 Nitrosylation of Human Glutathione Transferase P1-1 with Dinitrosyl Diglutathionyl Iron Complex in Vitro and in Vivo

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We have recently shown that dinitrosyl diglutathionyl iron complex, a possible in vivo nitric oxide (NO) donor, binds with extraordinary affinity to one of the active sites of human glutathione transferase (GST) P1-1 and triggers negative cooperativity in the neighboring subunit of the dimer. This strong interaction has also been observed in the human Mu, Alpha, and Theta GST classes, suggesting a common mechanism by which GSTs may act as intracellular NO carriers or scavengers. We present here the crystal structure of GST P1-1 in complex with the dinitrosyl diglutathionyl iron ligand at high resolution. In this complex the active site Tyr-7 coordinates to the iron atom through its phenolate group by displacing one of the GSH ligands. The crucial importance of this catalytic residue in binding the nitric oxide donor is demonstrated by site-directed mutagenesis of this residue with His, Cys, or Phe residues. The relative binding affinity for the complex is strongly reduced in all three mutants by about 3 orders of magnitude with respect to the wild type. Electron paramagnetic resonance spectroscopy studies on intact Escherichia coli cells expressing the recombinant GST P1-1 enzyme indicate that bacterial cells, in response to NO treatment, are able to form the dinitrosyl diglutathionyl iron complex using intracellular iron and GSH. We hypothesize the complex is stabilized in vivo through binding to GST P1-1.

S-Nitrosylation of protein thiol groups by nitric oxide is accepted as being among the most important posttranslational modifications (1). Such modifications can cause modulation of many different functions with recent examples including proteins involved in signaling cascades, apoptosis, ion channels, redox systems, and hemoproteins (2). It has been suggested that NO may play a role in iron homeostasis and/or metabolism based on observations of iron nitrosylation of non-heme iron proteins in bacteria (3) as well as in mammals (4–6). Iron-free proteins, such as albumin (7) and GSH reductase (8), can also become targets of nitrosylation in the presence of suitable amounts of iron and thiol ligand (mostly GSH under physiological conditions). In these cases the formation of iron-dithiol dinitrosyl complexes are readily detected by EPR spectroscopy. We have recently shown that human glutathione transferase (GST) P1-1 strongly binds dinitrosyl diglutathionyl iron complexes (DNDGIC) in vitro while maintaining its well known detoxifying activity toward dangerous compounds (9). A very high affinity for this complex was also found for other glutathione transferase classes (Mu, Alpha, and Theta), suggesting a common mechanism by which the more recently evolved GSTs may act as intracellular NO carriers or scavengers (10, 11).

The glutathione transferases (EC 2.5.1.18), historically also called glutathione S-transferase, catalyze the nucleophilic attack by reduced glutathione (GSH) on non-polar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. This classical conjugation reaction toward foreign compounds (e.g. cancer chemotherapeutic agents, insecticides, herbicides, carcinogens) and endogenous compounds (e.g. byproducts of oxidative stress) is considered part of a coordinated defense strategy together with other GST-dependent enzymes, the cytochrome P450s (Phase I enzymes) and some membrane transporters (Phase II) such as MRP1 and MRP2, to remove glutathione conjugates from the cell. In mammals there are three major families of proteins widely distributed in nature that exhibit glutathione transferase activity. Two of these, the cytosolic and mitochondrial GSTs, comprise soluble enzymes, whereas the third family is microsomal and is referred to as MAPEG (membrane-associated proteins in eicosanoid and glutathione) metabolism (12). The human cytosolic GSTs are dimeric proteins that can be grouped into at least seven gene-independent classes (Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta) on the basis of their amino acid sequence and immunological properties (13–16). Their three-dimensional structures do not differ significantly despite low sequence homology (17–20). Each subunit contains a very similar binding site for GSH (G-site) and a second one for the hydrophobic cosubstrate (H-site). Structural differences at the H-site confer a certain degree of substrate selectivity. Despite a common structure they appear to play multiple functions. For example, Zeta class GST Z1–1 is involved in the catalysis of phenylalanine (21), Pi class GST P1-1 and Mu class GST M1–1 are involved in signaling pathways through physical interaction with some kinases (22, 23), and Omega class GST O1–1 modulates calcium channels, thus protecting mammalian cells from apoptosis induced by Ca2+ mobilization (24).

