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Multiple Functions of Glutathione Transferases

A Study on Enzymatic Function, Regulatory Role and Distribution in Mouse and Man

BY

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ABSTRACT

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To cope with various endogenous toxin and xenobiotics nature has equipped the organisms with a proper protection system. Glutathione transferases (GSTs) are important components of the cellular defense against oxidative stress. These proteins appear to be suited for different tasks.

Based on catalytic activity of GSTs with monochlorobimane (MCB), a screening method was developed for identification of active GSTs in bacterial colonies and for characterization of combinatorial GST libraries.

Solvent viscosity effects on k_{cat} and k_{cat}/K_m on wild-type human GST A1-1 and phenylalanine-220 mutants indicate a physical step being the rate-limiting step in the catalytic mechanism.

Three residues that were under evolutionary selection pressure were identified in Mu class GSTs. By changing these residues in human GSTM2-2, a 1000-fold change of catalytic activity towards GSTM1-1 was accomplished.

Using peptide phage display, a peptide sequence was found that acts as non-substrate ligand for human GST M2-2. The peptide sequence was shown to be highly similar to the C-terminal region of c-Jun N-terminal kinase (JNK). JNK is a kinase linked to activating protein-1 (AP-1) transcriptional activity, which is part of the regulation of cell proliferation and apoptosis in response to cellular stress. Reporter gene assays in cell lines showed that human GST M2-2 coactivates the transcriptional activity of AP-1.

GSTs as part of the cellular defense against oxidative stress could be important in inflammatory processes. The distribution of GSTs in the intestine of both mice and human in abnormal inflammatory state was investigated immunohistochemically. Using an experimental mouse model, it was shown that mouse GST A4-4 is markedly induced while, the expression of Mu and Pi class GSTs is reduced in the colon of conventional and germ-free mice with extensive colitis. Moreover, the expression of mouse GST A4-4 was elevated with time when germ-free mice were exposed to normal bacteria flora. In contrast, Mu and Pi class GSTs showed decreased expression in the colon of germ-free mice associated with commensal flora. The Alpha, Mu and Pi class GST levels in mouse colon were increased when germ-free mice received *Lactobacillus* strain GG.

The distribution of Alpha, Mu and Pi class GST in the intestinal tissues of patients with Crohn's disease was investigated using immunohistochemistry. All the three classes were consistently expressed in the intestinal epithelium as well as in macrophage-like cells and smooth muscle tissue. The mucus secreting goblet cells, however, did not express Alpha class GST.

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To My Mother, the most courageous woman I know

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Birgitta I. Eklund, Maryam Edalat, Gun Stenberg, Bengt Mannervik (2002) Screening for recombinant glutathione transferases active with monochlorobimane. *Analytical Biochemistry,* Accepted.

II. Lisa O. Nilsson, Maryam Edalat, Pär L. Pettersson, Bengt Mannervik (2002) Aromatic residues in the dynamic C-terminal region of glutathione transferse A1-1 influence rate-determining steps in the catalytic mechanism *Biochimica et Biophysica Acta*, 1597:126-132.

III. Ylva Ivarsson, Aaron J. Mackey, Maryam Edalat, William R. Pearson, Bengt Mannervik (2002) Identification of residues in glutathione transferase driving functional diversification in evolution. Summitted for publication.

IV. Maryam Edalat, Sven Pettersson, Mats A. A. Persson, Bengt Mannervik (2002) Probing biomolecular interactions of glutathione transferase M2-2 using peptide phage display. Submitted for publication.

V. Maryam Edalat, Bengt Mannervik, Lars-Göran Axelsson (2002) Selective expression of detoxifying glutathione transferases in mice: effect of experimental colitis and the presence of bacteria. Submitted for publication.

VI. Maryam Edalat, Bengt Mannervik, Robert Löfberg, Åke Öst, Sven Pettersson, Lars-Göran Axelsson (2002). Distribution of glutathione transferases in the intestine of patients with Crohn's disease. Submitted for publication.

