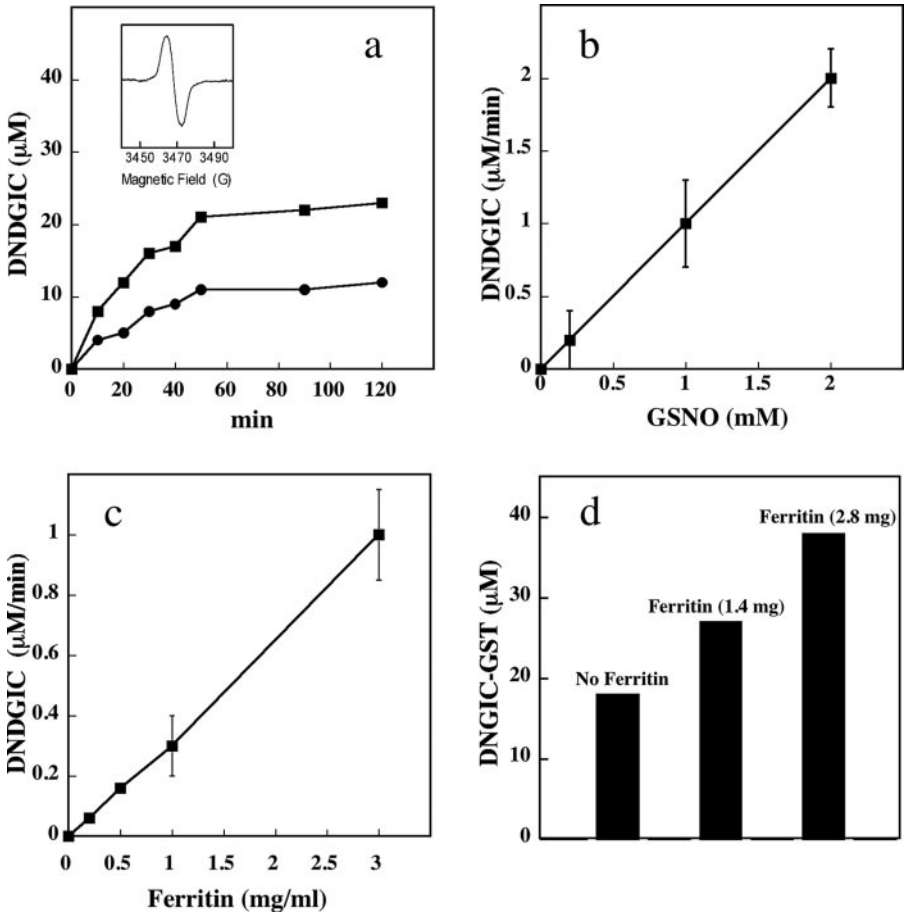


FIGURE 5. **DNDGIC formation from horse ferritin, GSH, and GSNO.** *a,* horse spleen ferritin was incubated in 1 ml of 10 mM GSH and 1 mM GSNO in 0. 1 M potassium phosphate buffer, pH 7.4, at 25 °C. At variable times, DNDGIC was measured by EPR measurements. ●, ferritin 1.4 mg/ml (final concentration) (estimated 4 mM total iron); ■, ferritin 2.8 mg/ml (final concentration) (estimated 8 mM total iron). The iron extracted by GSH and GSNO is about 0.3%. *b,* horse spleen ferritin (3 mg/ml) was incubated with variable amounts of GSNO and 10 mM GSH in 0.1 M potassium phosphate buffer, pH 7.4. At variable times the rate of DNDGIC was measured by EPR analysis or by the extent of GST inhibition, as described previously (10). *c,* variable amounts of horse spleen ferritin were incubated with 1 mM GSNO and 10 mM GSH in 0.1 M potassium phosphate buffer, pH 7.4. At variable times the rate of DNDGIC was measured by EPR analysis or by the extent of GST inhibition. *d,* rat liver homogenate was implemented with 1.4 or 2.8 mg/ml of horse spleen ferritin and incubated with 10 mM GSH and 1 mM GSNO in 0.1 M potassium phosphate buffer, pH 7.4. At various times the amount of DNDGIC was measured by EPR spectroscopy.

when the GST activity is almost reduced to zero, *i.e.* when the "buffer" capacity of GST is exhausted (Fig. 6*a*). In a different experiment, a fixed quantity of DNDGIC, over-stoichiometric to the endogenous GST pool, was incubated in homogenate previously implemented with variable amounts of GSTA1-1. Also in this case, the activity of GR is unaffected as long as the fixed DNDGIC concentration remains under-stoichiometric to the total GST level (Fig. 6*b*). These results demonstrate that Alpha GST acts as a potent protection system and allow us to predict that DNDGIC in hepatocytes in theory may accumulate a level up to 0.6 – 0.7 mM without doing any significant damage to the cell.