In this paper we report the crystallographic structure of GST P1-1

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5 The abbreviations used are: GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene; NONOate, 2-(N,N-diethylamino)-diazene 2-oxide; DEANO, diethylamine NONOate; DNDGIC, dinitrosyl diglutathionyl iron complex; GNIC, dinitrosyl glutathionyl iron complex; MES, 2-(N-morpholino)ethanesulfonic acid; WT, wild-type enzyme; GSNO, S-nitrosoglutathione.
with the DNDGIC bound in the active site, which together with site-directed mutagenesis studies demonstrate a crucial role for the catalytic residue Tyr-7 acting as a ligand for the iron complex in the active site (Scheme 1). These data provide direct support that GST’s can be nitrosylated in vitro. Further studies on intact *Escherichia coli* cells upon exposure to either GSNO or diethylamine NONOate suggest that human GST P1-1 can also be nitrosylated inside the cell.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Diethylamine NONOate (DEANO) was from Calbiochem. GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and other reagents used were from Sigma. DEANO solutions were prepared in phosphate-buffered saline buffer, pH 7.4, at room temperature; under these conditions the half-life of NO release is 16 min.

**GSNO Synthesis**—GSNO was prepared by a modification of the original published procedure (9). To 100 mM GSNO was added. Finally, 2 μl of 1 mM FeSO₄ solution containing 0.5 mM vitamin C was added. The final concentrations were 40 mM GSH, 4 mM GSNO, 4 mM FeSO₄, and 1 mM vitamin C in a 500-μl volume. After several hours on ice wrapped in aluminum foil to exclude light, the solution turned a strong yellow color that was stable, contrary to previous reports, for up to a week (9–11). DNDGIC cannot be isolated as a solid due to dimerization (30) but is stabilized in solution by the large excess of GSH present.

**Crystallization**—Wild-type human Pi class GST P1-1 was expressed and purified as previously described (25). The protein was crystallized using the hanging drop vapor diffusion method as described elsewhere (26). Briefly, a 2-μl drop of protein (concentration of 6.7 mg/ml in 1 mM EDTA, 1 mM mercaptoethanol, and 10 mM phosphate buffer, pH 7.0) was mixed with the same volume of reservoir buffer composed of 100 mM MES buffer, pH 5.5 or 6.0, 22% (w/v) polyethylene glycol 8000, 20 mM CaCl₂, 10 mM dithiothreitol, and 10 mM GSH. Crystals grew at 22 °C and reached a suitable size in approximately a week.

**Dinitrosyl Diglutathionyl Iron Complex Synthesis**—Wild-type crystals were transferred into a new drop containing 200 μl of the DNDGIC solution described above, 100 mM MES buffer, pH 6.0, 22% (w/v) polyethylene glycol 8000, and 20 mM CaCl₂. These crystals were soaked for several days.

**Data Collection and Processing**—The x-ray diffraction data were collected at the Advanced Photon Source (Chicago, Illinois), beam line 14-ID-B using a MAR165 CCD MARResearch detector. The wavelength was set to 0.99 Å. For cryoprotection the crystals were soaked for 2 min in the well solution containing 5% (v/v) methyl-2,4-pentanediol (MPD), then dipped briefly in well solution containing 10% (v/v) MPD. The crystals were then snap-frozen at 100 K in the cryostream. Diffraction data were processed and scaled with HKL (31). The crystals were shown to belong to a monoclinic lattice, with the space group C2, as seen previously for the wild-type GST P1-1 (26).

