Glutathione

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Chapter 11
Glutathione Transferases
From the Test Tube to the Cell

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11.1 INTRODUCTION

Enzymology is a cornerstone in the molecular life sciences. Besides providing fundamental understanding of the dynamics of life processes, enzymology has essential applications in drug discovery and biotechnology. Glutathione transferases (GSTs) are ubiquitous and versatile enzymes with crucial physiological functions and are excellently suited to explore many of the above-mentioned aspects. Based on the cutting-edge advances in molecular life sciences, it is now possible to move enzyme research from the test tube into the cell. Genes encoding GSTs in variant forms can be chemically synthesized, and expressed enzyme proteins can be produced and inserted directly into cells or produced intracellularly from transfected DNA. Organisms can be genetically engineered to acquire an altered "GSTome," and precision gene surgery can be performed with novel techniques such as CRISPR/Cas9.

11.2 BACKGROUND

11.2.1 The Versatility of GST Functions

Enzymes not only govern rates and specificities of metabolic reactions but also, via interactions with other biomolecules, are intimately involved in networks of cellular signal transduction. Particularly noteworthy in functional breadth and complexity are the GSTs, which have evolved to not only catalyze diverse chemical reactions but also serve as intracellular transporters of heme and other relatively small molecules, as well as to regulate the activity of protein kinases affecting the life expectancy of a cell (Josephy and Mannervik, 2006). Accordingly, GSTs are involved in different networks of interactions, which to different degrees may be entangled. Clearly, the cellular systems have to master parallel processing of a wide variety of inputs (Singh, 2015). Surprisingly, GST proteins can move in and out of live mammalian cells and participate in intercellular trafficking. As an example from pathology, parasites such as the human liver fluke *Opithorchis viverrini* secrete GSTs, which in the bile duct serve as mitogens that through AKT and ERK signaling can promote cell proliferation and thereby induce cholangiocarcinoma (Daorueang et al., 2012).

11.2.2 GSTs as Model System

The GSTs are promiscuous in their substrate acceptance and collectively they detoxify various xenobiotics or metabolic by-products that otherwise could be harmful. The protective effects of GSTs against xenobiotics are also exemplified by herbicide

resistance in plants (Cummins et al., 2011) and insecticide resistance in flies (Low et al., 2007). There are multiple forms of GSTs in higher organisms and both cytosolic and membrane-bound GSTs occur in different numbers. In humans 17 different genes encode cytosolic GSTs, and in poplar (*Populus trichocarpa*) 81 (Lan et al., 2009) and in potato (*Solanum tuberosum*) 90 (Islam et al., 2018) homologous GST genes have been annotated. The intrinsic catalytic promiscuity of GSTs makes evolution to high activity with new substrates facile by mutations of only a few amino acids in the GST protein (Pettersson et al., 2002). Enzyme evolution occurs both in natural systems and in protein engineering. Studies of GSTs are therefore well suited to illustrate and clarify a range of important aspects of enzymology including protein evolution for novel functions, drug discovery, behavior of enzymes in the intracellular milieu, relationships to diseases and cell death, as well as biotechnical applications.

More than 13,000 nonredundant GST gene sequences have been identified in the biosphere (Mashiyama et al., 2014) and at least 30,000 scientific papers on GSTs have been published such that any attempt to cover the "GSTome" (Mannervik, 2012) extensively would be preposterous. We have therefore made a more personal selection of topics that we consider significant for this chapter. Numerous recent reviews cover other aspects of GST research (Singhal et al., 2015; Hollman et al., 2016; Kumar et al., 2017; Mohana et al., 2017; Perperopoulou et al., 2017).

11.3 A NOTE ON NOMENCLATURE

GSTs discovered in hepatic tissues were catalyzing the conjugation of aromatic substrates (Booth et al., 1961; Combes et al., 1961) leading to the name glutathione *S*-aryltransferase. This designation was subsequently generalized to glutathione *S*-transferase (Boyland et al., 1969). However, the latter nomenclature was recognized as a misnomer, since sulfur is not transferred but a glutathionyl (GS^-) group. In fact, the enzymes catalyzing conjugation and addition reactions can be regarded as "glutathionyl transferases." The currently recommended nomenclature (Mannervik et al., 2005) designates the enzymes as glutathione transferase without the prefix "*S*−"; the abbreviation GST is still accurate.

In humans and other mammals, the GSTs have been grouped into membrane-bound (microsomal) and soluble (cytosolic) proteins. The former are members of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) family (Jakobsson et al., 1999) and are composed of three subunits and further described by Morgenstern et al. in Chapter 13. A mitochondrial GST is an outlier with a somewhat different protein fold than the soluble enzymes (Ladner et al., 2004).

The soluble GSTs are generally dimers, although some monomeric plant GSTs are known (Lallement et al., 2014). The dimeric GSTs occur in numerous classes of homologous sequences. Proteins of the same class can form heterodimers as well as homodimers (Mannervik et al., 1982) and can, thus, be identified, for example, as GST A1-1, GST A1-2, and GST A2-2 by their composition of subunits 1 and 2, which are encoded by the *GSTA1* and *GSTA2* genes of the Alpha class.

11.4 INACTIVATION OF TOXIC COMPOUNDS

11.4.1 Detoxification of Xenobiotics

GSTs catalyze the detoxification of xenobiotics, compounds foreign to the organism, including numerous mutagenic compounds that cause cancer, such as polyaromatic hydrocarbons studied by Boyland and coworkers (Boyland et al., 1969). The abundant forms of the human enzymes, GST A1-1, GST M1-1, and GST P1-1, are all active with the potently carcinogenic diolepoxide of benzo(*a*)pyrene (Robertson et al., 1986) resulting from oxidation by cytochrome P450 enzymes. The GST substrates also include toxic organic plant components, such as isothiocyanates present in edible vegetables (Zhang et al., 1995). However, isothiocyanates provide a positive feedback, since the compounds also prevent toxicity and carcinogenesis by induction of protective enzymes (including GSTs) via transcriptional gene activation (Dinkova-Kostova, 2013). In addition, GSTs inactivate alkylating anticancer drugs, reactions, which in tumor cells contribute to resistance against chemotherapy (Larsson et al., 2010).