DISCUSSION

This study gives a definitive demonstration of the profound interaction between the natural NO carrier DNDGIC and GSTs in intact hepatocytes and proposes a possible physiological significance. A first important finding is that this iron complex, when present at levels substoichiometric to GSTs, is almost exclusively sequestered by endogenous GSTs, even in a very complex protein milieu like a crude homogenate. In particular, in rat liver Alpha GSTs are the prime target of this interaction, whereas the Mu GSTs become effective only when the high affinity Alpha sites are saturated. This behavior could be predicted on the basis of the different dissociation constants for DNDGIC determined previously for each GST isoenzyme under purified conditions (11), but the present data demonstrate that the binding properties of these enzymes are unchanged in a complex protein system that approximates the *in vivo* conditions. Obviously, we cannot exclude that a small amount of DNDGIC may bind to other proteins, but we can conclude that more than 95% of the complex is bound to GST in a 1:1 stoichiometric interaction.

In addition we show that DNDGIC is formed and successively stabilized by GSTs in a similar way both in a crude liver homogenate and in intact hepatocytes exposed to GSNO. The unique stoichiometric binding/inhibition pattern of the GST-complex interaction reveals that the DNIC species formed in the cells is indeed the DNDGIC. This conclusion is important because the identity of intracellular DNIC species has never been established before. In hepatocytes DNDGIC is found entirely bound to GST and is never observed as the free complex. Preliminary data from our laboratory indicate that DNDGIC is formed and binds to GSTs also in other types of cells. Considering that GSTs are ubiquitous, and also Pi class GSTs bind the DNDGIC with high affinity, we propose that all the immobilized DNICs detected in biological systems through their characteristic EPR signal at $g = 2.03$ might be ascribed to intracellular DNGIC bound to GSTs.

Because of the very high amounts of GSTs in hepatocytes, the final level of DNDGIC is always substoichiometric to the GSTs pool. Inhibition data confirm that Alpha GST is primarily involved in this interaction also in intact cells. Interestingly, in the liver homogenate, a free form of DNIC produced by GSNO can be observed only when exogenous iron is added in amounts exceeding the GST concentration. Thus iron availability seems to be a crucial factor for DNIC accumulation in these multicomponent systems. In fact, experiments performed with purified horse ferritin indicate that this protein is the likely iron source for DNIC formation, but the iron released is only 0.3% of

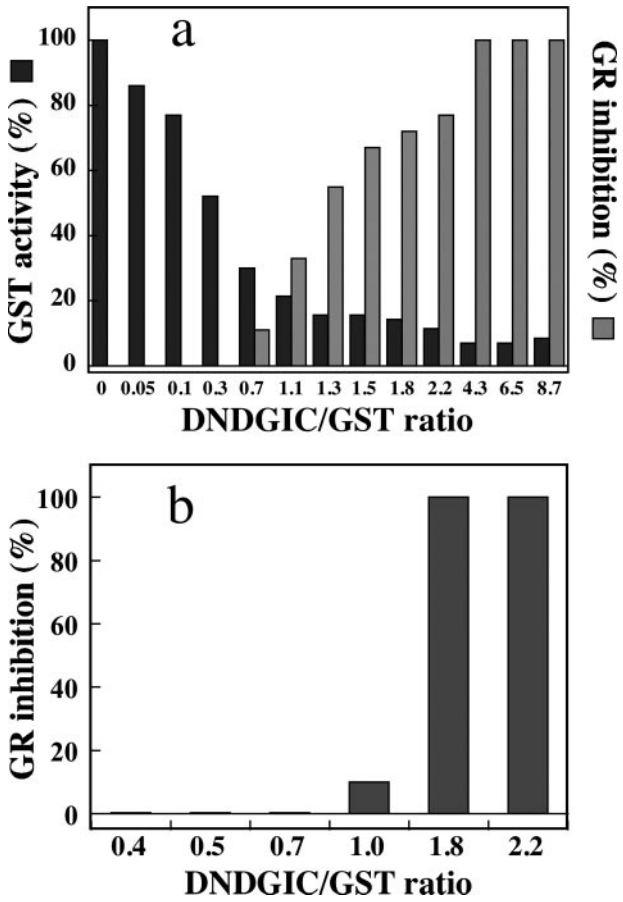
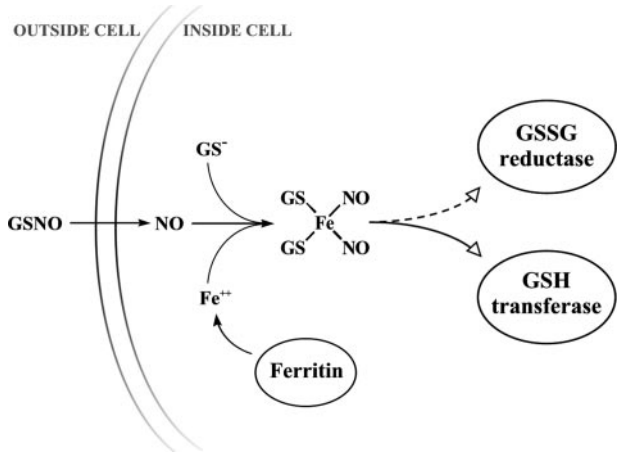


