Glutathione Transferase Superfamily Behaves Like Storage Proteins for Dinitrosyl-Diglutathionyl-Iron Complex in Heterogeneous Systems*

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Electron paramagnetic resonance and kinetics experiments have been made to determine the formation, stability, and fate of the natural nitric oxide carrier, dinitrosyl-diglutathionyl-iron complex (DNDGIC), in heterogeneous systems approaching in vivo conditions. Both in human placenta and rat liver homogenates DNDGIC is formed spontaneously from GSH, S-nitroso-glutathione, and trace amounts of ferrous ions. DNDGIC is unstable in homogenates depleted of glutathione S-transferase (GST); an initial phase of rapid decomposition is followed by a slower decay, which is inversely proportional to the concentration. In the crude human placenta homogenate, GSTP1-1, which represents 90% of the cytosolic GST isoenzymes, is the preferential target for DNDGIC. It binds the complex almost stoichiometrically and stabilizes it for several hours (t1/2 = 8 h). In the presence of an excess of DNDGIC, negative cooperativity in GSTP1-1 opposes the complete loss of the usual detoxicating activity of this enzyme. In the rat liver homogenate, multiple endogenous GSTs (mainly Alpha and Mu class isoenzymes) bind the complex quantitatively and stabilize it (t1/2 = 4.5 h); negative cooperativity is also seen for these GSTs. Thus, the entire pool of cytosolic GSTs, with the exception of the Theta GST, represents a target for stoichiometric amounts of DNDGIC and may act as storage proteins for nitric oxide. These results confirm the existence of a cross-link between NO metabolism and the GST superfamily.

A crucial step in biochemical research is the demonstration that a specific phenomenon observed in vitro may actually occur in vivo. In the accompanying paper (1) we have characterized the peculiar interaction in vitro of an endogenous NO carrier, the dinitrosyl-diglutathionyl-iron complex (DNDGIC),1 with representative members of the glutathione S-transferase (GST) superfamily. In this paper we extend these studies in order to verify the occurrence and the modality of this interaction in complex biological systems like crude tissue homogenates. The aim of the present paper is also to determine whether DNDGIC could be formed in multicompartment biological systems starting from GSNO, GSH, and trace amounts of ferrous ions. Although a homogenate is not representative of the environment found in the living cell, the use of homogenates allows direct measurements of GST activity, which at present cannot be determined in intact cells. A useful model for this investigation is the human placenta tissue which mainly expresses GSTP1-1, and the rat liver which represents a more complex system containing many different GST isoenzymes (2). Our results demonstrate that DNDGIC can be readily formed in homogenates and that GSTP1-1 is the main target of DNDGIC in the human placenta, whereas the entire pool of GSTs (mainly Alpha and Mu class GSTs) plays a similar role in rat liver. DNDGIC is greatly stabilized when bound to GSTs, and these enzymes may act as half-storage proteins for NO taking advantage of a common negative cooperativity induced by DNDGIC binding.

EXPERIMENTAL PROCEDURES

DNDGIC Synthesis—DNDGIC was prepared essentially as described previously (3). Briefly, 1 ml of 0.5 mM FeSO₄ (dissolved in degassed water to avoid rapid oxidation to ferric state) was incubated in 10 ml (final volume) of 0.1 M potassium phosphate buffer, pH 7.0, containing 20 mM GSH and 2 mM GSNO (25 °C). After 10 min, the reaction was almost complete, and the resulting stock solution of DNDGIC (50 μM) was stable at least for 3 h.

The stability of DNDGIC in a simple buffer or in homogenates was assayed in the presence of 10 mM EDTA. EDTA prevents the metal-catalyzed release of NO from GSNO (4) and thus the possible DNDGIC re-synthesis in the presence of an excess of GSH and GSNO. On the other hand 10 mM EDTA is unable to abstract ferrous ions from DNDGIC (data not shown).

EPR Detection of DNDGIC—Samples for EPR experiments were usually prepared using tissue homogenates or 0.1 M potassium phosphate buffer, pH 7.0, with DNDGIC added from a freshly made stock solution. EPR measurements were carried out at room temperature (22–25 °C) with a Bruker ESP900 X-band instrument (Bruker, Karlsruhe, Germany) equipped with a high sensitivity TM110-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) (5). For kinetics measurements standard round glass capillaries (1.10 mm inner diameter) with a working volume of 40 μl were applied, to allow rapid handling of samples. 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. High resolution spectra were recorded with 0.1 G modulation and 2 milliwatts power. Quantification of DNDGIC has been done by incubating GSH (20 mM) and GSNO (2 mM) with variable amounts of ferrous ions (from 2 μM to 20 μM) in 0.1 M potassium phosphate buffer, pH 7.0, in the presence or absence of crude homogenates.

