Minireview

REACTIVE INTERMEDIATES AND THE DYNAMICS OF GLUTATHIONE TRANSFERASES

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ABSTRACT:

Reactive intermediates are a continuous burden in biology and several defense mechanisms have evolved. Here we focus on the functions of glutathione transferases (GSTs) with the aim to discuss the quantitative aspects of defense against reactive intermediates. Humans excrete approximately 0.1 mmol of thioether conjugates per day. As the amount of GST active sites in liver is ~0.5 mmol, it appears that glutathione transferase catalysts are present in tremendous excess. In fact, the known catalytic properties of GSTs reveal that the enzymes can empty the liver glutathione (GSH) pool in a matter of seconds when provided with a suitable substrate. However, based on the urinary output of conjugates (or derivatives thereof), individual GSTs turn over (i.e., catalyze a single reaction) only once every few days. Glutathione transferase overcapacity reflects the fact that there is a linear relation between GST enzyme amount and protection level (provided that GSH is not depleted). Put in a different perspective, a few reactive molecules will always escape conjugation and reach cellular targets. It is therefore not surprising that signaling systems sensing reactive intermediates have evolved resulting in the increase of GSH and GST levels. Precisely for this reason, more moderately reactive electrophiles (Michael acceptors) are receiving growing interest due to their anticarcinogenic properties. Another putative regulatory mechanism involves direct activation of microsomal GST1 by thiol-reactive electrophiles through cysteine 49. The toxicological significance of low levels of reactive intermediates are of interest also in drug development, and here we discuss the use of microsomal GST1 activation as a surrogate detection marker.

Reacto Intermediate

Living beings are faced with a continuous burden of reactive chemical entities that are formed during biotransformation of foreign compounds, as well as from endogenous molecules. In fact, the wide spread occurrence of detoxication and repair enzymes testifies to the generality of this concept. With most reactive compounds being electrophiles, Ketterer (1988) introduced the useful chemical subdivision according to reactivity toward glutathione. Hard electrophiles like N-sulfonfylmethyl-4-aminoazobenzene react with nucleophilic N, O, and C in biological molecules whereas soft electrophiles like N-acetyl-p-benzoquinonimeine react more readily with sulfur (as in GSH)\(^1\). The former are more likely to be genotoxic as they can form DNA adducts. Since the primary defense of nature against electrophiles occurs by glutathione transferase (GST) catalyzed conjugation to glutathione, it would appear that soft electrophiles have exerted a selective pressure, in terms of their toxicology, during evolution. Epoxides, which vary widely in reactivity depending on the molecular context, are also of particular relevance since they are substrates for both epoxide hydrolases and glutathione transferases comprising a number of well known carcinogens [e.g., styrene oxide, benzo(a)pyrene diol epoxide] (Oesch, 1984; Coles and Ketterer, 1990). Overall, chemical modification can result in acute toxicity, genotoxicity, and cancer as well as autoimmune complications.

Glutathione Transferase

Glutathione transferases now include close to 20 human cytosolic forms and 5 that are membrane bound (for reviews, consult Andersson et al., 1994; Hayes and Pulford, 1995; Mannervik and Widersten, 1995; Armstrong, 1997). Their central importance in detoxication rests on the unique capacity for conjugation of a tremendous variety of reactive intermediates (Chasseaud, 1979). Besides, GSTs also display glutathione peroxidase activity and can thus protect from oxidative damage (Mosialou et al., 1993; 1995; Zinniak et al., 1997). The existence of a distinct “microsomal” glutathione transferase was proposed by Kraus (1975). In 1979, Morgenstern et al. showed that the glutathione transferase activity in rat liver microsomes toward

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1-chloro-2,4-dinitrobenzene (CDNB) could be stimulated up to 8-fold by sulphydryl reagents. This observation, together with subsequent work, has firmly established the concept that microsomal glutathione transferase 1 (MGST1) activation occurs with a large variety of electrophiles (of which some are reactive intermediates). During the last 20 years, MGST1 has been extensively studied regarding gene structure (Kelner et al., 1996, 2000; Iida et al., 2001), organ and species distribution (Estonius et al., 1999), molecular properties (Morgenstern et al., 1982, 1988; Weinander et al., 1997; Svensson et al., 2000), three-dimensional structure (Schmidt-Krey et al., 2000), genetic polymorphisms, in which humans lack only one of the many enzymes: 5-lipoxygenase-activating protein, leukotriene-C4 synthase, MGST1, MGST2, MGST3, and prostaglandin-E synthase, earlier known as microsomal glutathione transferase 1-like-1 (MGST1-L-1). The common denominator of the MAPEG superfamily is the ability to interact with lipid derivatives and to transform reactive lipid intermediates to physiological messengers (leukotrienes and prostaglandins) or unreactive products (lipid alcohols or hydroxyalkenal conjugates). Microsomal glutathione transferases 2 and 3 have both been shown to act as glutathione peroxidases and leukotriene-C4 synthases whereas only the former catalyzes the conjugation of the xenobiotic substrate CDNB (Jakobsson et al., 1999a). The role of these enzymes in xenobiotic metabolism requires further studies. MGST1 is the only MAPEG member, and indeed glutathione transferase, that undergoes activation in response to covalent modification by reactive intermediates.