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Abbreviations:

AD	Δ^5-androstene-3, 17-dione
AP-1	Activating protein 1
CAR	carrageenan
CD	Crohn's disease
CDNB	1-chloro-2,4-dinitrobenzene
DSS	dextran sulfate sodium
GSH	glutathione
GST	glutathione transferase
IBD	inflammatory bowel disease
JNK	c-Jun N-terminal kinase
MCB	Monochlorobimane
UC	ulcerative colitis
PG	prostaglandin

INTRODUCTION

In biochemistry, the chemistry of life, we seek to describe structure and function of life in molecular terms. What is the chemical structure of components of living organisms? How do biomolecules such as DNA, proteins, vitamins, hormones, sugars and lipids function? How do these components interact with each other and organize into cells, tissues and organisms? What is the chemical and molecular basis of reproduction, aging and death of an organism? What is the molecular mechanism behind disorders such as diabetes, Alzheimer's disease, cancer *etc*.? These are the type of questions that are frequently asked by the biochemist.

Proteins, the fundament of life

I would like to compare life to a cryptated book. When Watson and Crick solved the double helical structure of the deoxyribonucleic acid (DNA) and suggested how the genetic information could be encoded in the structure of DNA, the principle behind the crypt of the book of life was solved. However, we still cannot read this book because the words are not put in the right place and many words we still do not understand. The words in this analogy are symbolizing the proteins, which are the products of the genes. Proteins have many different functions: some proteins are structural proteins such as collagen; others are involved in transporting small molecules. Antibodies are proteins and most importantly enzymes, which act as the biological catalysts, are proteins. Now that the human genome is sequenced and the crypt is solved; we know what genes encode which proteins. Now we have to put the words together and make correct sentences and then put the sentences together in correct order.

Glutathione, the antioxidative peptide

Glutathione (GSH) is a tripeptide, which was first discovered by Sir Fredrick Gowland Hopkins in 1920s (Figure 1). GSH is used throughout all aerobic organisms and it is a low molecular thiol source with a millimolar concentration in the cells. Today along with β-carotene, ascorbic acid (vitamin C), α-tocopherol (vitamin E) and flavonoids *etc*. it is commonly referred to as an antioxidant (Cotgreave and Gerdes, 1998). Aerobic organisms possess an antioxidative defense to protect itself from oxidative stress or oxidative mediated processes, which are associated with both,

stimulated proliferation and tumor promotion (Cerutti, 1985; Burdon and Rice-Evans, 1989) and onset of apoptosis (van den Dobbelsteen *et al.*, 1996). Thus the oxidative mediated processes are very complex and dependent on many biochemical factors.

Moreover, the GSH/GSSG pair, with their high reduction potential participates in maintaining other cellular thiol in a reduced state. These reactions are catalyzed by glutathione reductase (GR) (Bellomo *et al.*, 1987), thioredoxin (TRX) and thioredoxin reductase (TRX red) system (Holmgren and Björnstedt, 1995) and glutaredoxin (GRX) (Holmgren, and Aslund, 1995) *etc.* Finally, GSH serves a substrate or cofactor in a number of GSH linked enzymes (see below).

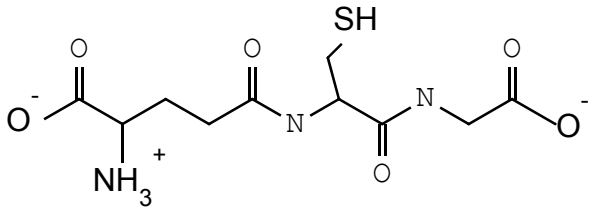

Figure 1. Chemical structure of glutathione, γ-Glu-Cys-Gly

Glutathione dependent enzymes

There are a number of GSH linked enzymes that are part of the cellular protection against both endogenous and xenobiotic toxic substances. Glutathione reductase (GR) catalyzes the reduction of GSSG using NADPH as a reductant (Krohne-Ehrich *et al.*, 1977). GR is important to keep the high cellular reductive potential. Selenium dependent glutathione peroxidase (GPxs) are other GSH-linked enzymes that catalyze the reduction of peroxides using GSH as the reducing agent (Arthur, 2000). Another important GSH dependent system is the glyoxalase I and II which are responsible for catalysing the conversion of methylglyoxal (a by-product in glycolysis) to lactic acid (Thornalley, 1993). Finally, last but not the least, glutathione transferases (GSTs) are also GSH dependent enzymes with many properties among which catalyzing the conjugation of GSH to various electrophilic compounds is one of the most investigated function.