**Structure Determination and Refinement**—Refinement began with the Pi class GST in the C2 space group (5GSS (26)) that had GSH and water molecules removed. Rigid body refinement in CNS (32) was used to compensate for any possible changes in crystal packing. The starting model gave an R-factor of 33.0% (R_free = 35.3%). The model was then refined by a round of simulated annealing using CNS. Because the asymmetric unit of the crystal contained two GST monomers, use was made of the non-crystallographic symmetry restraints on all non-hydrogen atoms in the initial rounds of the refinement. The model was rebuilt with TURBO (33), and GSH, MES, and water molecules were added. The model was further refined with cycles of positional and isotropically restrained B-factor refinement. After several rounds of refinement, the density for the iron complex was evident in a 2.7 ÅFo-Fc map and was subsequently built into the model. An absorption scan at the iron edge, at 7.13 keV or 1.74 Å, demonstrated the presence of the metal in the crystal. However, data collected from this crystal were limited to a resolution of 2.7 Å because of radiation damage at this wavelength. Thus, the data were recollected at 0.99 Å off a fresh crystal. After multiple rounds of refinement and rebuilding the final R-factor was 18.2% (R_free = 24.4%) for all data to 2.1 Å of resolution. The stereochemistry was analyzed with the program PROCHECK (34) and gave values either similar or better than expected for structures refined at similar resolu-
Expression Plasmids and Site-directed Mutagenesis—The plasmid pGST-1, producing large amounts of recombinant wild-type GST P1-1 in the cytoplasm of E. coli, has been described previously (25). Site-directed mutagenesis of Tyr-7 into Phe, His, and Cys residues was accomplished using the same strategy adopted for the plasmid pGST-1 except that the synthetic linkers with NcoI-TaqI-compatible ends were obtained by annealing the following complementary oligonucleotides:

5'H11032-CATGCCACCGTACACCGTTGTTTTCCTCCGTT and 5'H11032-CGAACCGGGAAACAACGGTGACCGTG (Phe-7); 5'H11032-CATGCCACCGTACACCGTTGTTTGCCTCCGTT and 5'H11032-CGAACCGGGAAACAACGGTGACCGTG (His-7); 5'H11032-CATGCCACCGTACACCGTTGTTTGCCTCCGTT and 5'H11032-CGAACCGGGAAACAACGGTGACCGTG (Cys-7).

Protein Expression and Purification—Native and mutant GST P1-1 enzymes were produced as described previously (25, 35). Briefly, E. coli strain TOP 10 cells, harboring plasmid pGST-1 or plasmids expressing Phe-7, His-7, or Cys-7 mutant enzymes (pGST-F7, pGST-H7, pGST-C7), were grown in Luria broth containing 100 μg/ml ampicillin and 50 μg/ml streptomycin. The expression of GST was induced by the addition of 0.2 mM isopropyl-1-thio-galactopyranoside when the absorbance at 600 nm was 0.5. Eighteen hours after induction cells were harvested by centrifugation and lysed as previously described (25).

TABLE ONE
Summary of data collection and structure refinement for the DNGIC-GST P1-1 complex
The values in parentheses are for the highest resolution bin.

<table>
<thead>
<tr>
<th>Data Collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>76.2</td>
</tr>
<tr>
<td>b (Å)</td>
<td>89.8</td>
</tr>
<tr>
<td>c (Å)</td>
<td>68.7</td>
</tr>
<tr>
<td>β (°)</td>
<td>97.6</td>
</tr>
<tr>
<td>Maximum resolution (Å)</td>
<td>2.1 (2.18-2.10)</td>
</tr>
<tr>
<td>No. of crystals</td>
<td>1</td>
</tr>
<tr>
<td>No. of observations</td>
<td>2,076,942</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>24,224 (1,811)</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>90.1 (68.0)</td>
</tr>
<tr>
<td>I/σ</td>
<td>27.1 (8.33)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>85.7 (13.5)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>6.1 (17.1)</td>
</tr>
</tbody>
</table>

Refinement
Non-hydrogen atoms
Protein                         | 3,260    |
DNGIC                           | 50       |
MES                             | 24       |
Solvent (H2O)                   | 242      |
Resolution (Å)                  | 2.1 (2.18-2.10) |
R_conv (%)                      | 18.2 (20.7) |
R_free (%)                      | 24.4 (28.4) |
Reflections used in R_conv calculations
Number                          | 22,998 (1729) |
Completeness (%)                | 85.8 (67.9) |
Root mean square deviation from ideal geometry
Bonds (Å)                       | 0.005    |
Angles (°)                      | 1.2      |
Mean B (protein) (Å²)           | 29.2     |
Main chain                      | 27.7     |
Side chain                      | 30.8     |
Iron                            | 43.3     |
NO                              | 33.1     |
GSH                             | 31.1     |
Mean B (solvent) (Å²)           | 33.0     |
Residues in most favored regions of Ramachandran plot (%) | 92.5 |
Residues in allowed regions of Ramachandran plot (%) | 5.8 |
Residues in generously allowed regions of Ramachandran plot (%) | 1.7 |
Residues in disallowed regions of Ramachandran plot (%) | 0 |