A Tremendous Overcapacity in Terms of Glutathione Transferase Catalysis

Examination of data from several sources point to an enormous overcapacity in the catalysis performed by glutathione transferases (here we discuss human liver). First, the concentration of enzyme active sites is approximately 0.2 mM (calculated from van Omme et al., 1990), which most times will exceed the concentration of reactive substrate. Second, the enzyme is charged with glutathione in the thiolate anion form, and hence the reaction would not be limited by slow glutathione recharging [which is especially relevant for MGST1 (Morgenstern et al., 2001)] or product release [which can be relevant for certain cytosolic GSTs (Johnson et al., 1993)]. Certainly, in a traditional assay with high substrate concentrations and low enzyme amount, these limits often apply. Third, at the normal GSH concentration in liver (5 mM), the GSTs can perform about 25 catalytic cycles before GSH is depleted. In theory then, GSH can be consumed in between 0.1 and 25 s depending on the substrate. Thus, any reactive drug and/or metabolite would be inactivated in this short time (provided that the total amount of the reactive molecule does not exceed the total amount of GSH). In the case when the amount of metabolite formed is also lower than the GST enzyme amount, metabolism would occur extremely fast in a single turn-over, and relatively few reactive molecules would escape detoxication.

However, if GSH is depleted, it is known that severe toxicity follows (Compordi et al., 1991). Glutathione transferases therefore cannot continuously operate at their maximal potential. Quite the opposite is the case if we estimate that what has been identified in human urine as glutathione-derived conjugates (0.1 mmol/day calculated from van Welie et al. (1991)) represents half the overall activity of GST conjugate synthesis and assume that the majority of conjugates are formed in the liver, one can calculate that any single glutathione transferase performs one catalytic cycle every 2nd day or so (based on a value of 0.5 mmol of enzyme). This stunning display of idleness gives a true measure of the overcapacity required for healthy life, namely, in terms of glutathione transferases, $10^5$- to $10^7$-fold [taking the time for a single catalytic turn-over, 1–0.01 s, with the average time between actual catalytic events, 172,800 s ($\approx 48$ h)]. Taken from another angle, if glutathione transferases were to use all GSH that is synthesized in liver, the catalytic overcapacity is still $10^5$-fold. Actually, comparing the output of glutathione conjugates and the synthesis rate reveals that approximately 0.2% of liver GSH is used for glutathione transferase catalysis. On a more sober note, what this tells us is that reactive intermediates by their very nature require a detoxification system with a large overcapacity. As the concentration of GSTs cannot exceed the concentration of xenobiotics in the cell, it is the capacity of GSTs to stabilize the strongly nucleophilic GSH thiolate and bind and conjugate hydrophobic molecules that provide this overcapacity. In evolutionary terms, products of oxidative stress could be particularly important since enzyme efficiencies close to the diffusion limit have been observed [e.g., hydroxyalkenals and certain GSTs (Jenson et al., 1986)], and humans actually excrete 5 μg of the mercapturic acid of 4-hydroxynonenal per day (corresponding to 15 nmol) (Alary et al., 1998).

Threshold Levels

Glutathione levels constitute a very important threshold that will determine the toxicity of a drug that forms reactive intermediates. For instance, after GSH depletion, resynthesis in rat liver requires 3 to 4 h. However, depletion of GSH requires high doses, which will not be approached in the case of potent drugs. For less potent drugs like acetaminophen, GSH can be depleted after which necrosis may occur. It follows that GSH levels are most relevant for acute toxicity.

In normal drug use, we are more concerned with the small levels of reactive intermediates formed and whether they pose any health risks. As is evident from mercapturic acid excretion, several drugs, chemicals, endogenous molecules, and constituents of foodstuffs give rise to reactive intermediates to which we are continuously exposed. From enzyme kinetic considerations, and provided a steady supply of GSH, a certain fraction of reactive intermediate will always escape conjugation, and there is no argument for a threshold in terms of detoxication capacity. That is not implying that conjugation is unimportant; on the contrary, it is well documented that the proportion of reactive benzo(a)pyrene diol epoxides reacting with DNA in a cellular system (Sundberg et al., 2002) is directly related to the amount and nature of glutathione transferases present (introduced by heterologous expression). In fact, the lack of threshold also means that small differences in glutathione transferase amount will directly translate into differences in reactive metabolite burden. This fact probably explains why genetic polymorphisms, in which humans lack only one of the many glutathione transferases, can actually have an impact (albeit small) on cancer frequency as demonstrated in epidemiological studies (Hayes and Strange, 2000; Strange et al., 2000, Mucci et al., 2001).