GST a protein with many features

GSTs were first discovered as enzymes 1961 (Booth *et al.* 1961). Later these proteins were also proposed to act as carrier proteins and were named ligandin

(Litwacks *et al.*, 1971). Today, despite 40 years of research the picture of what exactly the proteins of the superfamily do, is more complex than ever. GSTs are mostly known as the cellular catalysts of conjugation of the nucleophilic sulfhydryl group of glutathione to various electrophilic toxic compounds. However, today the catalytic function of GSTs is known not to be restricted to detoxication. Some GSTs isoenzymes seem to have a metabolic role in catalyzing synthesis of steroids and prostagandins (Petterson and Mannervik, 2000; Johansson and Mannervik, 2001; Beuckmann *et al.*, 2000). Lately, research on GSTs presented yet another function namely a regulatory role (Adler *et al.*, 1999; Cho *et al.*, 2001), which has added a new dimension to the importance of this large family of proteins.

Classification and structure of soluble GSTs

The superfamily of the glutathione transferases contains both cytosolic and membrane bound members. The human cytosolic or soluble GSTs are not only in the cytoplasm but may also be localized in the mitochondria or the nucleus (Johansson and B. Mannervik, 2001).

The mammalian soluble GSTs are so far divided into eight classes based on their amino acid sequences including Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta and Zeta (Mannervik *et al.,* 1985; Board *et al.*, 2000). Each class consists of one or several protein isoforms. The amino acid identity between two isoforms, within the same class, is more 50%, whereas the identity between classes is about 30% (Mannervik *et al.*, 1992).

The soluble GSTs are dimeric proteins with the molecular weight of approximately 50 kDa. GSTs occur both as homo- and heterodimers (Stockman *et al.*, 1985; Tsuchida *et al.*, 1990). GSTs are named using a letter corresponding to their class membership and Arabic numerals after the subunit composition (*e.g.* GST A1-1 is a homodimeric alpha class GST consisting of two subunit 1). Each subunit contains one active site with two binding pockets where the first binding domain is the glutathione-binding site (G-site) and the second domain is the pocket where the hydrophobic substrate binds (H-site). The G-site is highly conserved among different classes and isoenzymes. However, the functional properties of the amino acid residues of the H-site vary between different GSTs, accounting for substrate specificity. The crystal structure of mammalian GSTs representing each class has

been solved (Sinning *et al.*, 1993; Ji *et al.* 1992; Kanaoka *et al.*, 1997; Rossjohn *et al.*, 1998; Board *et al.*, 2000).

Evolution of GSTs

GSTs are believed to have evolved by fusion of functional domains (Mannervik, 1985) in a divergent manner. The evolutionary development of the GST superfamily from their enzymatic perspective can be observed by the structure of the active site. While the glutathione binding domain has the same overall fold as other glutathione linked enzymes such as glutathione peroxidase, glutaredoxin as well as thioredoxin (Dirr *et al.*, 1994), the structure of the hydrophobic site of glutathione transferases has evolved to provide different substrate specificity and subsequently catalytic diversity. Historically GST Theta is proposed to have branched off earliest followed by GST Sigma and GST Mu. GST Alpha and Pi are proposed to be the youngest GST classes (Armstrong, 1997; Pemble and Taylor, 1992).

Differential expression of GSTs

Studies of tissues in both rat and human have established that the expression pattern of GSTs is different in different organ (Johansson and Mannervik, 2001). Even within the same organ GST expression varies from tissue to tissue. For example, in the human kidney in proximal tubules, GST Alpha is the major class, while in distal tubules, GST Mu and GST Pi are dominating GSTs (Sundberg *et al.*, 1993).

Furthermore, the expression of GSTs seems to be more complicated as fetal tissues contain different GST pattern than adult tissues. Alpha class GST is expressed highly during the entire life, however, the expression level of GST P1-1 decreases at the end of prenatal period (Strange *et al.*, 1989). Additionally, the expression of GSTs can be both correlated to sex and age in some cases (Johansson and Mannervik, 2001). In summary, the distribution of different isoforms of GSTs is a complex subject and studies on this subject are far from completed.

Polymorphism in GSTs

Genetic polymorphism of some of GST classes in the human population has been well established. While some of the allelic variants are catalytically the same,

others have different phenotypic property. Table 1 shows five classes of GSTs with some of the identified polymorphisms.