11.4.2 Detoxification of Endogenously Produced Toxicants

Although GSTs were first recognized as major components in the cellular defense against xenobiotic electrophiles, it became obvious that their protective functions included toxic products arising from oxidative metabolism of lipids, nucleic acids, catecholamines, and other physiologically relevant molecules (Mannervik, 1986; Berhane et al., 1994). Two enzymes, in particular, seem to have evolved to conjugate and inactivate specific endogenous toxicants. GST A4-4 displays prominent activity with 4-hydroxynonenal and other alkenals formed by lipid peroxidation (Hubatsch et al., 1998). GST M2-2 is distinguished by remarkable activity with aminochrome and dopamine orthoquinone (Segura-Aguilar et al., 1997; Dagnino-Subiabre et al., 2000) arising from dopamine, as well as with similar orthoquinones derived from other catecholamines (Baez et al., 1997). The glutathione conjugation of orthoquinones counteracts their propensity to generate reactive oxygen species via extensive redox cycling, which is linked to Parkinson's disease and other degenerative conditions.

Notwithstanding established important roles of the enzymes in detoxification, the elimination of the three loci of the Mu, Pi, and Theta class GST genes, encoding 14 out of the 22 GST enzymes in the mouse has no obvious detrimental effects on normal development, well-being, or fertility of the animals (Xiang et al., 2014). The members of these classes may have their most important functions in the protection against environmental toxins and oxidative stress not imposed under laboratory conditions. The results are also indicative of robust backup functions, in agreement with the finding that the null alleles of human *GSTM1* and *GSTT1* have modest phenotypic consequences (Josephy, 2010). It was originally proposed that the frequent absence of the Mu class enzyme would influence the capacity of different individuals to metabolize and detoxify various carcinogenic polyaromatic hydrocarbons (Warholm et al., 1981), but subsequent genomic studies indicate that susceptibilities are dependent on a variety of gene sequence variations, and are not limited to the null genotype (Moyer et al., 2007).

11.5 ROLES IN INTERMEDIARY METABOLISM

11.5.1 Eicosanoid-Derived Signal Substances

Arachidonic acid and other polyunsaturated fatty acids are not only undergoing oxidative processes to give toxic electrophiles including 4-hydroxyalkenals but also the source of prominent cellular signaling molecules such as prostaglandins, leukotrienes, and thromboxanes, as outlined in Chapter 13. Leukotriene A_4 is an epoxide resulting from the action of 5-lipoxygenase on arachidonic acid, and leukotriene C_4 is the corresponding glutathione adduct. Soluble GSTs can catalyze this synthesis (Söderström et al., 1985), but the physiologically relevant conjugating enzyme is a separate membrane-bound leukotriene C synthase (Söderström et al., 1988). The leukotriene C synthase is established as a distinct enzyme in the MAPEG family.

However, another branch in the metabolism of arachidonic acid leads to the prostaglandins via cyclooxygenase-catalyzed oxidation to the 9,11-endoperoxide prostaglandin H_2, and this reactive product can give rise to a number of other signal molecules including the 9-hydroxy-11-keto derivative prostaglandin D_2 (Smith et al., 2011). Prostaglandin D_2 synthase was discovered as a cytosolic glutathione-dependent isomerase (Christ-Hazelhof et al., 1979) and has subsequently been called hematopoietic prostaglandin D_2 synthase (H-PGDS). A second nonhomologous PGDS catalyzes the same reaction, but has a different tissue distribution and is not dependent on glutathione (Smith et al., 2011). The latter enzyme shows homology to the members of the lipocalin family and is referred to as lipocalin-type PGDS (L-PGDS). By contrast, H-PGDS has prominent functional and structural similarities to the soluble GSTs and is a member of the Sigma class, encompassing members in animal species ranging from nematodes and insects to mammals. Like in the other glutathione-dependent isomerases in the GST, superfamily glutathione is not consumed but serves only as a cofactor.

11.5.2 Isomerization in Aromatic Amino Acid Catabolism

An early report of a glutathione-mediated *cis–trans* isomerization instrumental in the catabolism of aromatic amino acids indicated an unknown biochemical reaction (Edwards et al., 1956). The responsible enzyme was subsequently identified as the novel Zeta class GST Z1-1, ubiquitously present in organisms ranging from plants to mammals (Board et al., 1997). Obviously, GST Z1-1 plays a pivotal role in intermediary metabolism, preventing the accumulation of maleyl-acetoacetate and its by-products maleyl-acetone and succinyl-acetone in the body. Although not lethal, the disruption of the *Gstz1* gene in the mouse caused severe toxicity when the animals were challenged with dietary phenylalanine (Board et al., 2011).

11.5.3 Role in Steroid Hormone Production

Steroidogenesis begins with cholesterol and, via multiple steps, leads to production of steroid hormones such as progesterone and testosterone (Payne et al., 2004). One of the late steps in the synthesis of these hormones is formation of Δ^5-unsaturated

3-ketosteroids in a pyridine-nucleotide-dependent reaction catalyzed by 3β-hydroxysteroid dehydrogenase (Samuels et al., 1951), followed by a double-bond isomerization (Talalay et al., 1955). The double-bond isomerization of Δ^5-pregnene-3,20-dione (Δ^5-PD) to Δ^4-PD is the last step in progesterone biosynthesis. In the synthesis of testosterone, the double-bond isomerization from Δ^5-androstene-3,17-dione (Δ^5-AD) leads to the last precursor of testosterone, Δ^4-AD (Figure 11.1). The 3β-hydroxysteroid dehydrogenase has the prerequisite isomerase function, but the observed activity is modest in comparison with the high catalytic efficiency of the human or equine GST A3-3 (Johansson et al., 2002; Lindström et al., 2018). Indeed, in vitro suppression of the cellular GST steroid isomerase activity by either enzyme inhibitors or RNA interference diminishes progesterone production to a large extent (Raffalli-Mathieu et al., 2008). Furthermore, pharmacological administration of the glucocorticoid dexamethasone to stallions suppressed serum testosterone levels in parallel with both the GSTA3 mRNA concentration and Δ^5-AD isomerase activity in cytosolic testis extracts (Ing et al., 2014).