Another very important determinant of glutathione conjugation rate is the partitioning of lipophilic reactive intermediates into membranes (Ooi et al., 1994). In fact, the reactive intermediate can be redistributed and protected from solvolysis and conjugation (a negative aspect); on the other hand, sensitive cellular targets (apart from mem-
brane proteins) such as DNA would also be protected. If certain glutathione transferases would have access to the membrane pool of reactive intermediates is therefore of considerable interest. It is known that cytosolic GSTs are over-represented in membrane fractions compared with cytosolic marker proteins (Morgenstern et al., 1983). Certain forms are attached to the plasma membrane (Singh et al., 2002), and it has been shown that MGST1 has preferential access to a very fat-soluble substrate compared with cytosolic GSTs (Hargus et al., 1991). Clearly, partitioning on the cellular level can influence reactive intermediate disposition and thus will be an important field for further study. In summary, GSH levels constitute a global toxicity threshold whereas GST levels and intracellular dynamics of reactive intermediates determine toxicological outcome at low doses.

Glutathione Transferases Are Up-Regulated by Reactive Intermediates

Interestingly, cytosolic glutathione transferases, other protective enzymes, and glutathione synthesis (Hayes and McMahon, 2001; Ramos-Gomez et al., 2001) have been shown to be up-regulated by electrophiles through the Nrf2 transcription factor via the cis-acting antioxidant response element (indeed, also called electrophile-response element). Attesting to the importance of this regulatory mechanism, toxicity is augmented in the Nrf2 knockout mouse (Chan et al., 2001). Compounds that up-regulate GSTs via this pathway have been shown to possess anticarcinogenic properties. Therefore this is a field of great interest in cancer chemoprevention, which attempts to take advantage of the biological mechanisms that have developed (Talalay, 2000). As many of the chemopreventive substances actually are reactive electrophiles, it poses the challenging issue that drugs that give rise to reactive metabolites are not necessarily bad for health. However, sorting the safe from the harmful will be truly difficult.

Another peculiar form of regulation is the activation of MGST1 by electrophiles that react with cysteine 49, the only thiol in the enzyme. This activation has been demonstrated with pure enzyme, enzyme in cells, organs, and even in whole animals treated with compounds that give rise to reactive intermediates (Wallin and Morgenstern, 1990; Lundqvist and Morgenstern, 1992; Aniya and Naito, 1993; Yonamine et al., 1996). Activation can be envisioned as a useful regulation that has evolved naturally, but this concept has to be tested experimentally. We recently developed an experimental system where MGST1 is stably overexpressed in human adenocarcinoma cells and observed that these cells were protected from oxidative stress (unpublished). By using variants of the protein that are constitutively activated or lack the capacity to become fully activated, we hope to determine whether activation can indeed be of functional significance. In summary, reactive chemical entities can be sensed and can trigger appropriate transcriptional activation or, in the case of MGST1, even activate protective enzymes directly (Fig. 1).

Screening for Reactive Intermediates Using MGST1

Screening of reactive intermediates formed during metabolism can be performed relying on radioactivity and protein binding, trapping by small nucleophiles, enzyme inhibition, etc. Since MGST1 is activated by most alkylating agents tested, we and others have also developed the concept of using MGST1 activation as an enzyme-based method (Onderwater et al., 1999; Svensson et al., 2000). For instance, MGST1 is activated in liver microsomes during the metabolism of phenol and thio urea compounds (Wallin and Morgenstern, 1990; Onderwater et al., 1999). In hepatocytes, MGST1 is activated by CDNB and phorone (Lundqvist and Morgenstern, 1992) and in whole animals by phorone, allyl alcohol, and dibromo-ethane (Botti et al., 1982; Masukawa and Iwata, 1986; Haenen et al., 1988).

Cysteine 49, which is the target for electrophile activation of MGST1, was initially suggested to be particularly reactive (Morgenstern et al., 1979). However, later experiments showed that the thiol is, if anything, unreactive compared with GSH but resides in a hydrophobic pocket, which enhances the efficiency of modification (Svensson et al., 2000) probably explaining observations of covalent modification by reactive intermediates (Weis et al., 1992). If MGST1 evolved a mechanism to react with electrophiles, it is conceivable that this biological monitor for reactive intermediates might be relevant in...
Aniya et al. have shown that MGST1 is activated by oxidative stress induced by a variety of compounds (Aniya and Anders, 1992; Aniya and Daido, 1993; Aniya and Naito, 1993), and in addition, MGST1 can protect cells from oxidative stress. Therefore, a combination of assessing MGST1 activation and comparing cellular fate with and without overexpressed MGST1 might yield significant information on toxicity mechanism.

