Human Glutathione Transferase P1-1 and Nitric Oxide Carriers

A NEW ROLE FOR AN OLD ENZYME*

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S-Nitrosglutathione and the dinitrosyl-diglutathionyl iron complex are involved in the storage and transport of NO in biological systems. Their interactions with the human glutathione transferase P1-1 may reveal an additional physiological role for this enzyme. In the absence of GSH, S-nitrosglutathione causes rapid and stable S-nitrosylation of both the Cysγ and Cysα residues. Ion spray ionization-mass spectrometry ruled out the possibility of S-glutathionylation and confirms the occurrence of a poly-S-nitrosylation in GST P1-1. S-Nitrosylation of Cysγ lowers the affinity 10-fold for GSH, but this negative effect is minimized by a half-site reactivity mechanism that protects one Cysγ/dimer from nitrosylation. Thus, glutathione transferase P1-1, retaining most of its original activity, may act as a NO carrier protein when GSH depletion occurs in the cell. The dinitrosyl-diglutathionyl iron complex, which is formed by S-nitrosglutathione decomposition in the presence of physiological concentrations of GSH and traces of ferrous ions, binds with extraordinary affinity to one active site of this dimeric enzyme (Ki < 10⁻¹² M) and triggers negative cooperativity in the vacant subunit (Ki = 10⁻⁹ M). The complex bound to the enzyme is stable for hours, whereas in the free form and at low concentrations, its life time is only a few minutes. ESR and molecular modeling studies provide a reasonable explanation of this strong interaction, suggesting that Tyr7 and enzyme-bound GSH could be involved in the coordination of the iron atom. All of the observed findings suggest that glutathione transferase P1-1, by means of an intersubunit communication, may act as a NO carrier under different cellular conditions while maintaining its well known detoxifying activity toward dangerous compounds.

Glutathione transferases (EC 2.5.1.18) (GSTs)† are a superfamily of enzymes involved in the detoxication of the cell against toxic and carcinogenic compounds (1). The human cytosolic GSTs are dimeric proteins grouped into at least eight gene-independent classes (Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta, and Zeta) on the basis of their amino acid sequence, substrate specificity, and immunological properties (2–8). Their three-dimensional structures do not differ significantly despite low sequence homology (9–12). Their three-dimensional structures do not differ significantly despite low sequence homology (9–12). Each subunit contains a very similar binding site for GSH (G-site) and a second one for the hydrophobic co-substrate (H-site). Slight structural differences at the H-site confer a certain degree of substrate selectivity. For a more detailed review on the molecular properties of GSTs see Ref. 13.

The physiological role of GSTs does not appear unequivocal. A well known function of GSTs is to promote the conjugation of the sulfur atom of glutathione to an electrophilic center of endogenous and exogenous toxic compounds, thereby increasing their solubility and excretion (1). The Alpha class also displays a peroxidase activity with organic peroxides (14). Because of the considerable level of expression of GSTs in many tissues and their property to bind a number of large hydrophobic compounds, a possible role of “ligandins” (ligand carriers) has been also proposed (15). Recent advances suggest a role for GST P1-1 in the regulation of Jun kinase protein (a stress-activated protein that phosphorylates c-Jun) (16) and as a “tissue” transglutaminase-specific substrate in neural cells committed to apoptosis (17). It has also been reported that certain GSTs (class Theta) may inhibit the proapoptotic action of Bax (18), and the most recently discovered class Omega, GST O1-1, modulates calcium channels, thus protecting mammalian cells from apoptosis induced by Ca²⁺ mobilization (19). Thus, GSTs can be considered as multi-functional enzymes devoted to various aspects of cell defense.

Notwithstanding this versatility, GSTs have never been considered to be involved in the complex mechanisms of detoxification or storage of nitric oxide. NO is formed in organisms from endogenous or exogenous sources and triggers a number of cellular responses, including the regulation of blood pressure, the relaxation of smooth muscle, and the modulation of immunity (20, 21). In the central nervous system, NO is a neuronal messenger and is possibly responsible for the devel-