FIGURE 6. **GSTs protect glutathione reductase against DNDGIC.** *a,* rat liver homogenate (1:10 in 0.25 M sucrose) was incubated with variable amounts of DNDGIC (up to 8.7 M excesses on endogenous GSTs) and 1 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4 (1 ml final volume). The final concentration of GSTs was 4.5 μM. After 30 min, the activity of endogenous glutathione reductase was assayed as described under "Experimental Procedures." No inhibition was observed even at the highest DNDGIC concentration in the absence of NADPH, as reported previously (7). *b,* rat liver homogenate (1:10 in 0.25 M sucrose) was incubated with a fixed DNDGIC concentration (10 μM), 1 mM NADPH, and variable amounts of GSTA1-1 (up to 20 nmol/ml) in 0.1 M potassium phosphate buffer, pH 7.4 (1 ml final volume). After 30 min, the activity of endogenous glutathione reductase was assayed as described above.

its total iron content. Although rat liver ferritin displays a 10-fold higher propensity to iron mobilization (about 3% of its iron content), the level of DNDGIC never exceeds the GST concentration. In any case, both iron extraction from ferritin or transferrin and the formation of DNDGIC depend only on the presence of NO together with high levels of GSH; no other cellular component is required for the reaction. This means that DNDGIC is generated spontaneously, and its accumulation in hepatocytes exposed to a flux of NO simply cannot be avoided. It appears likely that NO-mediated mobilization of iron from ferritin to form DNDGIC could somehow be related to GST expression, to ensure that practically all DNDGIC is bound to GSTs. This may be critical for cell survival as DNDGIC is a potent inhibitor of glutathione reductase, causing the irreversible oxidation of Cys-63, a residue essential for catalysis. As proved here, this inactivation occurs only when DNDGIC is present as the free compound, *i.e.* when its concentration exceeds the binding capacity of the GST pool (0.6–0.8



SCHEME 1

mM). Thus GSTs, and in particular the Alpha class enzymes, represent a strong defense system in case of NO overloading or insult. Inhibition of GR is not the sole detriment coming from free DNDGIC. This complex may also be extruded from some cells through MRP1 pumps to cause iron and GSH depletion. The NO cytotoxicity promoted by macrophages against tumor cells (MCF7-VP) has been proposed to be due to this extrusion (36). In hepatocytes, the high level of GSTs and the strong affinity of the Alpha GST seem to oppose efficiently the MRP1-mediated extrusion of DNDGIC, as also suggested by the prolonged persistence (hours) of the DNGIC-GST complex inside the cells. Tumor cells that express lower levels of GSTs, and typically the Pi class GST with a lower affinity for DNDGIC, are likely less efficient in retaining the complex.

The results shown in this study may also explain the beneficial effect of NO against iron-mediated oxidative stress, observed previously in rat hepatocytes. Increased levels of labile iron (because of iron overload or to ethanol exposure) makes the cell more susceptible to oxidative stress. NO lowers the availability of the labile iron through DNDGIC formation (4). We can say now that this benefit is only possible because GSTs protect GR against the killer activity of DNDGIC, and at the same time because it avoids the extrusion of free DNDGIC that would cause iron depletion. Scheme 1 illustrates the basic principles of this protective mechanism. In this context it is interesting that preliminary results indicate that the sensitivity to NO of some parasites like *Plasmodium falciparum* could be related to the prevalent expression of GST classes with no affinity or scarce affinity for DNDGIC in these organisms. Overall, these results depict a scenario for the cell in which cytotoxic effects of NO could be determined by the intracellular levels of GSTs and by their intrinsic affinity for DNDGIC.

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Enzyme Catalysis and Regulation:

Glutathione Transferases Sequester Toxic Dinitrosyl-Iron Complexes in Cells: A PROTECTION MECHANISM AGAINST EXCESS NITRIC OXIDE

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J. Biol. Chem. 2007, 282:6364-6371.
doi: 10.1074/jbc.M609905200 originally published online December 30, 2006