From a chemical mechanistic perspective, it should be noted that there is a fundamental difference in the double-bond isomerization reactions catalyzed by GST Z1-1 and GST A3-3. The Zeta class enzyme is involved in a *cis–trans* rotational rearrangement enabled by a transient nucleophilic addition of glutathione to the double bond. By contrast, GST A3-3 utilizes the sulfur of glutathione as a base, which allows migration of the double bond in the B ring of the steroid to the A ring. This isomerization is facilitated by a hydrogen bond from the nitrogen of the glycine residue of glutathione to the 3-oxo group of the substrate (Dourado et al., 2014), also revealing a dual function of glutathione in catalysis.

Remarkably, GSTE14, a member of the Epsilon class, a GST class present in *Drosophila* but not in mammals, is involved in the biosynthesis of the main insect steroid

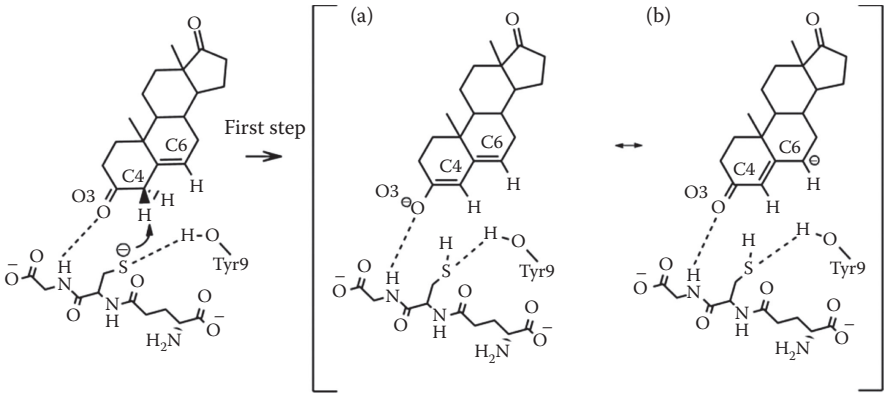

FIGURE 11.1 The first of three steps in the steroid isomerase reaction catalyzed by GST A3-3. The thiolate of glutathione serves as a base removing a proton from C4 of the substrate Δ^5-androstene-3,17-dione. In a concerted manner, the –NH– of the glycine moiety polarizes the O3 of the substrate to promote the dienolate intermediate (a), which facilitates the migration of the double bond between C5 and C6 from the B ring. Subsequently, the active-site Tyr9, following a rearrangement, protonates C6 (Dourado et al., 2014).

hormone ecdysterone (Chanut-Delalande et al., 2014; Enya et al., 2014).The examples aforementioned show that GSTs catalyze reactions relevant to normal physiological processes, thereby expanding the scope of GST enzymology beyond detoxification.

11.6 FUNCTIONAL GROUPS IN BOTH GLUTATHIONE AND PROTEIN EMPOWER GST CATALYSIS

It has been noted that the soluble GSTs can be divided into two main categories distinguishable by the active-site residue interacting with the sulfur of glutathione, one category displaying either serine or cysteine and the other featuring tyrosine (Atkinson et al., 2009). The Alpha class members belong to the second category, and based on site-directed mutagenesis substituting phenylalanine for tyrosine, it was concluded that the hydroxyl group of the latter was not strictly essential but contributed to catalysis, possibly by stabilizing the thiolate of enzyme-bound glutathione via hydrogen bonding (Stenberg et al., 1991).

Surprisingly, the ionization of the glutathione thiol was found not to be accomplished by the active-site tyrosine or any other residue of the protein, but to be due to the α-carboxylate of the γ-glutamyl group of glutathione itself (Widersten et al., 1996; Gustafsson et al., 2001). In fact, computational studies evidenced also the involvement of an active-site water molecule bridging the sulfur of glutathione with the carboxyl group (Dourado et al., 2008).

The GSTs with serine or cysteine in the active site have not been studied to the same extent as those featuring a tyrosine. However, serine is generally considered to stabilize the glutathione thiolate by hydrogen bonding in a similar fashion as tyrosine, whereas cysteine could be redox active and form a covalent bond with a reactant. The two members of the omega class are the only human GSTs presenting cysteine in the active site, and formation of a mixed disulfide with glutathione has been demonstrated as a reaction intermediate in catalysis (Brock et al., 2013).

11.7 REGULATION OF GST GENE EXPRESSION

A key player in the regulation of GST gene expression is the nuclear factor erythroid-2-related factor 2, Nrf2, which is responsible for activation of the transcription of over 500 genes in the human genome, most of which have cytoprotective functions. This nuclear transcription factor binds to the antioxidant response element ARE of the DNA to promote mRNA synthesis (Suzuki et al., 2015). The Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm functions as a negative regulator by binding of Nrf2 and thereby destining Nrf2 to ubiquitinylation and proteasomal degradation in the absence of chemical stress. Electrophiles and oxidants, including GST substrates, bind to Keap1, thus releasing Nrf2 to escape degradation and enter the nucleus as a result of exposure to the toxicants. The cadre of enzymes induced in general provide protection of the exposed tissue to carcinogenesis and other pathophysiological conditions. However, in neoplastic cells, the Nrf2/Keap1 system may actually promote the carcinogenic process and thereby have undesired consequences (Suzuki et al., 2015; Pandey et al., 2017).

An added level of complexity is the modulation of Nfr2 and Keap1 by a number of microRNAs (Cheng et al., 2013) indicating a mechanism for fine-tuning the expression of the diverse GSTs and other enzymes at the posttranslational level.