† The abbreviations used are: GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DNDGC, dinitrosyl-diglutathionyl-iron complex(es); DNGIC, dinitrosyl-glutathionyl-iron complex(es); DNIC, dinitrosyl-iron complex(es); ESR, electron spin resonance; GSNO, S-nitrosoglutathione.
opment of various diseases (22–25). NO has a very short life time in the cell, and its stabilization and transport are promoted by specific NO carriers of low molecular mass, which are formed by the interaction of nitric oxide with ferrous ions (dinitrosoyl-iron complexes (DNIC)) (26–28) or with GSNO (S-nitrosogluthathione (GSNO)) (29–32). DNIC bind to proteins such as albumin forming paramagnetic NO-Fe-protein complexes that can be detected by ESR spectroscopy (33). Similar complexes, known as 2.03 complexes, because of the g value of their characteristic ESR spectra, have been observed in cells and tissues exposed to NO, but nothing is known about the proteins involved (34–37). Recently, kinetic studies showed that both GSNO and dinitrosoyl-diglutathionyl-iron complex (DNDCIG) are competitive inhibitors of GSTs, and they have been proposed as activity modulators of these enzymes (38, 39). Starting from these preliminary findings, we have now investigated the interaction of these compounds with GST P1-1, using ESR spectroscopy, site-directed mutagenesis, mass spectrometry, and molecular modeling. Our results reveal a surprising and sophisticated mode of interaction suggestive of a new role for this enzyme in the cell as a NO reservoir or NO scavenger protein.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids and Site-directed Mutagenesis**—The plasmid pGST-1, producing large amounts of recombinant wild-type GST P1-1 in the cytoplasm of *Escherichia coli*, has been described previously (40). The expression plasmid p8Seq-1, reported previously (41), was used to generate the single-stranded DNA template to be used for site-directed mutagenesis of Cys47 and Cys101 residues, according to the method described by Kunkel et al. (42) with minor modifications.

**Protein Expression and Purification**—Native and mutant GST P1-1 enzymes were produced as described previously (40, 41). Briefly, TOP 10 *E. coli* cells harboring plasmid pGST-1 or plasmid expressing Cys47 and Cys101 mutant enzymes (pGST-A47 and pGST-A101) were grown in LB medium containing 100 μg/ml ampicillin and 50 μg/ml streptomycin. The synthesis of GST was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside when the absorbance at 600 nm was 0.5. Eighteen hours after induction, the cells were harvested by centrifugation and lyzed as described previously (40). Native and GST mutant enzymes were purified by affinity chromatography on immobilized glutathione (43). After affinity purification, the native and the mutant enzymes (C47A and C101A) were homogeneous as judged by SDS-polyacrylamide gel electrophoresis (44). Protein concentration was determined by the method of Lowry et al. (45).

**Kinetic Studies**—The enzymatic activities were determined spectrophotometrically at 25 °C with 1-chloro-2,4-dinitrobenzene (CDNB), as a co-substrate, following the product formation at 340 nm, ε = 9600 M⁻¹ cm⁻¹ (46). Spectrophotometric measurements were performed in a double-beam Uvicrom 940 spectrophotometer (Kontron Instruments) equipped with a thermostatted cuvette compartment. Initial rates were measured at 0.1-s intervals for a total period of 12 s after a lag time of 5 s. Enzymic rates were corrected for the spontaneous reaction.

Apparent kinetic parameters *K*m and *K*m were determined in 0.1 M potassium phosphate buffer, pH 6.5, and 0.1 mM EDTA, containing a fixed concentration of GSH (10 mM) and variable concentrations of CDNB (0.1–2 mM). The collected data were fitted to the Michaelis-Menten equation by nonlinear regression analysis using the GraphPad Prism (GraphPad Software, San Diego, CA). In a more accurate analysis, kinetic data were also fitted to Equation 1, which considers two different enzyme populations characterized by different *K*m values for GSH.

\[
\frac{V_{\text{max}}[S]}{K_m[S]} = \frac{V_{\text{max}}[S]}{K_m[S]} + \frac{V_{\text{max}}[S]}{K_m[S]}
\]

(Eq. 1)

The apparent *K*m was determined at fixed CDNB concentration (1 mM) and variable GSH concentrations (from 0.02 to 20 mM). Kinetic parameters reported in this paper represent the mean of at least three different experimental data sets.

**Preparation of GSNO**—GSNO was prepared as described previously (47). Briefly, a few drops of HCl were added to a solution containing equimolar amounts of GSH and sodium nitrite until pH 1.5 was reached. After standing for 5 min at room temperature, the red GSNO was neutralized with NaOH. GSNO displays an absorption maximum of 750 M⁻¹ cm⁻¹ at 332 nm and appears to be stable for a few days at room temperature. Appropriate aliquots of freshly synthesized compound were stored at −80 °C and used when necessary, after checking their absorbance at 332 nm.