*D Rmerge = Σ½[I(i) - <I(i)>]/Σ½[I(i)], where I(i) is the intensity for the ith measurement of an equivalent reflection with indices h,k,l.

**R_conv = Σ[I(i)] - F_calc)/Σ[I(i)] F_calc, where F_calc are the observed and calculated structure factor amplitudes, respectively.

* R_free was calculated with 5% of the diffraction data that were selected randomly and not used throughout refinement.
and GST mutant enzymes were purified by affinity chromatography on immobilized glutathione (36). After affinity purification, the native and the mutant enzymes (Y7F, Y7H, and Y7C) were homogeneous as judged by SDS-PAGE (37). Protein concentration was determined by the method of Lowry et al. (38).

**Kinetic Studies**—The enzymatic activities were determined spectrophotometrically at 25 °C with CDNB as cosubstrate following the product formation at 340 nm, $\varepsilon = 9600 \text{ M}^{-1} \text{ cm}^{-1}$ (39). Spectrophotometric measurements were performed in a double beam Uvicon 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. Enzymatic rates were corrected for the spontaneous reaction.

Apparent kinetic parameters, $k_{\text{cat}}$, $K_{m}^{\text{CDNB}}$ were determined in 0.1 M potassium phosphate buffer, pH 6.5, and 0.1 mM EDTA, containing fixed concentrations of GSH (10 mM) and variable concentrations of CDNB (0.1–2 mM). The collected data were fitted to the Michaelis-Menten equation by non-linear regression analysis using the GraphPad Prism (GraphPad Software, San Diego, CA). The apparent $K_{m}^{\text{GSH}}$ was also determined at a fixed CDNB concentration (1 mM) and variable

**FIGURE 1.** Crystal structure of the GST P1-1 DNDGIC complex. **A**, stereo diagram of the final $2F_o - F_r$ electron density map of the DNGIC complex at 2.1 Å of resolution centered about the active site. The DNGIC ligand, GSH, and Tyr-7 were omitted from the final calculation, and the map is contoured at 0.75σ. **B**, stereo diagram showing the coordination geometry of the active site.
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Steady-state kinetic properties of wild type and Tyr7 mutant enzymes
See “Experimental Procedures” for experimental conditions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(K_m^{\text{GSH}})</th>
<th>(K_m^{\text{GSNO}})</th>
<th>(k_{\text{cat}})</th>
<th>(K_i^{\text{DNDGIC}})</th>
<th>(pK_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.15 ± 0.04</td>
<td>1.2 ± 0.1</td>
<td>38 ± 2</td>
<td>(&lt;1.5 \times 10^{-9})</td>
<td>6.2</td>
</tr>
<tr>
<td>Y7F</td>
<td>0.16 ± 0.03</td>
<td>1.8 ± 0.1</td>
<td>0.13 ± 0.05</td>
<td>0.94 \times 10^{-6}</td>
<td>8.14</td>
</tr>
<tr>
<td>Y7H</td>
<td>0.35 ± 0.08</td>
<td>1.24 ± 0.12</td>
<td>0.08 ± 0.01</td>
<td>7.44 \times 10^{-7}</td>
<td>8.46</td>
</tr>
<tr>
<td>Y7C</td>
<td>0.64 ± 0.14</td>
<td>1.11 ± 0.02</td>
<td>0.73 ± 0.12</td>
<td>1.33 \times 10^{-6}</td>
<td>7.74</td>
</tr>
</tbody>
</table>