In cellular systems there is generally crosstalk between different signaling pathways leading to modulation of the resulting outcomes (Singh, 2015). In this connection and with reference to the role of Alpha class GSTs and steroid hormone biosynthesis, it should be noted that the expression of the enzymes is regulated by the steroidogenic factor 1 (SF-1) in human cells (Matsumura et al., 2013).

11.8 PROTEIN ENGINEERING AND DIRECTED MOLECULAR EVOLUTION OF GSTS

11.8.1 Beyond the Genetic Code

In the vein of the earliest applications of site-directed mutagenesis applied to enzymes (Winter et al., 1982), the functions of numerous amino acid residues in various GSTs have been explored, as exemplified by the active-site tyrosine in human GST A1-1 (Stenberg et al., 1991). Drawing on the relatively small size of fluorine (van der Waals radius 1.35 Å) versus hydrogen (1.2 Å), steric perturbations resulting from fluorine substitution should be relatively small. This was exploited in the successful site-specific replacement of the active-site tyrosine by four different fluorinated tyrosines in order to influence the pK_a of the hydroxyl group and to provide evidence for its proposed function of hydrogen bonding to the glutathione thiolate in the active site (Thorson et al., 1998). The role of the active-site tyrosine was also suggested by a nonspecific substitution of all 14 tyrosines in rat GST M1-1 by 3-fluorotyrosine (Parsons et al., 1996). In a similar investigation, substituting 5-fluoro-tryptophan for all four tryptophan residues resulting in a 4-fold increased turnover number, apparently due to an enhanced rate of product release (Parsons et al., 1998).

11.8.2 Cooperative Protein Subunits

Most soluble GSTs are dimeric proteins and the question whether an isolated subunit could be catalytically functional has frequently been asked. A set of 10 mutations were therefore introduced in the subunit–subunit interface of human GST P1-1 to prevent dimerization and the monomeric protein was produced (Abdalla et al., 2002). Based on physicochemical parameters, the GSTP1 monomer was properly folded, but the protein showed no enzyme activity. Binding studies suggested that the H-site was functional and could bind hydrophobic substrates, whereas the binding of glutathione was impaired in spite of the fact that none of the G-site residues had been mutated (Abdalla et al., 2002). This lack of activity of the monomer is in accord with other studies suggesting that the two subunits of the functional GST P1-1 cooperate, possibly via networks of water molecules (Hegazy et al., 2004; Hegazy et al., 2006). GSTs are not typical allosteric enzymes, but several studies demonstrate that GSTs can display cooperativity under certain physical conditions (Caccuri et al., 1999) or

with select substrates (Lien et al., 2001), as well as in the sequestration of the toxic nitric oxide derivative dinitrosyl-diglutathionyl-iron complex (Bocedi et al., 2016).

11.8.3 Active-Site Mimicry in Engineered GSTs

Based on the premise that the substrate selectivity of GSTs is largely governed by the amino acids in the H-site, several successful attempts have been made to mimic the high activity of a chosen GST by installing corresponding residues in the H-site of a homologous low-activity enzyme of the same GST class. The catalytic efficiency of human GST M1-1 is 2700-fold higher than that of GST M2-2 in the conjugation of *trans*-stilbene oxide, and the mutation Thr210→Ser in GST M2-2 rendered the enzyme selectively more active with the same substrate by 200-fold (Ivarsson et al., 2003). Apart from mimicking the structure of the active site of GST M1-1 and its activity with *trans*-stilbene oxide by this point mutation, investigating all 19 possible residue-210 substitutions in GST M2-2 revealed that a point mutation in the active site can enable or disable alternative catalytic reactions without necessarily altering already established activities with other substrates (Norrgård et al., 2006). This finding demonstrates a significant evolutionary plasticity useful for the emergence of diverse activities of the same protein.

Another example of mimicry is the generation of the GIMFhelix mutant of GST A1-1, comprising the replacement of four amino acids and the C-terminal helix by those present in GST A4-4 (Nilsson et al., 2000). The properties of the mutant were similar to the typical high catalytic activity of GST A4-4 with 4-hydroxyalkenals and the characteristic low pK_a (~7) of the active-site tyrosine. Analyses of crystals demonstrate the structural similarities of GIMFhelix and GST A4-4 (Balogh et al., 2009).

A third case involved the augmentation of the steroid isomerase activity of human GST A2-2, which is lower by three orders of magnitude than that of GST A3-3 (Pettersson et al., 2002). Five residues in GST A2-2 were changed into the corresponding active-site residues of GST A3-3 enabling the steroid substrate to bind in a catalytically favorable orientation (Tars et al., 2010).

11.9 EVOLUTION OF CATALYTIC ACTIVITIES IN NEW DIRECTIONS

11.9.1 Engineering GSTs for Fine-Chemical Synthesis

GSTs are generally promiscuous in their acceptance of alternative substrates, and catalyze alkylation, arylation, thiocarbamoylation, transacylation, reduction, transnitrosylation, isomerization, and various addition reactions with different efficiencies dependent on their structure (Kurtovic et al., 2008). For possible biotechnical applications, it may therefore be possible to enhance a desirable function by protein engineering and in vitro evolution. For example, the catalytic activity of GST M2-2 with indene 1,2-oxide, relevant to the synthesis of the drug Crixivan (indinavir), was enhanced approximately 100-fold by iterative saturation mutagenesis (Norrgård et al., 2011). The highest indene 1,2-oxide activity was obtained with the double mutant Thr210→Gly/Ile10→Cys. However, with five alternative substrates,

undergoing mechanistically different reactions, other mutants displayed higher activity. It is noteworthy that the stereoselectivity of epoxide reactions can be manipulated by simple chemical modifications of suitable side chains in the GST structure (Ivarsson et al., 2007).