**Synthesis of DNDCIG**—Dinitrosoyl-diglutathionyl-iron complex was synthesized according to the following procedure. Suitable amounts of ferrous ions (FeSO₄, ranging from 10 to 50 μM) were added to a mixture containing 20 mM GSH and 2 mM GS-NO in 0.1 M phosphate buffer, pH 7.4, and 25 °C. The synthesis of the complex was completed in the first 15–20 min and gives an extinction coefficient of 3000 M⁻¹ cm⁻¹ at 403 nm. Dinitrosoyl-dicysthiinyliron complex, under slightly different conditions, displays an identical extinction coefficient at 395 nm (48).

**Interaction of GSNO with GST P1-1**—GST P1-1 or Cys mutant enzymes (ranging from 0.03 to 1 mg/ml) were incubated with 2 mM GSNO in 0.1 M phosphate buffer, pH 7.4, at 25 °C. At various times aliquots were taken and assayed for enzymatic activity with CDNB as co-substrate. In a different set of experiments, 20 mM GSNO was added to the mixture containing the above concentrations of GSNO and protein with or without 1 mM EDTA.

**UV Difference Spectra**—Difference spectra were performed with a double beam Uvicrom 940 spectrophotometer (Kontron Instruments) equipped with a thermostatted cuvette compartment. Spectral data of free and bound DNDCIG complex were recorded in the range 290–500 nm at 25 °C at pH 7.4 using quartz cuvettes (1-cm light path).

**Interaction of DNDCIG with GST P1-1**—Inhibition of GST P1-1 by DNDCIG was measured by incubating variable amounts of DNDCIG (from 0.5 to 9 μM) with 0.1 mg/ml (4.4 μM of active sites) of GST P1-1. After 2 min 10-μl aliquots (final concentration of GST P1-1 is 44 nM active sites) were assayed for activity at pH 6.5 (final volume, 1 ml) in the presence of 10 mM GSH and 1 mM CDNB. Thus, the final concentration of DNDCIG in the activity sample ranged from 5 to 90 nM. In a second experimental set, GST P1-1 (44 nM of active sites) was incubated at pH 7.4 with variable amounts of DNDCIG (from 0.1 to 22 μM). After 2 min the pH was adjusted to 6.5, the GSH concentration was brought to a final concentration of 10 mM, and 1 mM CDNB was then added for activity measurement.

**Electron Spin Resonance**—ESR spectra were recorded with an ESP300 instrument (Bruker, Karlsruhe, Germany) operating at X-band frequency. Low temperature measurements (100 K) were made using a standard TE₉₀₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-...
ligand of DNGIC occupied the same site as the GSH ligand in the crystal structure of the enzyme-GSH complex. It was immediately noted that the hydroxyl moiety of Tyr7 was in covalent bonding distance of the iron atom. A model of the complex covalently bound via Tyr7 was constructed. The resultant model was energy minimized with CNS (52). Initially harmonic restraints of 10 kcal/mol were applied to the protein atoms, but subsequently the restraints were removed in the final stages of minimization. The final energy minimized model exhibited no significant structural differences with the crystal structure of the enzyme. Graphic representation was produced by the computer program MOLSCRIPT (53).

RESULTS AND DISCUSSION

Interaction of GSNO with GST P1-1 in the Absence of GSH—GSNO is a competitive inhibitor for GST P1-1 (38, 39), but in the absence of GSH this compound also causes a time-dependent partial inactivation of the enzyme. After 10 min of incubation, the activity is reduced to about 70% of its initial value and remains unchanged even after 1 h of incubation (Fig. 1). The presence of 1 mM EDTA in the incubation mixture does not affect significantly the extent and the rate of inactivation. Treatment of the inactivated enzyme with 100 mM 1,4-dithiothreitol restores completely the original activity. The modified enzyme does not follow strictly Michaelian kinetics and shows an apparent \( K_m \) value for GSH of 0.45 mM, which is three times higher than in the native enzyme and accounts completely for the observed loss of activity, using the standard GSH concentration of 1 mM (46). Cys47 and Cys101, the most reactive among the four cysteines of each subunit (41, 54), are possible targets of minimization. The final energy minimized model exhibited no significant structural differences with the crystal structure of the enzyme. Graphic representation was produced by the computer program MOLSCRIPT (53).