GST A2, and GST M1 alleles (GSTM1*A and GSTM1*B) have the same catalytic properties as their respective wild-type forms. GST P1, GST Z1 allelic variants have different catalytic properties. Moreover, GST M3, another Mu class enzyme, has two variants GSTM3*A and GSTM3*B. The B allele contains a three base deletion in intron 6, which is associated with transcription factor YY1 and other transcription factors (Inskip *et al.*, 1995). The difference in transcription region indicates different expression levels of these GSTM3 alleles (Inskip *et al.*, 1995).

It should be mentioned that the GSTT1 null allele as well as the GSTM1 null allele are believed to be the consequence of gene deletion. Furthermore, it has been proposed that there might be allelic variations at position 126 of GST T1-1 (Sherratt *et al.*, 1997; Jemth and Mannervik, 1997).

Table 1. Some of the GST variants

GST Class	Gene	Allelic variants	Amino acid residues effected	References
Alpha	GSTA2	GST A2*A	Thr112/Glu210	Rhoads, 1987
Alpha	GSTA2	GST A2*B	Ser112/Ala210	Röhrdanz, 1992
Mu	GSTM1	GSTM1*A	Lys173	Widersten,1991
Mu	GSTM1	GSTM1*B	Asn173	Widersten,1991
Mu	GSTM1	GSTM1*0	No protein	Seidegård,1988
Pi	GSTP1	GSTP1*A	Ile105/Ala114	Kano, 1987
Pi	GSTP1	GSTP1*B	Val105/Ala114	Board, 1989
Pi	GSTP1	GSTP1*C	Val105/Val114	Ahmad, 1990
Pi	GSTP1	GSTP1*D	Ile105/Val114	Ali-Osman, 1997
Theta	GSTT1	GSTT1*A	WT	Pemble, 1994
Theta	GSTT1	GSTT1*0	No protein	Pemble, 1994
Zeta	GSTZ1	GSTZ1*A	Lys32/Arg42	Blackburn,2000
Zeta	GSTZ1	GSTZ1*B	Lys32/Gly42	Blackburn,2000
Zeta	GSTZ1	GSTZ1*C	Glu32/Gly42	Blackburn,2000
Zeta	GSTZ1	GSTZ1*D	Glu32/Gly42/Met82	Blackburn,2000

The detoxication function of GSTs

As enzymes, GSTs are involved in many different detoxication reactions. They are commonly referred to as phase II enzymes (Williams, 1947). They catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic toxic compounds. The GSH conjugates are excreted as mercapturic acids by the phase III metabolic pathway (Ishikawa, 1992). The substrates mentioned below are some of the physiologically interesting substrates.

GST P1-1, GST M1-1 and GST A1-1 have been shown to catalyze the inactivation process of α, β unsaturated carbonyls. One example of α, β unsaturated carbonyls is acrolein, a cytotoxic compound present in tobacco smoke (Figure 2). Exposure of cells to acrolein produce single strand DNA breaks (Crook *et al.,* 1986). Introduction of GST P1-1 to HepG2 cells gave a significant protection (Berhane and Mannervik, 1990; Castro *et al.*, 1990).

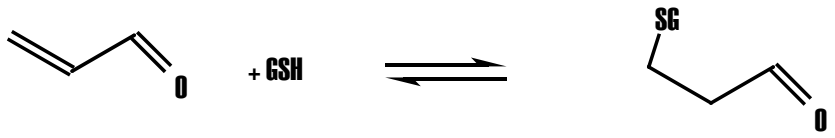

Figure 2. Inactivation of acrolein catalyzed by GSTs.

Another class of α, β unsaturated carbonyls are propenals, which are generated by oxidative damage to DNA (Figure 3). (The detoxication role of GSTs has been demonstrated *in vivo*) The toxicity of thymine propenal in HeLa cells was substantially reduced when the cells received GST P1-1 and GSH (Berhane *et al.*, 1994).

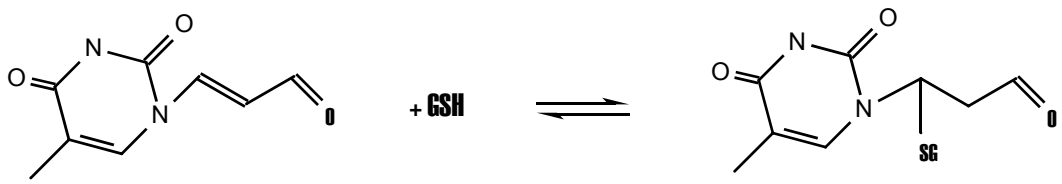

Figure 3. GST P1-1 catalyzes the conjugation of GSH to thymine propenal.