11.9.2 Evolution of GSTs for Prodrug Activation

Human GSTs have been shown to activate different thiopurine prodrugs to release 6-mercaptopurine, which subsequently serves as an antimetabolite interfering with nucleotide metabolism and nucleic acid biosynthesis. Azathioprine (Imuran), 6-[(1-methyl-4-nitro-1H-imidazol-5-yl)thio]-1H-purine, has been in clinical use for more than half a century. It is activated most efficiently by GSTs A1-1, A2-2, and M1-1, whereas 10 other human GSTs were significantly less effective or lacked detectable activity (Eklund et al., 2006). Other prodrugs releasing 6-mercaptopurine are *cis*-6-(2-acetylvinylthio)purine and *trans*-6-(2-acetylvinylthio)-guanine, but with these compounds GSTs A1-1 and A2-2 are not particularly active, whereas GST M1-1 is an efficient catalyst (Eklund et al., 2007). Clinical data show that *GSTM1 positive* patients are more liable to adverse side effects of azathioprine than patients presenting with the *GSTM1 null* genotype (Stocco et al., 2007). This observation can, at least in part, be attributed to higher total GST activity resulting in elevated release of 6-mercaptopurine and more extensive consumption of glutathione in the presence of the GST M1-1 enzyme.

For potential therapeutic purposes (see Section 11.14), it was considered worthwhile to enhance the GST activity with azathioprine. GST A2-2, the most efficient enzyme with this substrate (Eklund et al., 2006), was therefore subjected to various mutational strategies (Modén et al., 2014). The allelic gene variant *GSTA2*E*, encoding the most efficient GST protein, was engineered by a structure-based approach in which three of its H-site residues were mutated. The resulting focused mutant library consisting of 864 possible amino acid combinations was screened with azathioprine, and several highly active triple-point mutants were isolated (Zhang et al., 2012b). The most active variant displayed 70-fold higher catalytic efficiency than the parental GSTA2-2*E enzyme.

In order to map the theoretically available evolutionary trajectories leading, one mutation at a time, from the parent GST A2-2 to the most efficient mutant, all six intermediate mutants were constructed and assayed with eight alternative substrates in addition to azathioprine (Zhang et al., 2012a). Conspicuously, all of the six trajectories showed a monotonically increasing activity with azathioprine, but monotonically decreasing activities, or peaks and valleys, with some of the other substrates in the multidimensional fitness landscape. Furthermore, epistatic effects of the mutations on catalytic activity were noted, which were variable in sign and magnitude depending on the substrate used, showing that epistasis is a multidimensional quality (Zhang et al., 2012a).

11.10 EVOLVING QUASI-SPECIES OF ENZYMES

The examples of mutagenesis aforementioned are based on well-reasoned rational aspects, but in nature, mutations are largely caused by stochastic processes. The

rationale of the latter is that mutants are more or less randomly produced and those found useful are retained. Based on the studies of populations of evolving RNA viruses, Eigen and coworkers (Eigen et al., 1988) proposed that survival of the fittest should apply not to an individual but to a population of related mutants called the quasi-species. The concept of quasi-species was adopted for the evolution of GSTs in mutant libraries obtained by DNA shuffling of homologous sequences (Emrén et al., 2006; Runarsdottir et al., 2010). Importantly, this approach incorporates information about activities with several alternative substrates, a multivariate scheme that in many aspects reflects natural evolution. Both the design of iterative mutagenesis for new generations of enzymes and the analysis of evolutionary trajectories in multivariate dimensions are facilitated by regarding the quasi-species, rather than the "best" enzyme variant, as the relevant evolving unit (Mannervik et al., 2009).

11.11 INFOLOGS AS NOVEL INFORMATION-OPTIMIZED MUTANTS FOR ENZYME EVOLUTION

Natural evolution is based on stochastically generated quasi-species. By contrast, a rational primary-structure-guided approach of directed enzyme evolution has been designed (Govindarajan et al., 2015). Suitable amino acid substitutions are selected by phylogenetic analysis and combined, via chemical gene synthesis, into a set of maximally information-rich gene variants called infologs. The relative contribution of each substitution is determined across multiple catalytic dimensions, providing the basis for predictive functional models with broad applicability for bioengineering (Musdal et al., 2017). This novel method for enzyme engineering combines machine learning and synthesis of a modest number of genes and provides multivariate modeling of protein sequence-function in a cost-effective manner.

11.12 EXPRESSION OF GSTS IN PLANTS FOR PHYTOREMEDIATION

A variety of biochemical and biological approaches are drawing on enzymes that catalyze the biotransformation of organic pollutants, such as pesticides, explosives, and other xenobiotic substances occurring in the environment (Abhilash et al., 2009). Appropriate biotransformation render these chemical compounds nontoxic and facilitate their elimination. Genomics studies have demonstrated that GSTs in rice, during various stages of development, can counteract many of the stress challenges indicated earlier (Jain et al., 2010) suggesting that plants could be engineered with suitable GSTs and be used for phytoremediation. For example, overexpression of GSTL2 in rice provided resistance to the herbicides glyphosate and chlorsulfuron (Hu, 2014), and experiments involving transgenic overexpression of rice GSTU4 afforded tolerance to salinity and oxidative stress in *Arabidopsis thaliana* (Sharma et al., 2014). These and other investigations demonstrate the importance of GST enzymes to resistance against various chemical challenges, not only in animals but also in plants.

An example relevant to phytoremediation applications involves the explosive 2,4,6-trinitrotoluene (TNT). The environmental pollutant TNT has for decades

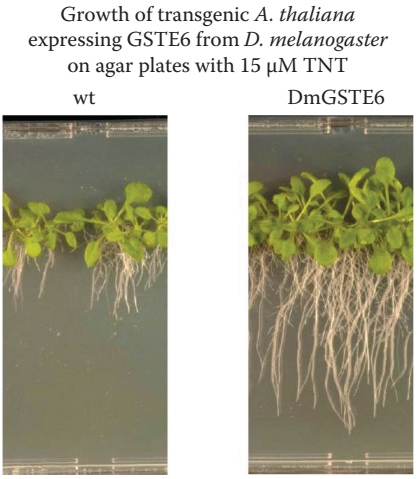

FIGURE 11.2 Comparison of *Arabidopsis thaliana* wild-type plantlets (left) and transgenic plantlets expressing *Drosophila melanogaster* GSTE6 (right) grown in the presence of 1,3,5-trinitrotoluene (TNT) showing the protective effect of the GST transgene. (From collaborative study with the laboratory of Neil Bruce, York University, UK; cf. Tzafestas et al., 2017.)

spread over large areas as a result of large-scale military and industrial activities. TNT can be metabolized by GST-catalyzed glutathione conjugation, and *A. thaliana* plantlets overexpressing one of its GST enzymes deplete the growth medium of TNT and inactivate the compound (Gunning et al., 2014). However, the plant GSTs have modest activity and GSTs optimized by methods of biochemistry and molecular genetics are in demand (Figure 11.2).