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§ The abbreviations used are: DNDGIC, dinitrosyl-diglutathionyl-iron complex; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.
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 (25 °C). The reaction was followed spectrophotometrically at 340 nm where the GSH-2,4-dinitrobenzene adduct absorbs (ε = 9,600 M⁻¹ cm⁻¹).

Detection of DNDGIC by GSTA1-1 Inhibition—Variable amounts of DNDGIC (from 0.2 to 2 μM) were incubated at 25 °C with 4 μM GSTA1-1 in 0.1 M potassium phosphate buffer, pH 7.0, and 10 mM EDTA. After 5 min, 10-μl aliquots were assayed for enzymatic activity with 10 mM GSH and 1 mM CDNB in 0.1 M potassium phosphate buffer, pH 6.5. Spectrophotometric determinations at 340 nm started within 10 s from addition of the substrates. A strict linearity of inhibition was observed within a complex:enzyme ratio between 0.05 and 0.5 (see Fig. 1).

Preparation of Crude Homogenates—Crude human placenta (20 g) was cut in small pieces and washed 5 times with 200 ml of physiological solution. The washed tissue was then homogenized in a Waring blender with 20 ml of 0.1 M potassium phosphate buffer, pH 7.0. The resulting homogenate was centrifuged at 15,000 rpm for 15 min. The supernatant, containing 2–3 μM of GSTP1-1, was termed “crude human placenta homogenate.” Rat liver crude homogenate was prepared starting from 10 g of Sprague-Dawley female rat liver washed 2-fold with 200 ml of physiological solution. The tissue was homogenized with 30 ml of 0.1 M potassium phosphate buffer, pH 7.0. After centrifugation at 13,000 × g (15 min), the resulting supernatant, termed “crude rat liver homogenate,” contained the entire pool of GST isoenzymes corresponding to about 56 μM GST.

Preparation of GST-depleted Homogenates—GST-depleted homogenates were obtained using affinity chromatography on immobilized GSH (6). 10 ml of crude human placenta homogenate was passed through a column (1 × 4 cm) of glutathione-Sepharose matrix. This single step procedure of affinity chromatography allowed us to retain on the column more than 95% of the transfectase activity, and the resulting eluate was termed “GST-depleted placenta homogenate.” An identical procedure was made for the rat liver homogenate, with the exception that the first eluate was again passed 2 times through the same column. This procedure was necessary to retain quantitatively the Alpha class GST that showed limited affinity for this specific matrix. The final eluate was termed “GST-depleted liver homogenate,” although it contains a small amount of endogenous Theta class GST which is not retained by this specific column. Protein concentration was determined by the method of Lowry et al. (7).