Toxicity and Localization of the Glutathione Conjugate

Microsomal glutathione transferase 1 does not protect cells from one of its favorite substrates, the arylating agent CDNB (unpublished). In fact, this substance yielded significant information on detoxication by GSTs in general. Cells transfected with high amounts of very active cytosolic GST were actually more vulnerable to CDNB than control cells (Diah et al., 1999). Only when efficient glutathione conjugate export pumps were also present in the cells did increased glutathione conjugation afford protection. This nicely illustrates the principle that the conjugate itself can be more toxic to a cell than the electrophilic compound. It is also known that certain conjugates are more reactive than the parent electrophile [e.g., vicinal dihalogen compounds (Dekant, 2001)] or form harmful metabolites upon further processing by cytochrome conjugate β-lyase, which has been reported to result in kidney damage [e.g., hexachlorobutadiene (Dekant, 2001)].

Here an observation by Ketterer’s group comes to mind (Briviba et al., 1993). When they microinjected a fluorescent glutathione conjugate (of monobromobimane) into cells, it was efficiently transported into the nucleus. Whether this is a protective mechanism to relieve inhibition in the cytoplasm, a signaling mechanism influencing gene transcription, or simply a reflection of the normal transport of glutathione and conjugates in cells remains a challenging issue for future research.

Reactive Intermediates and Autoimmunity

Bioactivation of drugs may lead to the formation of drug-protein adducts. According to the hapten-hypothesis, these drug-modified proteins may be recognized as nonself or neoantigens and thereby trigger an immunological reaction against the drug-hapten, the carrier protein, or both (Kenna et al., 1987; Park et al., 1998). The enzymes involved in the formation or inactivation of reactive intermediates should be at particular risk of haptenation, and it is therefore not surprising that a number of drug-metabolizing enzymes have been identified as immunological targets in specific cases of idiosyncratic drug toxicity, such as drug-induced hepatitis (Manns and Obermayer-Straub, 1997; Park et al., 1998). One of several examples hereof is halothane hepatitis. The major enzyme responsible for halothane bioactivation to trifluoroacetychloride is cytochrome P450 2E1 (CYP2E1), which in turn becomes alkylated and thereby immunogenic (Eliasson and Kenna, 1996; Eliasson et al., 1998). Around 70% of patients with halothane hepatitis express high titers of anti-CYP2E1-autoantibodies as well as antibodies to a number of trifluoroacetylated microsomal proteins (Kenna et al., 1987; Eliasson and Kenna, 1996). Reduced GSH protects endoplasmic reticulum proteins from trifluoroacetylation (Eliasson et al., 1998), but whether this is catalyzed by MGST1 is not known. We have investigated whether there is an anti-MGST1 immune response in halothane hepatitis. Interestingly, there is indeed evidence of significant anti-MGST1-IgG titers in at least 20 to 30% of sera from patients with halothane hepatitis (Fig. 2). A cytosolic GST has recently been identified as a soluble liver antigen in some cases of autoimmune hepatitis (Wesier-
illustrated in the up-regulation of glutathione transferases by reactive molecules, which in turn can protect from chemical-induced toxicity. Prediction and testing for compounds that form reactive intermediates thus remains an important research challenge not only in drug safety but also since dietary or synthetic reactive compounds might find increasing use for instance in cancer prevention.

References


Ralf Morgenstern was born in Uppsala, Sweden and received his Ph.D. in 1984 at the University of Stockholm under the supervision of Prof. Joseph DePierre. The research was focused on glutathione transferases and the metabolism of polycyclic aromatic hydrocarbons. In 1988 he began at the Department of Toxicology at Karolinska Institutet, which later was incorporated into the Institute of Environmental Medicine at the same university. He has contributed to the understanding of membrane bound glutathione transferases, oxidative stress, and chemical carcinogenesis.

Rosanna Rinaldi was born in Bari (Italy). She received her Ph.D. jointly at the Department of Pharmacology and Human Physiology at the University of Bari and the Institute of Environmental Medicine at Karolinska Institutet (Stockholm, Sweden) in 2000. Dr. Rinaldi then began work as a postdoctoral fellow at the Karolinska Institutet under the supervision of Prof. Ralf Morgenstern and was recently awarded a new position at the University of Foggia (Italy). Her research interests involve oxidative stress, membrane bound glutathione transferase, and cellular mechanisms of toxicity.