FIGURE 2. Dependence of \(k_{\text{cat}}\) on pH. The experimental values of \(k_{\text{cat}}\) were normalized for a useful comparison among the different mutant enzymes. The true \(pK_a\) values are reported in TABLE TWO. The experimental values refer to Y7H (○), Y7C (●), Y7F (●●), and wild type (○), respectively.

results to those shown in Figs. 3 and 4. As an example, after 15 min of exposure to 0.5 mM GSNO or DEANO, the GST inactivation was about 20% (despite 35% with 2 mM GSNO), whereas the ERP signal was only slightly decreased in comparison with that shown in Fig. 3Ba (2 mM GSNO exposure). E. coli cell exposure to 10 mM GSNO (a sledge hammer) only reduces GST activity 40%. To check if 2 mM GSNO concentration could inhibit cell growth, we followed bacterial growth of cells exposed to 2 mM GSNO for 24 h at 600 nm, and we found no significant difference with E. coli untreated cells under the same conditions (data not shown).

EPR Detection of Dinitrosyl Diglutathionyl Iron Complex—EPR spectra were recorded using 80-μl samples in flat glass capillaries (inner cross-section 5 × 0.3 mm) to optimize instrument sensitivity as previously described (40). All measurements were made at room temperature with an ESP300 X-band instrument (Bruker, Karlsruhe, Germany) equipped with a high sensitivity TM107 TC-mode cavity. Spectra were recorded over a 200 G range using 20 milliwatt power, 2.0 G modulation, and a scan time of 42 s. Typically 4–16 single scans were accumulated to improve the signal-to-noise ratio. High resolution spectra were recorded with 0.1-G modulation and 2 milliwatts of power. Quantitation of DNDGIC was done by incubating GSH (20 mM) and GSNO (2 mM) with variable amounts of ferrous ions (from 2 to 20 μM) under anaerobic conditions in 0.1 M potassium phosphate buffer, pH 7.4.

RESULTS

Human GST P1-1 Binds the Dinitrosyl Diglutathionyl Iron Complex at the Active Site through Tyr-7—Overall, the crystal structures of the wild-type enzyme with either GSH or the DNDGIC complex bound are very similar. Superposition of the α-carbon atoms of the GSTP1-1-DNDGIC structure and the wild-type structure (5GSS) yielded a root mean square deviation of 0.27 Å for the 416 residues included in the calculation, indicating the structures are virtually identical. There are some significant, albeit small, movements of atoms close to the DNDGIC ligand with some backbone atoms of the GSH molecule having moved up to 0.7 Å, the hydroxyl group of Tyr-108 moved 0.4 Å, and Phe-8 moved 0.7 Å. The most important change is that in the complex Tyr-7 binds to the iron atom through its phenolate group and in doing so displaces one of the GSH ligands (Fig. 1 and Scheme 1). The remaining iron-bound GSH ligand binds in the G-site in an almost identical manner to that observed in the structure of the GST complex of human GST P1-1 (26). The iron atom is coordinated in a distorted tetrahedral geometry with angles between the four iron ligands (Tyr-7, GSH, and the two nitroso groups) of between 101° and 118°. Other notable interactions include Tyr-108, which forms a water-mediated hydrogen bond to the oxygen of one of the nitroso groups and a water-mediated contact between one of the nitroso groups and Asn-204.

To confirm the importance of Tyr-7 in the coordination of the iron atom of DNDGIC, this residue was mutated by site-directed mutagenesis into Phe, His, or Cys, and the corresponding mutants were expressed in E. coli. The first mutant (Y7F) was already produced and characterized.

GSH concentrations (from 0.02–10 mM). Kinetic parameters reported in this paper represent the mean of at least three different experimental data sets.

Cell Growth and NO Treatment of Intact Cells—Single colonies of freshly plated E. coli strain TOP 10 harboring plasmid pGST-1 or pGST-F7 were used to inoculate 25 ml of overnight cultures. These cultures were diluted 1:100 into Luria-Bertani medium containing 100 μg/ml ampicillin and 50 μg/ml streptomycin sulfate, grown at 37 °C to an A600 value of 0.5, and induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside. Cells (1 liter) were grown at 37 °C for 4 h, divided in 4 aliquots (0.25 liter each), and treated as follows. (a) Two aliquots were incubated with either 2 mM GSNO or 50 μM FeSO4, one aliquot was incubated with 2 mM GSNO and 50 μM FeSO4, and the last aliquot was used as the control. All the aliquots were incubated under the same conditions, at 37 °C for 15 min. (b) Another experimental set was established to monitor the time course of DNDGIC complex formation in which two aliquots were incubated with either 2 mM GSNO alone or 2 mM GSNO plus 50 μM FeSO4 at different times (5, 15, 30, 60 min).