RESULTS

DNDGIC Formation in GST-depleted Human Placenta Homogenate—DNDGIC is readily formed in vitro starting from GSH, GSNO, and trace amounts of ferrous ions (3). The reaction probably occurs after release of nitric oxide through an iron-mediated reaction between GSNO and GSH. The effects of heterogeneous biological systems on the formation and stability of DNDGIC have been evaluated by reacting these natural compounds in human placenta homogenates. DNDGIC may be monitored through the typical EPR spectrum of this complex which shows a characteristic shape with a maximum at g = 2.03 (Fig. 1a). The detection limit of this procedure is about 1 μM concentration for EPR analysis performed at 25 °C. Alternatively, a more sensitive procedure has been used based on the strong competitive inhibition of GSTA1-1 by DNDGIC (1). (Fig. 1b). Human placenta represents a peculiar tissue as it contains mainly a single isoenzyme, GSTP1-1, which accounts for more than 90% of all cytosolic GSTs. In a first experimental approach, a GST-depleted homogenate was employed to avoid any possible influence of GSTP1-1 on the formation and stability of DNDGIC. In this milieu, after incubation of 20 mM GSH, 2 mM GSNO, and 10 mM ferrous sulfate, the complex was quantitatively formed within 10 min (half-time of formation = 2.7 min), and apparently, it was stable for at least 1 h (Fig. 2a). This finding was not obvious as a lot of endogenous compounds could sequester or oxidize ferrous ions. The synthesis of DNDGIC proceeds about 10 times slower than in a simple buffer (Fig. 2a), suggesting that unknown components of the homogenate bind or oxidize reversibly the exogenous ferrous ions. The apparent stability of DNDGIC in the presence of an excess of GSH and GSNO both in solution and in the GST-depleted homogenate is probably the consequence of a steady-state condition, by which the decomposition of the complex is balanced by re-synthesis from the excess of reagents. In fact, when authentic DNDGIC is incubated in a simple buffer, it is unstable and decomposes at increasing rates when the complex concentration is lowered (Fig. 2d). The kinetics are complicated, showing an initial fast phase which is mainly evident at low concentrations, followed by a second phase of slower decay. At 2, 5, and 10 μM DNDGIC concentrations, the overall t1/2 values are 12, 17, and 21 min, respectively. In the placental GST-depleted homogenate, a more prominent fast decomposition is observed at the lowest concentrations, whereas the second phase is similar to that observed in the simple buffer (Fig. 2b). Interestingly, EPR analysis, performed under specific conditions where free and bound complexes can easily be distinguished (1), reveals that most of the DNDGIC incubated in the GST-depleted homogenate remains as free complex, although a small amount (about 10–15%) behaves like a different paramagnetic species bound to unknown proteins (Fig. 4a).
GSTP1-1 Is the Primary Target for DNDGIC in Human Placenta Homogenate—Preliminary evidence that in human placenta homogenate GSTP1-1 may act as the preferential target for DNDGIC is given by the extent of enzyme inhibition observed during incubation of 20 mM GSH, 2 mM GSNO, and variable ferrous ion concentrations (up to 10 excesses over the endogenous GSTP1-1). As shown in Fig. 3a, GSTP1-1 undergoes a time-dependent and iron-dependent loss of activity. The inhibition does not occur in the presence of 1 mM EDTA and is reversed by addition of 10 mM KCN, confirming that it is really caused by DNDGIC binding (3). Despite the presence of 20 mM GSH, which competes with DNDGIC for the same G-site, a significant inhibition is observed even when the added iron is stoichiometric to the enzyme (2 μM), indicating that DNDGIC is quantitatively formed at these very low concentrations and that it binds strongly to GSTP1-1. In addition, loss of activity does not exceed 50% of the original activity even at an enzyme:iron ratio of about 1:20, a behavior that corresponds to the biphasic inhibition pattern observed for the DNDGIC-GSTP1-1 interaction (1, 3). Interestingly, a slower inhibition of about 30% of the original activity was also observed in the absence of added iron. This is most likely due to DNDGIC being formed from the constitutive free iron of the homogenate or to mobilization of iron from endogenous sources. A more accurate determination of the modality and stoichiometry of this interaction has been obtained by direct incubation of variable amounts of authentic DNDGIC (from 0.1 to 20 μM) in the crude placenta homogenate that contains 2 μM of endogenous GSTP1-1. The extent of the transferase inhibition reflects a 1:1 complex-enzyme interaction (Fig. 3b) up to 50% of residual activity. The inhibition behavior parallels that found when GSTP1-1 and DNDGIC were incubated in a simple buffer (Fig. 3b), providing strong evidence that in the homogenate GSTP1-1 is the primary target for DNDGIC despite the presence of thousands of different low molecular mass compounds and proteins. We must emphasize that these inhibition data are obtained using the assay procedure “under non-equilibrium conditions” which gives a snapshot of the binding situation as it occurs in the homogenate mixture and not in the final activity solution (1). Increasing the DNDGIC concentrations over a 10 times excess leads to further inhibition due to binding of the complex also to the second subunit, thus confirming the presence of a negative cooperativity that lowers the affinity of the second G-site. By using the activity assay procedure under equilibrium conditions and taking into account that DNDGIC is a competitive inhibitor toward GSH, KI values of 10−9 and 7 × 10−9 M have been calculated for the high affinity and low affinity G-sites, respectively (experiment not shown), close to those calculated for the purified enzyme (1). EPR spectra, recorded after the incubation of DNDGIC in the placental homogenate, confirm the appearance of a protein-bound paramagnetic species very similar to that obtained in a purified system after binding of DNDGIC to GSTP1-1 (Fig. 4a). Note that in this experiment, the GST concentration has been increased artificially from 2 to 8 μM to obtain sufficiently good EPR spectra to allow comparison.