The insect enzymes GSTE6 and GSTE7 from *Drosophila melanogaster* were demonstrated to be orders of magnitude more efficient in the detoxification of TNT than the available plant GSTs (Mazari et al., 2016). The gene encoding GSTE6 was therefore expressed in *A. thaliana* to obtain a phytoremediation model system. Plants transgenetically expressing the *Drosophila* GSTE6 were more resistant to TNT than both unmodified plants and the *Arabidopsis* lines overexpressing the plant GSTU24 and GSTU25 (Tzafestas et al., 2017). Also, the uptake of TNT from the growth medium was enhanced in plants expressing the transgene. For actual field applications plants more robust than *Arabidopsis* will obviously be required.

11.13 INTERCELLULAR TRAFFICKING OF GSTS

In a study of protein transduction domains using a *Schistosoma japonicum* GST as intended cargo to be delivered into COS7 cells, the GST protein was unexpectedly taken up even in the absence of an added protein transduction domain (Namiki et al., 2003). Similar cellular GST uptake was demonstrated with the cell lines HeLa, NIH3T3, and PC12. In an investigation using members of different classes within the GST structural superfamily and numerous additional cell lines, the results were extended (Morris et al., 2009). Experiments also indicated that the GSTs enter cells

through an energy-dependent process involving endocytosis, and GST protein was found to colocalize with transferrin in the cells implying that the endocytosis process involves clathrin-coated pits. Further, incisive studies of truncated forms of human GST M2-2 showed that the globular C-terminal domain (GST-C) is responsible for the cell translocation. In particular, it was noted that alteration of the conformational stability of GST-C, governed by the α6-helix, can significantly influence cellular uptake efficiency (Morris et al., 2011). GST-C has possible medical applications in the treatment of heart arrhythmia, since it binds selectively to the ryanodine receptor RyR2 (Hewawasam et al., 2010; Samarasinghe et al., 2015) and influences contractility and calcium transients in cardiomyocytes (Hewawasam et al., 2016).

We have verified the remarkable phenomenon of GST uptake in several cell lines including neuroblastoma SH-SYS5 cells (Figure 11.3). The wild-type GST proteins are taken up in a catalytically functional state in the SH-SYS5 cells, as demonstrated with human GST M2-2 and the neurotoxic orthoquinone substrate aminochrome (Cuevas et al., 2015). Uptake of GST M2-2 provided protection against cell death caused by aminochrome, and the protective effect was counteracted by antibodies directed to the enzyme. Remarkably, the protective outcome was obtained not only by administration of the purified recombinant GST M2-2, but also by the enzyme secreted into the culture medium by astrocytoma U373MG cells. GST M2-2 occurs as a constitutive enzyme in the U373MG cells, in which it acts as an endogenous protective agent (Huenchuguala et al., 2014). The conclusion is that the two cell types can communicate via excreted GST M2-2 such that U373MG cells can protect SH-SYS5 cells against the toxic aminochrome (Cuevas et al., 2015). By extrapolation, astrocytes could similarly protect neurons via intercellular GST trafficking in the nervous system.

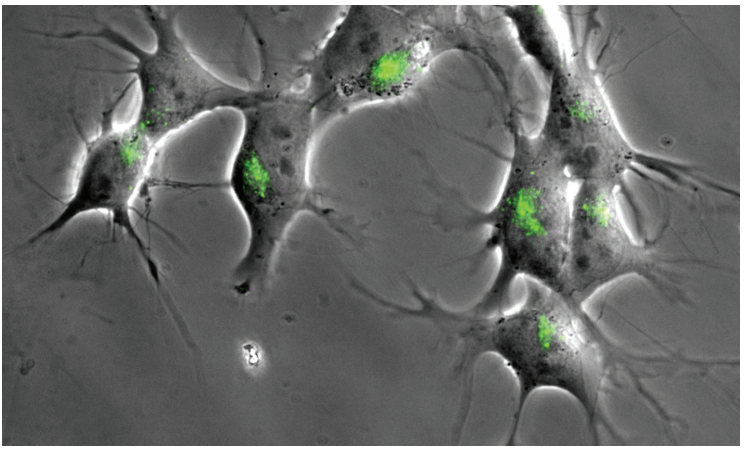

FIGURE 11.3 Uptake of human GST T1-1 in living neuroblastoma SH-SYS5 cells. The cells were incubated for 1.5 h in DMEM with 400 nM hGST T1-1 labeled with Oregon Green 488 (OG) in 37°C and 5% CO_2. Trypan Blue was used to quench any extracellular fluorescence remaining after washing of the cells.

Extracellular vesicles, first described in the form of prostasomes in prostatic fluid (Ronquist et al., 1985), occur in several physiological settings. The vesicles could be single-membrane exosomes (Van Niel et al., 2018) or multivesicular bodies (Von Bartheld et al., 2011) and may be relevant to normal as well as pathological states, including blood coagulation, inflammation, neuronal communication, and tumorigenesis. The GST excretion described earlier may possibly occur via exosomes or other membrane vesicles, and the uptake into cells may be effected via the reverse process. Considering the well-known ligandin function of GSTs (Litwack et al., 1971) intercellular trafficking of bound ligands might thus be mediated by GST proteins.