At the end of the incubation cells of different aliquots were harvested by centrifugation for 15 min at 7000 rpm, washed with 10 mM phosphate buffer, pH 7.0, containing 0.1 mM EDTA, and after centrifugation resuspended in a suitable volume of 10 mM phosphate buffer, pH 7.0, for electron paramagnetic resonance (EPR) analysis. The same cells were also lysed by sonication, and cell membranes were removed by centrifugation at 14,000 rpm for 10 min, and the resulting supernatant was tested for GST activity assay, protein concentration, and further EPR analysis. A similar set of experiments was also carried out using 0.5 mM DEANO (final concentration) as the NO donor instead of GSNO. We used the concentration of 2 mM GSNO throughout this work, which is the same reported previously for the DNDGIC synthesis in vitro (9). However, we have also exposed E. coli cells to different concentrations of GSNO (in a range between 0.5 and 10 mM) and obtained quite similar...
GST P1-1 Is Inhibited by NO inside the Cell—We have tested the effect of NO in a simple cellular model by exposing TOP10 E. coli cells, overexpressing human GST P1-1, to 2 mM nitrosothiol for 15 min. EPR spectroscopic analysis of the intact cells showed a characteristic signal of a protein-bound dinitrosyl dithiol iron complex (Fig. 3Aa), and a GST assay of the same cell extracts indicated a 35% decrease of enzymatic activity compared with both the E. coli cells alone and the E. coli cells treated with FeSO₄ (Fig. 4). Simultaneous exposure of the cells to both GSNO and ferrous ions did not increase the extent of the inactivation (Fig. 4) nor the intensity of EPR signal (data not shown).

The Lifetime of the Dinitrosyl Diglutathionyl Iron Complex Bound to GST P1-1 Is Dependent on Iron Availability in the Cell—We observed the lifetime of the DNDGIC complex bound to the protein by exposing E. coli cells to GSNO for different times at each time point, carrying out EPR spectra of intact cells and monitoring GST activity in the same cell extracts. It appears that the maximum extent of enzymatic inactivation (about 40%) can be reached after 5 min of GSNO exposure followed by
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![Graph A: Residual Activity (%) vs Time (min)](image)

![Graph B: EPR Signal Intensity vs Time (min)](image)

**DISCUSSION**

In this paper, we provide compelling evidence that GSTs can bind NO donors (such as DNDGIC) at the active site using as a fourth ligand, the catalytic Tyr (Tyr-7 in GST P1-1), thus confirming the hypotheses proposed in previous papers (9–11). The crystal structure provides the first structural view of the DNDGIC complex and first view of it bound to a protein and, therefore, may be of value for other studies concerning these biological complexes.

The structure of this NO donor bound at the active site shows that 1) one GSH ligand is displaced and that the remaining GSH ligand of the complex binds in the G-site in an almost identical manner to that of the structure of GSH bound to GST; 2) iron binds in the complex in a distorted tetrahedral geometry with angles between the four iron ligands, Tyr-7, GSH, and the two nitroso groups, between 101° and 118°; 3) the protein side chain that is involved in covalent attachment with the complex is Tyr-7; 4) that other significant residues involved in the binding of the complex are Tyr-108 and Asn-204, both of which form water-mediated hydrogen bonds with the oxygen of one of the nitroso groups. Site-directed mutagenesis studies of Tyr-7 are consistent with the crystallographic data. Change of this residue is detrimental for the binding of DNDGIC at the active site, as demonstrated by the strong decrease of $K_i^{DNDGIC}$ (Table Two) and by EPR spectra, which show that the equilibrium between the bound and free form of DNDGIC complex is shifted toward the free form in all Tyr mutants (Fig. 3A). In previous studies (42) the importance of Tyr-7 in catalysis was emphasized but not its role in GSH binding (because there were no changes in $K_m$ or $K_p$ values between WT and Tyr-7 mutant enzymes). The results reported here suggest a specific and new function of Tyr-7 in stabilizing DNDGIC in the active site through a covalent binding to the iron atom.