GSTP1-1 Stabilizes DNDGIC in the Placenta Homogenate—The stability of DNDGIC bound to GSTP1-1 has been determined by using the EPR spectroscopy and, alternatively, by measuring the extent of the GSTP1-1 inhibition in the homogenate due to DNDGIC. As shown in Fig. 4b, both these analytical approaches indicate a dramatic stabilization of this complex when it interacts with the G-site. A half-life of about 480 min has been calculated at a concentration of 2 μM of DNDGIC which must be compared with that found in the GST-depleted homogenate (t1/2 = 2.8 min) (Fig. 4b) and also to that calculated in the sample buffer (t1/2 = 12 min)

Formation and Instability of DNDGIC in GST-depleted Rat Liver Homogenate—The rat liver homogenate is a second model used to check both formation and stability of DNDGIC in heterogeneous systems. In this context, rat liver is a more complex tissue than human placenta as it contains at least 14 GST isoenzymes belonging to the Alpha, Pi, Mu, and Theta classes.

Fig. 2. Synthesis and degradation of DNDGIC in GST-depleted placenta homogenate. a, 20 mM GSH and 2 mM GSNO were incubated with 10 μM ferrous sulfate in 1 ml of GST-depleted human placenta homogenate containing 0.1 M potassium phosphate buffer, pH 7.0 (25 °C) (●). DNDGIC formation was assayed by the enzymatic procedure (see “Experimental Procedures”). Time course of DNDGIC formation in a simple buffer is also reported (○). b, variable amounts of DNDGIC were incubated in 1 ml of GST-depleted placenta homogenate containing 0.1 M potassium phosphate buffer, pH 7.0, and 10 mM EDTA (25 °C). At fixed times, suitable aliquots were incubated for 5 min with 1 nmol of GSTA1-1 in 0.5 ml of potassium phosphate buffer, pH 7.0. After incubation, DNDGIC was quantitated on the basis of GSTA1-1 inhibition. ■, 2 μM DNDGIC; ▲, 5 μM DNDGIC; ●, 10 μM DNDGIC. c, same experiment as in b except that DNDGIC was incubated in GST-depleted rat liver homogenate. ■, 2 μM DNDGIC; ▲, 5 μM DNDGIC; ●, 10 μM DNDGIC. d, same experiment as in b in the absence of homogenate. ■, 2 μM DNDGIC; ▲, 5 μM DNDGIC; ●, 10 μM DNDGIC. Degradation of DNDGIC in the homogenates, followed by EPR measurements, gave identical results (not shown).
bated with variable amounts of ferrous sulfate (from 2 to 20 μM) in 1 ml of crude human placenta homogenate containing 0.1 mM potassium phosphate buffer, pH 7.0. The endogenous GSTP1-1 was 2 μM. At fixed times, the residual GST activity was assayed on 10-μl aliquots diluted in 1 ml of 0.1 M of potassium phosphate buffer, pH 6.5, containing 10 mM GSH and 1 mM CDNB. •, no FeSO₄ added; □, 2 μM FeSO₄; ○, 10 μM FeSO₄; ●, 20 μM FeSO₄. b, variable amounts of authentic DNDGIC (from 0.1 to 20 μM) were incubated in 1 ml of crude human placenta homogenate containing 2 μM GSTP1-1 and 10 mM EDTA (■). At fixed times 10-μl aliquots were assayed for activity determination after dilution in 1 ml of potassium phosphate buffer, pH 6.5, containing 10 mM GSH and 1 mM CDNB. The same experiment with 2 μM GSTP1-1 in the absence of homogenate is also reported (○).

(2) which account for 3–5% of the cytosolic proteins. In this case, the use of a GST-depleted homogenate is a useful tool to evaluate the net influence of the entire pool of the endogenous GSTs on the formation and stability of the complex. In this GST-depleted system, DNDGIC is quantitatively formed starting from 20 mM GSH, 2 mM GSNO, and 10 μM ferrous ions. The kinetics of formation and apparent stability are similar to that observed in the placenta homogenate (data not shown). Incubation of authentic DNDGIC in the GST-depleted rat liver homogenate results in a fast degradation of the complex (Fig. 2c). The kinetics of decomposition show first a very fast and concentration-independent phase that causes the disappearance of about 20–25% of DNDGIC in a few seconds. In a second step, a slower degradation occurs that is concentration-dependent. The Theta class GST, present in the depleted homogenate, represents only 0.02% of the soluble liver protein (8) and therefore a small amount (about 0.5 μM) when compared with the final concentration of DNDGIC used in these experiments (from 2 to 10 μM).