Mass Spectrometric Analysis of NO-modified GST P1-1—To gain direct evidence of S-nitrosylation of human GST P1-1 we have carried out ion spray ionization mass spectrometry of the modified GST P1-1, and the results are shown in Fig. 2. Ion spray mass spectra of the unmodified protein (Fig. 2a) showed two main peaks corresponding to the molecular masses of 23,226 and 23,356 Da, respectively. The former molecular mass corresponds to that calculated from its cDNA, whereas the latter is due to the failed removal of N-terminal methionine, and both are consistent with data reported previously (40, 56).

In the Fig. 2b, we observe two series of peaks with mass increases of 30 and 1 Da, in addition to the unmodified proteins (23,226 and 23,356 Da, respectively), suggesting nitrosylation of GST P1-1. In particular, it appears that the first species (23,226 Da) exhibits molecular masses of M+ + 30 and M+ + 60, respectively; the second species (23,356 Da) shows the same mass increases of +30 ± 1 and +60 ± 1 Da and, to lesser
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It is well known that saturating amounts of GSH will be made to unravel the mechanism of GST P1-1 investigation because it may be due also to a methionine oxidation in the species with N-terminal methionine requires further investigation because it may be due also to a methionine oxidation. Further mass spectrometry studies on Cys mutant enzymes will be made to unravel the mechanism of GST P1-1 modification.

Interaction of GSNO with GST P1-1 in the Presence of GSH—It is well known that saturating amounts of GSH protect from the chemical modifications caused by thiol reagents. Cys, located at the end of the mobile helix-2, is exposed to the solvent only in the apo-enzyme, whereas it is buried in the holoenzyme (55, 57, 58). When GSNO (2 mM) is incubated with 0.1 mg/ml GST P1-1 in the presence of 20 mM GSH and 1 mM EDTA, no inactivation can be observed within 1 h of incubation (Fig. 3). However, in the absence of EDTA, a fast inactivation occurs that leaves 50% of the original activity after 10 min of incubation (Fig. 3). Similar half-site inhibition is observed at lower GST concentrations, but the extent of this phenomenon is reduced by increasing the enzyme concentration. For example, at 1 mg/ml, it does not exceed 5% (Fig. 3). The cause of this inhibition will be clarified below and likely depends on metal traces present in the buffers.

Dinitrosyl-Diglutathionyl Iron Complex Is Formed in the GSH-GSNO System with Ferrous Ions—When GSNO (2 mM) and GSH (20 mM) are incubated at 25 °C in the presence of substoichiometric amounts of ferrous ions (10–50 μM), a fast reaction occurs, signaled by the appearance of an UV absorption peak, centered at about 400 nm, which is suggestive of the formation of the dinitrosyl-diglutathionyl iron complex (48) (Fig. 4). Kinetics of this process cannot be described satisfactorily by a simple mono-exponential function but fit better to a two phase exponential function (Fig. 5). ESR spectra confirm the presence of a paramagnetic species with ESR signals at g = 2.0036 (33). The protocol described here provides a convenient procedure for a quantitative and stabilized preparation of DNDGIC, based on slow release of NO by GSNO in the presence of physiological concentrations of GSH and substoichiometric iron. The apparent stability of DNDGIC is likely due to a steady-state equilibrium coming from simultaneous decomposition and resynthesis of the complex.

Binding of DNDGIC to GST P1-1—DNDGIC has been described as a competitive inhibitor of GST P1-1 toward GSH (39), but a more careful investigation reveals different and unexpected properties of interaction with this enzyme. Substoichiometric amounts of DNDGIC yield a degree of inhibition that corresponds exactly to an interaction of 1:1 active site-inhibitor. 50% of the original activity is reached when the molar amount of DNDGIC corresponds to 50% of the active sites (Fig. 7a). Increasing concentrations of DNDGIC (up to 4-fold excess over free active sites) does not produce any further
significant inhibition. This behavior can be explained by a very strong interaction of the complex with one of the two binding sites of the dimeric enzyme. By assuming that DNDGIC causes competitive inhibition, a quantitative evaluation of the dissociation constant for the DNDGIC-GST complex ($K_i$) can be made observing that the 50% inactivated enzyme maintains its unchanged. Even in this case, as observed for Cys 47 nitrosylation, this behavior seems to be a mechanism by which the enzyme protects itself against complete inactivation because of DNDGIC binding.

The preliminary finding of a particular inhibition obtained by reacting the enzyme with GSNO and GSH without the addition of exogenous iron (Fig. 3) can be now rationalized. In fact, about 2 mM of DNDGIC are formed using the spurious contaminating iron present in the buffer. This low DNDGIC concentration (which will be confirmed by ESR analysis) is enough to cause 50% inhibition when reacted with 0.1 mg/ml GST P1-1 (4.4 nmol/ml active site) or with lower enzyme concentrations but not with 1 mg/ml enzyme concentration.