In studying the DNDGIC formation inside the cell we have used E. coli cells because they possess a unique class of GST (GST B1-1) unable to bind this complex (10) and with no detectable CDNB activity in the cytosolic extracts. Therefore, the GST activity (4 units/mg) measured in the extracts of E. coli cells grown at 37 °C and 4 h after isopropyl 1-thio-β-D-galactopyranoside induction (see "Experimental Procedures") accounted entirely for the human recombinant GST P1-1 activity. Experiments with E. coli cells overexpressing human GST P1-1 demonstrated for the first time the in vivo formation of the DNDGIC complex upon exposure to 2 mM GSNO (Fig. 5B) (see "Experimental Procedures" for the range of GSNO concentrations used). The partial but significant inhibition of GST P1-1 in the same cell extracts (Fig. 4) suggests that the DNDGIC complex is tightly bound to the GST P1-1 active site, and only cysteine or, more efficiently, potassium cyanide (in large excess with respect to the concentration of the protein bound DNDGIC complex) is able to abolish this strong interaction and restore the enzymatic activity (Fig. 4). We have previously shown that the in vitro formation of the DNDGIC complex is independent of the presence of GST P1-1 (i.e. there is no catalytic effect by GST) (9), and this is also the case when DNDGIC synthesis occurs in bacteria upon exposure to NO donors. Indeed, Fig. 3Bb shows a faint EPR signal of such a bound complex also in the absence of GST P1-1. The presence of the overexpressing GST P1-1 in bacteria is crucial to stabilize this complex; in fact, free DNDGIC has a very short half-life in solution and is never observed in the cells. Also, the partial inactivation of GST P1-1 observed in E. coli extracts can be explained on the basis of previous in vitro experiments of GST P1-1 inhibition (9) and present structural findings. We have previously reported that DNDGIC binding to GST P1-1 exhibited negative cooperativity so that only one active site in the dimer is occupied by ligand (9). The GST P1-1-DNDGIC complex crystallizes with one dimer, and thus, two active sites per asymmetric unit in the crystal. The occupancy of the DNDGIC ligand in each site is ~0.5, which is consistent with the scenario that the ligand binds to only one site in solution, but the complex crystallizes so that the GST dimer packs into the asymmetric unit in the two possible orientations so that each active site appears half occupied.

We have already shown that GSNO causes in the absence of GSH S-nitrosylation of both the Cys-47 and Cys-101 residues of GST P1-1, whereas in the presence of GSH and traces of ferrous ions there is always formation of DNDGIC, which binds to the active site with extraordinary affinity by competitive inhibition with respect to GSH. Because bacterial cells contain iron proteins and GSH (at millimolar concentrations) it is very likely that the DNDGIC complex inhibits GST P1-1 in a similar fashion also in vivo. The recovery of GST activity after KCN treatment...
of E. coli extracts containing DNDGIC-GST (Fig. 4) is consistent with this suggestion. In fact, CN− ions are able to bind ferrous ions to give the extremely stable [Fe(CN)₆]⁻⁴ complex (9), and in this way they effectively destroy the active site-bound DNDGIC complex and restore the enzymatic activity. Another important piece of evidence comes from the heterologous expression of Y7F mutant inside the bacterial cells. EPR analysis of the intact cells after GSNO exposure showed only a modest signal of DNDGIC complex similar to that obtained in bacterial cells exposed to 2 mM GSNO and lacking the expression vector for GST P1-1 (data not shown) and no detectable GST activity, confirming the crucial importance of Tyr-7 in both the binding of DNDGIC and the catalytic role of this residue also in vivo. We have attempted purifying the human GST P1-1 bearing the bound complex from E. coli cells by affinity chromatography with GSH as ligand. Unfortunately, the high pH, 10.0, of the elution buffer destabilized the strong interaction between GST P1-1 and DNDGIC complex (data not shown), and we could not isolate the protein bearing the DNDGIC complex.