Alkenals, which are formed by oxidative damage to lipids, are the third class of α, β unsaturated carbonyls that are inactivated by GSTs (Berhane *et al.*, 1994). GST A4-4 is the most efficient enzymes in conjugating GSH to 4-hydroxynonenal, which is produced by lipid peroxidation of biomembranes (Figure 4) (Esterbauer *et al.*, 1982; Hubatsch *et al.*, 1998).

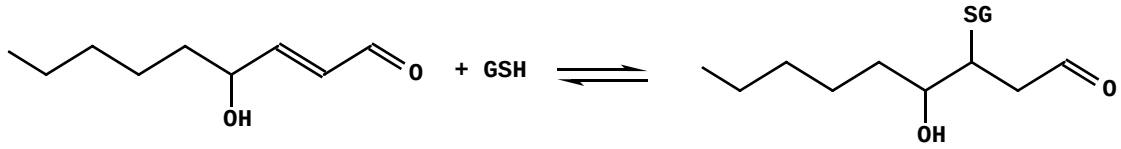

Figure 4. Addition of GSH to 4-hydroxynonenal catalyzed by GST A4-4

Besides catalyzing GSH conjugation reactions, GSTs possess selenium independent peroxidase activity using GSH to reduce hydroperoxides of phospholipids to alcohols (Hayes *et al.*, 1999; Zhao *et al.*, 1999). GSH peroxidase activity of GSTs is thought to prevent hydroperoxides to be engaged in free radical reactions during oxidative stress.

The metabolic function of GSTs

Alpha class GSTs and in particular GST A3-3 catalyze the isomerization of Δ^5-androstene-3, 17-dione to, Δ^4-androstene-3, 17-dione one of the steps in steroid synthesis (Figure 5) (Benson *et al.*, 1977; Pettersson and Mannervik, 2001; Johansson and Mannervik, 2001).

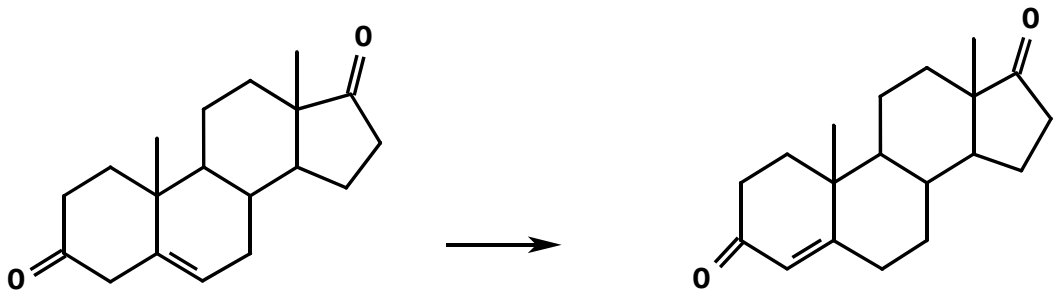

Figure 5. Isomerization of Δ^5-androstene-3, 17-dione to, Δ^4-androstene-3, 17-dione catalyzed by GST A3-3.

GSTs are also involved in the biosynthesis of prostaglandins (PGs). Human GST M2-2 has been isolated as a prostaglandin E synthase in the brain cortex (Beuckmann *et al.*, 2000). Interestingly human GST M3-3 also displays the same activity while GST M4-4 does not. Moreover, rat GST A1-1 and GST A3-3 catalyze the reduction of PGH_2 to PGF_2 (Chang *et al.*, 1987; Ujihara *et al.*, 1988). The Sigma class of GST has shown to catalyze the isomerization reaction of PGF_2 to PGD_2. PGD_2, PGE_2 and PGF_2 act as hormones that bind to G-protein coupled receptors. These receptors in turn regulate other hormones and neurotransmittors. PGF_2 are believed to stimulate mitogen activated protein kinases (Rao *et al.*, 1994). Prostaglandin D_2 and E_2 are unstable and will easily be converted to prostaglandin J_2 and A_2, respectively. The derivatives of PGJ_2 and PGA_2 inhibit NFκB by inhibition of IκB kinase, resulting in an anti-inflammatory response (Rossi A *et al.*, 2000). NFκB is a family of transcription factors that regulate the transcription of genes important for inflammatory processes (Barnes and Karin, 1997). In unstimulated cells NFκB interacts with IκB in the cytoplasm. However, upon stimulation IκB kinase phosphorylates IκB, which results in release of NFκB and relocalisation to the nucleus (Baichwal and Baeuerle, 1997). There are interesting speculations that GSTs might block other anti-inflammatory pathways by catalyzing the conjugation of GST to PGJ_2 (Hayes and Strange, 2000).