**Competition with Serum Albumin**—DNDGIC binds to serum albumin with high affinity, and this protein could be a reservoir of S-nitrosylating species (33). Experiments of competition of this protein with GST P1-1 may give an estimation of the relative affinity of the two proteins against DNDGIC. We found that even 50 mg/ml of serum albumin does not affect at all the inhibition of 0.1 mg/ml GST P1-1 because of the interaction with 2 mM of DNDGIC, thus indicating that GST P1-1 has at least 500 times more affinity than albumin for DNDGIC.

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broad (Fig. 4), but the subsequent addition of 20 mM GSH restores the descending limb below 400 nm (spectrum not shown). Thus, at least three similar but not identical DNDGIC forms can be distinguished: the free mononuclear complex, the dinitrosyl-iron complex bound to the enzyme in the presence of an excess of GSH, and a third species obtained after removal of the excess of GSH. Notably, the bound complex is stable for hours, and only 24 h after Sephadex chromatography it spontaneously decomposes with the full recovery of the enzyme activity. This represents a remarkable stabilization of the bound complex when compared with its fast decomposition in the free form (33).

It has been observed that the DNDGIC analogue dinitrosyl-dicysteinyl iron complex binds tightly to bovine serum albumin, and one or both cysteines involved in the iron complex may be replaced by protein sulphydryls or imidazoles (33). Titration of the amount of GSH involved in the DNDGIC-GST P1-1 complex may be informative if a similar event also occurs in GST P1-1. After binding of 44 μM DNDGIC to 88 μM GST P1-1 (final volume, 1 ml), the 50% inactivated enzyme was passed through a G-25 Sephadex column to remove the excess of GSH. The enzyme displays the same half-activity, and the absorbance at 403 nm confirms that about 40 nmol of the complex are still bound to the enzyme. By adding 10 mM potassium cyanide, the spectral band of DNDGIC at 403 nm disappears within a few minutes, and the enzyme recovers the original full activity. In fact, CN− ions bind tightly to ferrous ions to give the extremely stable [Fe(CN)₆]⁻⁴ complex (K_{stab} = 10⁻³⁵ at 291 K). After cyanide treatment, the amount of free GSH has been titrated by adding 1 mM chloro-dinitrobenzene, a good co-substrate for GST, and by measuring the amount of GS-DNB product formed (ε = 9,600 m⁻¹ cm⁻¹ at 340 nm). The result is 40 nmol of GSH detected, which corresponds exactly to 50% of that expected if the bound DNDGIC would retain the same original composition, thus indicating that one protein group replaced one GSH molecule as iron ligand. Note that no GSH can be titrated without previous potassium cyanide treatment, indicating complete removal of the free GSH by gel chromatography. Both C47A and C101A mutant enzymes give a very similar affinity and inhibition pattern by DNDGIC as the native enzyme, so the Cys⁴⁷ and Cys¹⁰¹ sulphydryls are not involved in the stabilization of the iron complex in the G-site.

**ESR Studies**—At 77 K, DNDGIC shows an anisotropic ESR signal of axial symmetry with g factors gₓ = 2.04 and gᵧ = 2.01 (Fig. 6A, trace a). When 20 μM DNDGIC are reacted with 44 μM GST P1-1 in the presence of 20 mM GSH, the spectrum at 77 K changes slightly, indicating a shift toward a more defined axial symmetry (Fig. 6A, trace b). This spectrum is probably due to the bound complex that still retains two GS⁻ ions as iron ligands. When the excess of GSH is removed by G25 Sephadex chromatography, the ESR spectrum changes, with a gₓ = 2.03 and gᵧ = 2.01, which corresponds to the monoglutathionyl complex (Fig. 6A, trace c), as confirmed by titration data (see above). Readdition of 10 mM GSH to this species, shifts again the ESR spectrum toward that shown by the authentic dигlutathionyl species (data not shown).

As expected, the anisotropy of the ESR signal of the bound complex does not change significantly at 298 K, because of the slow tumbling rate of the enzyme, whereas the free complex exhibits a quite isotropic signal at g = 2.03 with multiple lines hyperfine structure (Fig. 6B). Thus, as also indicated by UV analysis, three slightly different paramagnetic forms can be
Identified: the DNDGIC in a free form, a DNDGIC bound to the GST P1-1 observed with GSH in excess, and a third species in which one GSH molecule has probably been replaced by a ligand provided by the protein or by a solvent molecule.