Alpha and Mu Class GSTs Are the Primary Targets for DNDGIC in the Rat Liver Homogenate—The female rat liver used in our experiments expresses multiple GST isoenzymes, but the Alpha class and Mu class GSTs are prevalently represented (56 and 43%, respectively) (2), whereas GSTP1-1 is less than 1%. Even the Theta class GST does not exceed the 1% of all GSTs (8). Specific activities and the relative concentrations of all cytosolic rat liver GSTs have been determined previously (9), so a quantitative evaluation of the interaction of DNDGIC with endogenous GSTs can be made despite the high complexity of this system. When GSH (20 mM) and GSNO (2 mM) were incubated in the presence of variable amounts of ferrous ions in the crude rat liver homogenate, a time-dependent inhibition occurs, which can be reversed completely by 10 mM KCN. The activity decreases to about 30% of the original level at about 3–4 mM excesses of ferrous ions over GSTs (Fig. 5a). Interestingly, in the absence of exogenous iron, a slower inhibition to about 60% of residual activity also occurs, likely due to DNDGIC formed by the constitutive free iron of the homogenate. A more detailed analysis has been obtained by incubating authentic DNDGIC in the same crude rat liver homogenate (Fig. 5b). The inhibition pattern displays an evident biphasic behavior similar to that found in the placenta homogenate, although many different GST isoenzymes are present in this system. The first phase ends at about 25% of the original activity when the added DNDGIC is stoichiometric to all GSTs. The second step of inhibition is seen only when the complex: GST ratio exceeds 30 (not shown). In vitro GSTA1-1, differently from GSTM2-2 and GSTP1-1, is almost inactive already when DNDGIC is bound to only one subunit of this dimeric enzyme (1). Because the Alpha class GSTs in rat liver account for about 40% of the total transferase activity, a residual activity of about 30% is expected when DNDGIC half-saturates all endogenous GSTs. This theoretical value agrees well to that found in our experiments. These conclusions have been confirmed by EPR experiments. After incubation of DNDGIC (half-stoichiometric to all endogenous GSTs) in the crude rat liver homogenate, no free complex was detected. The EPR spectrum reported in Fig.
 aliquots in 0.1 M potassium phosphate buffer, pH 6.5, containing 10 mM FeSO₄; crude rat liver homogenate (diluted up to a 5 M final concentration). At fixed times, the residual GST activity was assayed by diluting 3-μl aliquots in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM GSH and 1 mM CDNB (1 ml final volume). After 5 min, 10-μl aliquots were assayed for activity determination after dilution in 1 ml of potassium phosphate buffer, pH 6.5, containing 10 mM GSH and 1 mM CDNB. The same experiment with rat liver GSTs, purified by affinity chromatography (5 μM final concentration of GSTs), is also reported (●).

6α does not correspond to either DNDGIC-GSTA1-1 or DNDGIC-GSTM2-2, but it is compatible with the sum of two different paramagnetic species corresponding to the DNDGIC-GSTA1-1 and DNDGIC-GSTM2-2 complexes in a ratio of 56:43; this ratio reflects their relative abundance in the tissue.

Stability of DNDGIC When Bound to GST Forms—As observed in human placenta homogenate, DNDGIC is strongly stabilized in rat liver homogenate when it is bound to all different GSTs (Fig. 6a). The decomposition of the bound DNDGIC (2 μM) displays a t½ of about 280 min, a value that should be compared with that found in the GST-depleted homogenate (6 min) (Fig. 6b).