The ligandin function of GSTs

Glutathione tranferases, also called ligandin, have been demonstrated to bind hydrophobic compounds non-covalently acting as binding and transport proteins. The X-ray structures of GSTs suggest that there might be a third binding site between the subunits, designed for the ligandin function of these proteins (McTigue *et al.*, 1995; Ji *et al.*, 1996). The non-enzymatic binding capacity of GSTs to a broad range of lipophilic ligands including bile acid, heme fatty acids, steroids and peptides has been proposed to serve the same function as albumin in the blood stream (Litwack *et al.*, 1971; Tipping *et al.*, 1978; Danielson and Mannervik, 1985). Alpha class GST efficiently bind bilirubin (Soltes *et al.*, 1989), while members of GST Mu class have been shown to bind heme and take part in the transport of heme from mitochondria to the endoplasmatic reticulum (Senjo *et al.*, 1985).

The regulatory function of GSTs

The most recent studies on GSTs have demonstrated that GST P1-1 interacts with c-Jun N-terminal kinase 1 (JNK1) suppressing the basal kinase activity (Adler *et al.*, 1999). The K_d for this protein-protein interaction has been found to be in the nanomolar range (Wang *et al.*, 2001). Introduction of GST P1-1 elicits protection and increased cell survival when the cells are exposed to H_2O_2 (Yin *et al.*, 2000). However, GST P1-1 does not elicit the same protection against UV-induced apoptosis (Adler *et al.*, 1999). Furthermore, mouse GST M1-1 has been shown to bind to apoptosis signal-regulating kinase 1 (ASK1) and inhibit its kinase activity (Cho *et al.*, 2001). In contrast to GST P1-1, mouse GST M1-1 seems to protect cells against both UV-and H_2O_2-induced cell death. Moreover, mouse GST A4-4 has also been proposed to interact with JNK and prevent cells from 4-hydroxynonenal induced apoptosis (Cheng *et al.*, 2001).

GSTs, pain or gain?

Many studies have established a link between cancer susceptibility and GST deficiency (Seidegård *et al.*, 1986; Moisio *et al.*, 1998; Salagovic *et al.*, 1999). For example the GSTM1null variant is associated with higher risk of developing proximal colon cancer and the GSTT1 null allele with colorectal cancer (Hadfield *et al.*, 2001; Rebbeck, 1997). On the other hand, GST P1-1 is highly expressed in rapidly proliferating cells such as embryonic cells and many tumor cells (Kantor *et al.*, 1991; McKay *et al.*, 1993; Wang *et al.*, 1997). The high expression level of GST P1-1 could be explained by the more recently discovered regulatory function of GST P1-1 (Adler *et al.*, 1999). Interaction between GST P1-1 and JNK1 seems to have a growth regulatory function. However, the high expression of GST P1-1 may also be a therapeutic obstacle in cancer treatment, since it catalyzes inactivation of alkylating agents (Chen *et al.*, 1994; Wang *et al.*, 1985).

Beside involvement in cancer diseases, GSTs are associated with protection against neurodegenerative disorders. Parkinson's disease and schizophrenia are believed to be the result of degeneration of dopaminergic neurons, which is partly caused by reactive oxygen species (ROS), generated in the redox cycling of *ortho*-quinones (Baez *et al.*, 1997). GST M2-2 has been proposed to protect neurons by catalyzing the conjugation of GSH to aminochrome.

Furthermore, in other diseases such as multiple sclerosis (MS), GSTs might influence in the degree of severity of MS by protecting the cells from ROS (Mann *et al.*, 2000).