**Molecular Modeling—**A view of the active site of the covalent DNGIC (as obtained after removal of the excess GSH) is shown in Fig. 8. The model shows that: (i) one of the GSH ligands of DNDGIC can dock into the G-site and adopt the canonical extended conformation seen in crystal structures of GST-GSH complexes, (ii) Tyr7 is close enough to displace the other GSH ligand to generate a stable enzyme-inhibitor complex, and (iii) the NO moieties of the complex form van der Waal’s interactions with Ile104 and Tyr108. In addition, there are possible polar interactions with Tyr108 and the main chain nitrogen of Gly205.

**Concluding Remarks—**The findings reported above propose a novel role for GST P1-1 in its interaction with physiological NO carriers like GSNO and DNDGIC. In the absence of GSH, Cys47 and Cys101 represent the target residues for GSNO interaction. The modification that occurs on these residues may be restricted only to S-nitrosylation reaction. Different modifications like S-glutathionylation of cysteine residues, as observed in sarcoplasmic reticulum Ca-ATPase (62) and in neurogranin/RC3 and neuromodulin/GAP-43 (63), can be ruled out on the basis of mass spectrometry data. The nitrosylation of Cys101 does not cause any activity perturbation, whereas the modification of Cys47 results in a decrease in affinity for GSH to 1.2 ms

However, when one Cys47 has been nitrosylated, negative cooperativity masks the Cys47 residue of the second subunit, which remains unmodified even after prolonged incubation times with GSNO. The nitrosylated enzyme displays only slightly increased apparent $K_m$ value for GSH, and then it retains a large fraction of its detoxicating potential under physiological GSH concentrations. Thus, GST P1-1, in case of cellular GSH depletion, may act directly as NO protein carrier without detriment to its detoxicating activity. This corresponds to certain cellular conditions such as strong oxidative stress, etc. Under normal physiological conditions (millimolar GSH concentrations and metal traces), GSNO does not react directly with the enzyme but through the dinitrosyl-diglutathionyl iron complex. In fact, DNDGIC is rapidly formed by GSNO decomposition at millimolar GSH concentrations and micromolar ferrous ions. The interaction of this complex with GST P1-1 proceeds through a selected and sophisticated binding mechanism that can be summarized as follows, on the basis of UV and ESR data and molecular modeling: (i) In the presence of excess of GSH, DNDGIC binds to the G-site of the enzyme. One of the two GSH molecules is stabilized by the classical interactions of the G-site with its natural substrate; the NO moieties are stabilized by van der Waal’s and possible polar interactions with protein atoms. All of these interactions account for the very high affinity of the enzyme for this complex ($K_m < 10^{-12}$).

When the excess of GSH is removed, one of the two GSH molecules of the bound DNDGIC is lost from the complex, and one protein residue, possibly the hydroxyl group of Tyr7, is probably involved in the coordination of the iron atom. (ii) Binding of DNDGIC triggers a structural modification in the vacant subunit, which lowers its affinity for the complex by about 3 orders of magnitude ($K_m = 2 \times 10^{-9}$).

In conclusion, GST P1-1 appears to be a protein precisely designed for a specific and sophisticated interaction with the natural compound DNDGIC. Interestingly, serum albumin, previously suggested as a physiological DNDGIC carrier, displays more than 500 times lower affinity for this complex when compared with GST P1-1.

Because of the wide distribution of this cysteolic enzyme in several tissues, this property suggests a new putative role for this enzyme in the cell as a DNDGIC carrier protein. Interestingly, ESR studies revealed that, in animal tissues, DNDGIC is bound to unknown proteins with apparent molecular mass in the range 50–120 kDa (64). In some cases, i.e. in the rat aorta (65), such complexes give ESR signals different from DNDGIC in the free form, but very similar to those seen after reaction with GST P1-1.

Our preliminary data suggest that other recently evolved GSTs, i.e. Alpha and Mu isoenzymes, interact with DNDGIC like the Pi enzyme. However, the older bacterial enzyme, which is closer to the ancestral precursor of GSTs and lacks the crucial Tyr residue in the active site, does not interact with DNDGIC. Thus, we believe that the present data, obtained for the human GST P1-1, may represent only a first piece of a more complex scenario that can redefine and enlarge the physiological role of the recently evolved GSTs.

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