It has been reported previously that nitric oxide (administered to the cells as nitrosothiol) can cross the cell membrane and be regenerated as nitrosothiol inside the cell (43). This appears to have occurred in our experiments, where upon exposure to exogenous GSNO we observed a rapid formation of DNDGIC complex inside the cell. Very similar results have been obtained using DEANO as an exogenous NO donor; therefore, nitric oxide alone without the GSH moiety can enter into the E. coli cells. The critical events follow; that is, the recruitment of intracellular iron by nitrosylation of iron proteins (e.g. iron-sulfur proteins (44)) and utilization of intracellular GSH, leading to the synthesis of the DNDGIC complex in vivo (at micromolar concentrations as indicated by EPR spectra sensitivity). The importance of thiols (mostly GSH) in favoring this interaction between NO and iron has been pointed out (or as reductant to remove iron proteins or as a ligand for iron coordination along with NO) (5), and as result there is always formation of DNDGIC complexes. Our studies add a significant piece of knowledge to this mechanism; this complex, with a very short life in solution, once formed may be trapped and stabilized by GST P1-1 (Fig. 3B).

Other experiments using EPR analysis and GST activity assays gave an insight into the lifetime of the DNDGIC complex when bound to GST P1-1 (Fig. 5, A and B). The strong and specific binding of DNDGIC GST complex to P1-1 is reversible and dependent on iron availability inside the cell; in fact, unless iron is supplied from outside of the cell, there is a recovery of nearly all GST activity and loss of EPR signal within 1 h (Fig. 5, A and B). This last result is surprising since results obtained previously in vitro show the DNDGIC complex remains stable for several hours when bound to GST proteins (9). It is possible, as suggested by others, that in bacterial cells iron-sulfur cluster proteins are used as a temporary source of iron to counteract the exogenous NO until there is activation of enzymatic systems able to destroy this DNDGIC complex and repair the proteins depleted of iron. We already know that a number of enzymes can be involved in NO metabolism, especially in bacteria, where upon exposure to high amounts of NO there is protein S-nitrosylation and in turn induction of genes controlling nitrosant metabolism (e.g flavohemoglobin) (45). Our findings provide an additional glimpse of this event; on the basis of a high affinity of nitric oxide for iron, several iron proteins can be selected targets of nitrosylation with dangerous consequences; the GSH molecule, present at large levels (millimolar concentration), favors the rapid formation of more stable nitrosothiol species (DNDGIC complexes), which in turn are sequestered by GSTs, present in several tissues at micromolar concentrations. We have already described in vitro a sophisticated device based on intersubunit communication by which GST proteins can trap these complexes with one subunit while maintaining its well known detoxifying activity toward dangerous compounds with the other subunit (9), and we would speculate that GSTs can act in a similar way also in vivo. In fact, despite the exposure of E. coli cells to large amounts of GSNO (up to 10 mM), we have observed only a partial inhibition of the overexpressed GST P1-1 inside the cell (up to 40% inactivation), consistent with the data obtained in vitro. The next question to be addressed is, is there export of these complexes outside the cell or destruction inside? A previous report (5) suggests the possibility of a transporter located in the membrane that is able to extrude such complexes, and one could argue that membrane pumps (Phase 3), which promote the efflux of toxic GSN conjugates outside the cytosol, also play a role in the extrusion of these complexes. There is no evidence for this at moment; for example, a tightly regulated GSNO detoxification pathway has been reported in E. coli cells but not in eukaryotic cells, suggesting a possible destruction of such complexes inside the cell (45). Our experimental model (E. coli cells overexpressing human GST P1-1) is based on the fact that large amounts of GSNO (at least micromolar concentrations) enter the cell containing high GSH concentrations, iron, and overexpressed GST. Whether these conditions could have physiological relevance in eukaryotic cells in some instances (e.g inflammatory diseases), where GSTs are expressed in higher concentration, remains to be established.

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REFERENCES

DNDGIC Stabilization by Human Glutathione Transferase P1-1