PRESENT INVESTIGATION

Screening for recombinant glutathione transferases active with monochlorobimane (Paper I)

In protein engineering several combinatorial methods have been developed to alter the function of a protein (Stemmer, 1994). Regardless of which method is used to obtain combinatorial protein libraries, the selection or screening method is very critical to achieve the goal of making new proteins with novel properties. In other words it is of great importance that within a reasonable period of time, as many mutated variants as possible can be examined for the new property. GSTs are great scaffolds from a protein redesign point of view. Even Mother Nature has used GST structures for multiple purposes, from structural protein in the eye lens of octopus (Chuang *et al.*, 1999) to enzymes with different catalytical properties (Mannervik, 1985) to binding proteins ("ligandin") (Litwack *et al.*, 1971) and even proteins that are involved in gene regulatory processes (Adler *et al.*, 1999). Therefore, good screening methods are necessary to evolve new GST functions *in vitro*.

In paper I the ability of GSTs to catalyze the addition of GSH to monochlorobimane, MCB (Fig.6), which results in a fluorescent product, was used to develop a colony assay to identify active GST mutants in combinatorial libraries. This conjugation reaction is catalyzed by many cytosolic GSTs, demonstrated in paper I. The conjugate (GS-bimane) is fluorescent under long wavelength UV light. Halogenated bimanes have been used to quantitatively determine GSH levels in cells (Rice *et al.*, 1986) and to measure GST activity (Hulbert *et al.*, 1983).

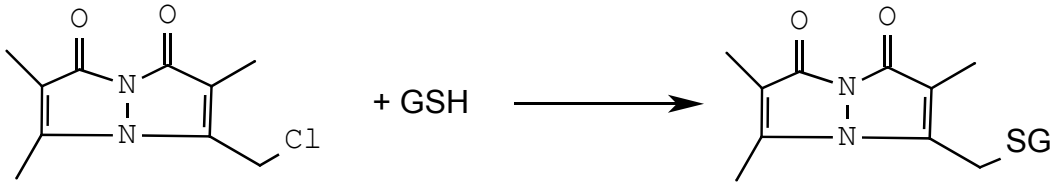

Figure 6. Conversion of non-fluorescent MCB to fluorescent glutathione adduct catalyzed by GST.

The screening was conducted by spraying MCB, dissolved in acetonitrile, on bacterial colonies grown on agar plates. The colonies were then exposed to long wavelength UV light. The bacteria containing active GST immediately give

fluorescence, which lasts for 20 min. Furthermore, this method was applied to a mixture of the bacterial colonies that contained the catalytically inactive P1 monomer (Abdalla, A.-M, Bruns, C. M., Tainer, J. A., Mannervik, B., and Stenberg, G.) and the active dimeric enzyme GST P1-1. Using MCB screening, it was possible to distinguish one colony containing active GST P1-1 out of at least 1000 inactive colonies, which demonstrate the sensitivity of the method.

As an example of application, a library (T1/T2) (Broo *et al.* 2002) consisting of shuffled DNA encoding GST T1-1 (which is catalytically inactive with MCB) and GST T2-2 (which catalyze GSH conjugation to MCB) was used to identify mutants with MCB activity. All of the fluorescent colonies displayed detectable MCB activity in lysates. However, only 70% of the same lysates showed activity with 1-menaphthyl sulfate (MS), which is a substrate for wild-type GST T2-2. This shows that the T1/T2 library is diverse and lacks a strict correlation between MCB and MS activities.

In summary, the screening method developed in Paper I is a useful approach to identify and characterize catalytically active GST mutants in a combinatorial library. Another application is in the sub-cloning of DNA encoding GST, where the MCB screening could be used as an alternative to blue-white selection, which is based on β-galactosidase.

Aromatic residues in the dynamic C-terminal region of glutathione transferase A1-1 influence rate-determining steps in the catalytic mechanism (Paper II)

In human GST A1-1 the active site becomes more structured upon the binding of the substrates. In the hydrophobic site the C-terminal part form an α-helix that docks against the bulk of the protein upon the binding of the eletrophilic substrate. Phe 220 is a highly conserved residue in the C-terminal region of alpha class GSTs. Phe 220 both contributes to glutathione binding and interacts with the second substrate. In Paper II, mutants F220A, F220T were shown to decrease the catalytic efficiency with three substrates, 1-chloro-2,4-dinitrobenzene (CDNB), 1-chloro-2-trifluoromethyl-4,6-dinitrobenzene (*o*-CF_3-CDNB) and Δ^5-androstene-3, 17-dione

(AD). An additional mutation in the C-terminal segment of GST A1-1, F220A/F222A, reduced the catalytic activity even more with CDNB and AD.