11.14 BIOMARKER APPLICATIONS OF GSTS

Early studies of carcinogenesis in rat liver demonstrated that foci (Kitahara et al., 1984) and preneoplastic nodules (Jensson et al., 1985) express a protein, now known as GST P1-1, which is not present in normal hepatocytes. Even if the same GST phenotype was not observed in the development of human hepatocarcinoma, the use of anti-GST P1-1 antibodies to detect hepatic lesions was found useful in immunochemical tests of potentially genotoxic agents in the rat model system. However, many human neoplasias other than primary liver cancer do express elevated GST P1-1 concentrations, and diagnostic tests for other tumors have been proposed (Kodate et al., 1986). The finding that different regions of the kidney diverge in their composition of the multiple GSTs present opportunities for differential diagnosis for various lesions (Rozell et al., 1993) using body fluids in additioin to histology (Hao et al., 1994). However, the release of GSTs is quite variable and can depend on various factors, not all of which indicate disease.

A more robust method applicable to diagnosis of prostate cancer is based on measurement of the methylated promoter region of the *GSTP1* gene, which is a signature for the downregulation of the enzyme in this tumor (Wu et al., 2017). Prostate cells are shed in the urine, and their DNA can be analyzed for the hypermethylation characterizing the cancer cells.

11.15 ANTIBODY DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

A recent promising development in oncology is the use of biologicals, in particular monoclonal antibodies with specificity for epitopes that distinguish tumors from normal tissues (Bhutani et al., 2013). Some therapeutic antibodies themselves afford significant antitumor activity, but new generations of antibodies carry payloads such as a drug, a radionuclide, or a toxin in order to achieve enhanced therapeutic effects (Teicher et al., 2011). Examples of antibodies conjugated with proteins include the use of ADEPT that releases the toxic agent from a prodrug to the targeted tumor (Afshar et al., 2009; Tietze et al., 2009). ADEPT has the potential to significantly improve drug efficacy and reduce adverse side-effects (Sharma et al., 2017).

It should be possible to target cellular tumor receptors by binding proteins fused with a highly active GST followed by administration of the prodrug. The high GST activity will give a focused and concentrated release of the active cytotoxic drug in the tumor tissue. Among the several novel GST-activated drugs (Ruzza et al., 2013), the prodrug TLK286/Telcyta has a particular advantage (Morgan et al., 1998). The activation of Telcyta occurs without involving glutathione as a cosubstrate and is therefore independent of the ambient concentration of glutathione. By contrast, the reactions of GSTs with most other substrates require glutathione in a concentration-dependent manner.

The underlying rationale for the development of the glutathione derivative Telcyta was its selective affinity for GST P1-1 (Lyttle et al., 1994), the GST enzyme that is often overexpressed in cancer cells (Mannervik et al., 1987). Linking the GST to a target-seeking antibody would increase the selectivity of action and obviate the requirement of high expression of the enzyme in the targeted tumor (Figure 11.4). The prodrug is activated by the GST to release an active phosphoramide mustard similar to the alkylating agent released from cyclophosphamide, a drug widely used in cancer chemotherapy. Telcyta has undergone multiple clinical trials in cancer patients and not demonstrated toxicity above that of other alkylating drugs.

It can be assumed that GST bound extracellularly can fulfill its assigned role to kill the target cell, but also that the liberated toxic drug will cause a bystander effect on proximal tumor tissue (Dachs et al., 2009). Unpublished experiments (B. Sjödin and B. Mannervik) show that scFv-anti-CD123 fused with a GST protein is taken up by neuroblastoma cells in culture (cf. Figure 11.3). Specific uptake into the receptor-presenting cells would make the treatment especially powerful.

In order to target diverse tumors, we have invented a generalized tripartite therapeutic toolbox consisting of different combinations of prodrug (substrate)–GST enzyme–binding protein (Figure 11.5). A designated GST could be engineered for high efficiency with a preferred prodrug, as in the example of GST A2-2 and azathioprine (Section 11.9.2). The redesigned GST is then coupled to an antibody or another binding protein with selective affinity for the tumor target.

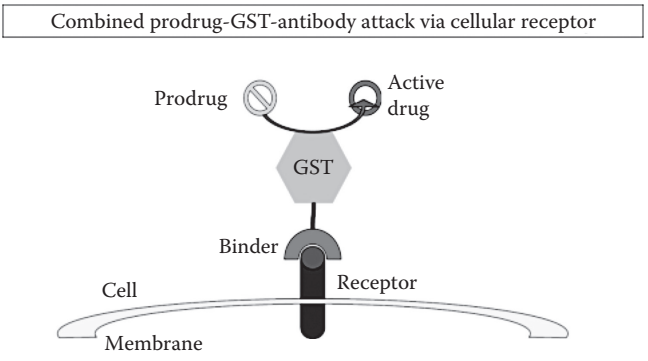

FIGURE 11.4 Scheme of directed combination-treatment using a prodrug activated by a GST linked to a binding protein, which selectively recognizes a receptor or other epitope on the target cell. The active drug will be focused to the cell and the neighboring tissue.

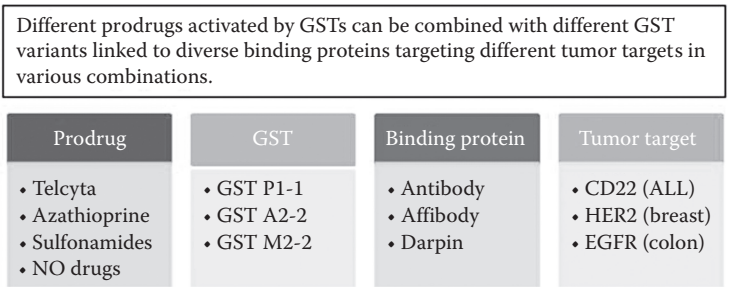

FIGURE 11.5 Molecular tripartite toolbox composed of alternative prodrugs, selective activating GSTs, and alternative binding proteins recognizing different tumors. Using different combinations of the components, the therapeutic applications can be tailored for individual requirements.