**DISCUSSION**

The results presented in this paper demonstrate that GSTs can represent the prime target for DNDGIC interaction in a multicomponent system. Kinetics and EPR data clearly indicate that in GST-depleted homogenates from both human placenta and rat liver, DNDGIC is formed quantitatively starting from an excess of GSH, GSNO, and micromolar amounts of ferrous ions. This is not the sole pathway for DNDGIC biosynthesis; direct production of the free NO by NO synthase can also replace the GSH-GSNO system (10). In the absence of GSTs, most of this complex remains in an unbound form and undergoes a fast time- and concentration-dependent decomposition. On the contrary, in the crude human placenta homogenate GSTP1-1 represents the prime target for DNDGIC, and in rat liver homogenate, a similar role is taken by the entire pool of GSTs (mainly Alpha and Mu class GSTs). DNDGIC, even stoichiometric to the endogenous GSTs, is quantitatively bound to the high affinity G-sites, whereas the second binding site becomes active only at higher DNDGIC concentrations. Negative cooperativity as well as thermodynamic dissociation constants for GSTP1-1 in the crude homogenates correspond to the results found for the purified enzyme (1). Thus, the modality of interaction of this transferase with DNDGIC seems to be unperturbed even in the presence of thousands of different organic compounds and proteins. A similar scenario is observed in the rat liver where the Alpha and Mu class GSTs are the prevalent isoenzymes. In this case, a residual activity of 25% was obtained when DNDGIC binds to the high affinity binding sites of these enzymes, close to the expected value calculated on the basis of their relative amount and considering the peculiar overinhibition of the Alpha class GST by DNDGIC (1). This finding could be related to previous observations in intact vascular tissues; after stimulation of NO production, high mass dinitrosyl-iron complexes have been observed but not characterized (11). Interestingly, the involved proteins have molecular mass within 50–120 kDa, a range compatible with the molecular mass of GSTs (11). Our data also indicate a dramatic stabilization of DNDGIC when it is bound to GSTs. In fact, the free complex decomposes in a short time, but it is stable for hours when it is bound to GSTs. Thus, GSTs display an evident propensity to act as storage proteins for DNDGIC. It may be finalized to produce a reservoir for the slow release of NO. Alternatively, it could represent only a sequestration mechanism to avoid that an excess of NO could induce cell damages. Because of the oxidative stress caused by the free iron, it could
be also a detoxification mechanism toward the free iron present in the cell.

Whatever the conclusion, all members of the GST superfamily appear to be proteins under a specific evolutionary pressure in the direction of an optimization of this interaction. In fact, only the more recently evolved GSTs (Alpha, Pi, and Mu isoenzymes) display an optimized binding mechanism that allow them to bind quantitatively stoichiometric amounts of DNDGIC in a short time (1).

Conversely, the Theta class GST, close to the ancestral precursor of all GSTs, displays lower affinity and slower binding kinetics (1). This behavior parallels that observed for GSH binding that appears not optimized in the Theta class GST (12, 13).

How can the enzymatic role of GSTs against toxic alkylating compounds coexist with a role of binding protein toward DNDGIC which abolishes its conjugating activity? We emphasize that the steady-state concentration of DNDGIC in normal tissue is unknown but is probably under the micromolar level. In fact, it has been detected by EPR spectroscopy only after stimulation of NO synthase or after administration of NO-producing compounds. Thus, the steady-state concentration of DNDGIC in non-stimulated tissues is lower than 1 μM which represents the detection limit of the EPR procedure. On the other hand, GSTs are largely expressed in all tissues, reaching 3–5% of all cytosolic proteins in hepatocytes. This means that the cytoplasmic concentration of these enzymes in rat liver may approach the 0.1 mM range, a value 100 times higher than the estimated concentration of DNDGIC in non-stimulated tissues.

In human placenta, the concentration of cytosolic GSTP1-1 is 5–10 μM, which is still over-stoichiometric to DNDGIC. Thus, the endogenous GSTP1-1 in the placental cells and the entire pool of GSTs in the rat liver cells could act as DNDGIC storage proteins without any relevant loss of the conjugating activity essential for cell survival. In case of overproduction of NO, the negative cooperativity triggered by DNDGIC, found in purified systems as well as in crude homogenates, appears to be an important mechanism finalized to preserve a residual conjugating activity. Thus GSTs can be defined more appropriately, as half-site binding proteins for DNDGIC. The overinhibition found in the half-bound GSTA1-1 does not oppose this idea. This specific enzyme is often expressed in tissues together with significant amounts of Mu or Pi GSTs that prevent a complete loss of the transferase activity at high DNDGIC concentrations. This enzyme coexpression may be important as the Alpha class GST shows 10-fold higher affinity than Mu and Pi isoenzymes (1) and represents the prime target for DNDGIC among all GSTs. In the case of NO or DNDGIC overproduction, a relevant loss of the anti-cancer activity of GSTs is expected only in tissues where the expression of the Alpha class GST is predominant. Interestingly, it has been reported that the homozygous deletion of the GSTM1 gene (occurring in 50% of Caucasians) is associated with increased risk of cancer (14).

The cross-link between NO metabolism and the GST superfamily, proposed here for the first time 30 years after from the discovery of this enzyme, seems to be supported by previous interesting observations. The expression of GSTP1-1 in head and neck squamous cell carcinomas correlated strongly with the expression of nitric-oxide synthase and nitrotyrosine, and these findings demonstrate that GSTP1-1 is up-regulated in conjunction with the NO-generating nitric-oxide synthase isoform and implicate GSTP1-1 in protecting these cells from the cytotoxic effects of high concentrations of NO found in the tumor bed (15).

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