11.16 FUTURE DIRECTIONS

11.16.1 Studies at the Molecular Level

GSTs can conveniently be obtained via gene synthesis and heterologous expression and be subjected to structural and functional investigations in combination with redesign and molecular evolution. Fluorophore-labeled GSTs as well as fluorogenic substrates (Shibata et al., 2013) will enable single-molecule characterization of the interactions of GSTs with alternative substrates as well as inhibitors, both *in vitro* and in living cells (Xie et al., 2008). Of particular importance are the effects induced by the intracellular milieu on catalysis and the interaction with inhibitors (Fu et al., 2014).

11.16.2 Studies at the Cellular Level

Introduction of GST proteins into living cells via endocytosis or via transfection from eukaryote expression vectors perturbs the composition of the proteome by mechanisms that appear unrelated to the catalytic activities of GSTs, which can be verified by treatments with incapacitated mutants. Networks of cellular signaling may be influenced by GSTs and studies of proteomes as well as transcriptomes can explore these newly discovered phenomena in cells. Functionally important complexes between GSTs and c-Jun N-terminal kinase (JNK), apoptosis signal-regulating kinase 1 (ASK1), and other protein kinases have been reported (Singh, 2015). Decreased GST levels can be accomplished by gene silencing or elimination performed by the CRISPR/Cas9 approach (Jinek et al., 2012). By eliminating defined GSTs, the possible compensatory effects of other enzymes can be evaluated.

11.16.3 The Fruit Fly as a Model Organism

Drosophila melanogaster has emerged as one of the most effective biological systems for investigations of gene function in eukaryotes, and is increasingly used to model human diseases. Remarkably, a study of *Caenorhabditis elegance, D. melanogaster*,

and mouse (*Mus musculus*) demonstrated that a subset of GSTs, but not the entire GSTome in each species was overexpressed in long-lived animals (McElwee et al., 2007). An integrated and comprehensive investigation of the GSTs in flies could clarify key issues related to longevity assurance and concomitantly provide essential information on biochemical processes preventing various degenerative diseases. The various GSTs are differentially expressed and the enzyme composition changes from tissue to tissue and during ontogenesis. Overexpression of GSTE7 via injection of a plasmid carrying the corresponding gene into fly embryos has already been accomplished (Mazari et al., 2014). Intriguingly, the transgenic females overexpressing GSTE7 demonstrated an enhanced egg-laying both in the absence and the presence of the toxic allyl isothiocyanate (Figure 11.6). The effect on the oviposition rate is independent of the presence or absence of toxic allyl isothiocyanate, and surprisingly also obtained with the catalytically incapacitated mutant enzyme GSTE7_S12F. The results demonstrate consequential cellular activities of GSTs other than catalysis of chemical reactions.

Underlying the expression of the proteome is the transcriptional activation of genes. Methods are now available for characterization of the global transcriptome via sequence analysis. It is even possible to study transcripts by noninvasive capturing and sequencing of mRNA from live single cells (Lovatt et al., 2014). Apparently,

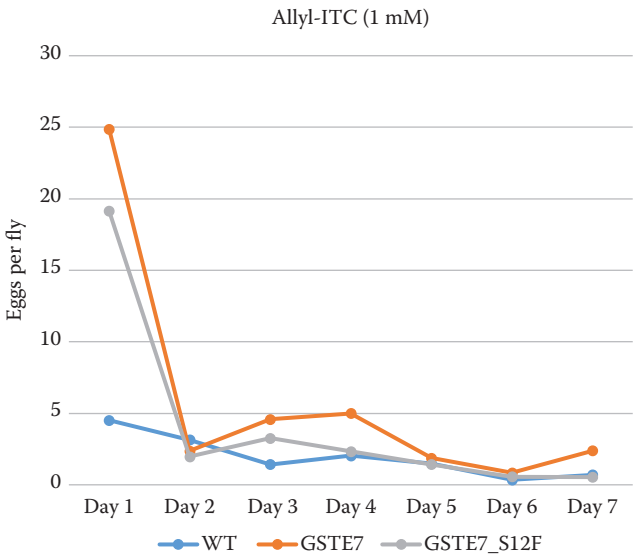

FIGURE 11.6 Significant increase in egg-laying frequency in *Drosophila melanogaster* following transgenesis with the GSTE7 gene. Overexpression of catalytically active GSTE7 as well as the catalytically incapacitated mutant GSTE7_S12F enhanced oviposition. In the experiment shown, the toxic GST substrate allyl isothiocyanate was added to the standard fly food, but the same effect of transgenesis was obtained in the absence of allyl isothiocyanate. The similar effects of the active GSTE7 and the mutant show that catalytic GST activity was irrelevant for enhanced oviposition. (Unpublished data from A.M.A. Mazari, O. Dahlberg, B. Mannervik and M. Mannervik; cf. Mazari et al., 2014.)

the tissue microenvironment shapes the transcriptomic landscape of individual cells. Mosaicism of GST expression in kidney epithelial cells has been observed (Rozell et al., 1993). Incisive examination of the GSTome using transcriptome profiling of single cells resident in their natural microenvironment would help to illuminate the multitude of GST functions.

11.17 SIGNIFICANCE OF GST RESEARCH

Enzymes are key players in all cellular processes in every living organism. In spite of a wealth of knowledge, the entire functional scope of enzymes is still incompletely understood. For example, approximately half of all pharmaceutical drugs are directed against enzymes, but the interactions of targeted enzymes with the full complement of molecules encountered in the cellular context is largely unchartered. The traditional biochemical approach involves isolation of the enzyme of interest and examination of its interaction with its cognate ligands. However, in the intracellular milieu, a protein is surrounded by thousands of different molecules, and we now understand that every enzyme molecule has to cope with both cognate and noncognate partners. Noncognate interactions may give rise to malfunctioning biochemical systems and disease, and in the field of pharmacology, a lack of specificity may cause adverse side reactions that jeopardize the beneficial effects of a drug. Furthermore, numerous application in biotechnology are based on enzymology, and the design of enzymes for new purposes still presents a challenge. Many of these questions are relevant to the GSTs as such. However, the enzymes also lend themselves to research that goes beyond glutathione biochemistry and illustrate principles and phenomena of general significance.

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