Furthermore, the rate limiting step in the enzymatic activity of wild-type GST A1-1, and mutants F220A, F220A/F222A was investigated by addressing the effect of viscosogen on kinetic parameters k_{cat} and k_{cat}/K_m with both CDNB and AD. If mutation affects k_{cat}, it suggests that chemical transformation of substrate or the release of the product is rate-limiting (Brouwer and Kirsch, 1982; Hardy and Kirsch, 1984; Fersht, 1999). To distinguish the chemical step from the physical step, the relative steady-state turnover rate constant k^o_{cat} / k_{cat} versus relative solvent viscosity η/η^o should be plotted. If the slope of such graph is 1.0 the physical step is rate-limiting, but if the slope is zero the rate-limiting step is the chemical reaction. In paper II the effect of viscosogen on k_{cat} for CDNB suggests that the physical step is rate-limiting. This result is consistent with the pre-steady state kinetics, earlier published (Gustafsson *et al.*, 2001). Moreover, the viscosogen influenced the isomerization reaction in a similar manner. In conclusion, Phe 220 and Phe 222 are important residues in the C-terminal region of GST A1-1, which influence the rate-detemining steps in catalytic mechanism of substitution and isomerization reactions.

Identification of residues in glutathione transferase driving functional diversification in evolution (Paper III)

There are several approaches from rational to random that areused today to evolve proteins with new catalytic properties (Scrutton *et al.*, 1990; Stemmer, 1994; Zhao *et al.*, 1998). Since adaptation of the active site as well as the overall structure might be needed to accomplish new activities, rational redesign of the active site may not be efficient enough. Combinatorial approaches require large libraries and good selection methods. By understanding the rules of the evolution and applying them for protein redesign the odds of success might be better.

An alternative approach to engineer new enzymes has been presented in paper III. We have shown that by mutating the positions that have been under selection pressure during evolution protein redesign may be achieved easier and more efficient. Positions 104, 130 and 210 were identified as residues that have been driven by positive selection as being important for functional evolution of the Mu class GST.

Theses three residues in GST M2-2 were mutated to change the substrate specificity towards GST M1-1. Positions 104 and 210 are close to the active site while position 130 is far from the active site. A replacement of Thr with a Ser in position 210 elicited 1000-fold improvement in specific activity with *trans*-stilbene oxide (tSO), a GST M1-1 specific substrate. Additional mutations at positions 104 and 130 increased tSO even more.

Probing iomolecular interactions of glutathione transferase GST M2-2 using peptide phage display (Paper IV)

The non-enzymatic binding function of GSTs ("ligandin) was proposed by Litwack et al.(1971) suggesting GSTs to be intracellular carrier proteins that bind hydrophobic compounds. Structural data suggest a third binding pocket where the non-substrate ligands bind (McTigue *et al.*, 1995; Ji *et al.*, 1996). Molecules such as bile acid, heme, steroids, and fatty acids are among the non-substrate ligands of GSTs. In 1997, Kemmer and co-workers reported that rat GST M1-1 binds a cyclolinopeptide A analogue and a linear rennin-inhibiting peptide (Kemmer *et al.*, 1997). Since Mu class GST in rat binds peptides we chose to study the same class of human GSTs. However, human GST M1-1 with its polymorphic gene was not our first choice. Therefore, the peptide binding properties of another human Mu class member, such as GST M2-2, seemed more appealing.

Two random peptide libraries (a 9- and a 15-mer) displayed on phage (Lundin *et al*., 1996) were screened to identify peptides with affinity to GSTM2-2. Phage display is a powerful technology that was first introduced by George Smith in the mid 1980s (Smith, 1985). By fusing DNA encoding a peptide or a protein to the gene III that encodes a minor capsid protein of filamentous bacteriophage M13, it was shown to be possible to express that foreign segment on the surface of the phage. The principle behind phage display is that the phenotype is linked to its genotype. This means that, in our case, every displayed peptide has an addressable tag via the DNA encoding a peptide carried by the phage. Based on the original approach introduced by Smith two peptide libraries were constructed. The peptide libraries were tested for binding using a selection procedure that is commonly known as biopanning (Figure 7).

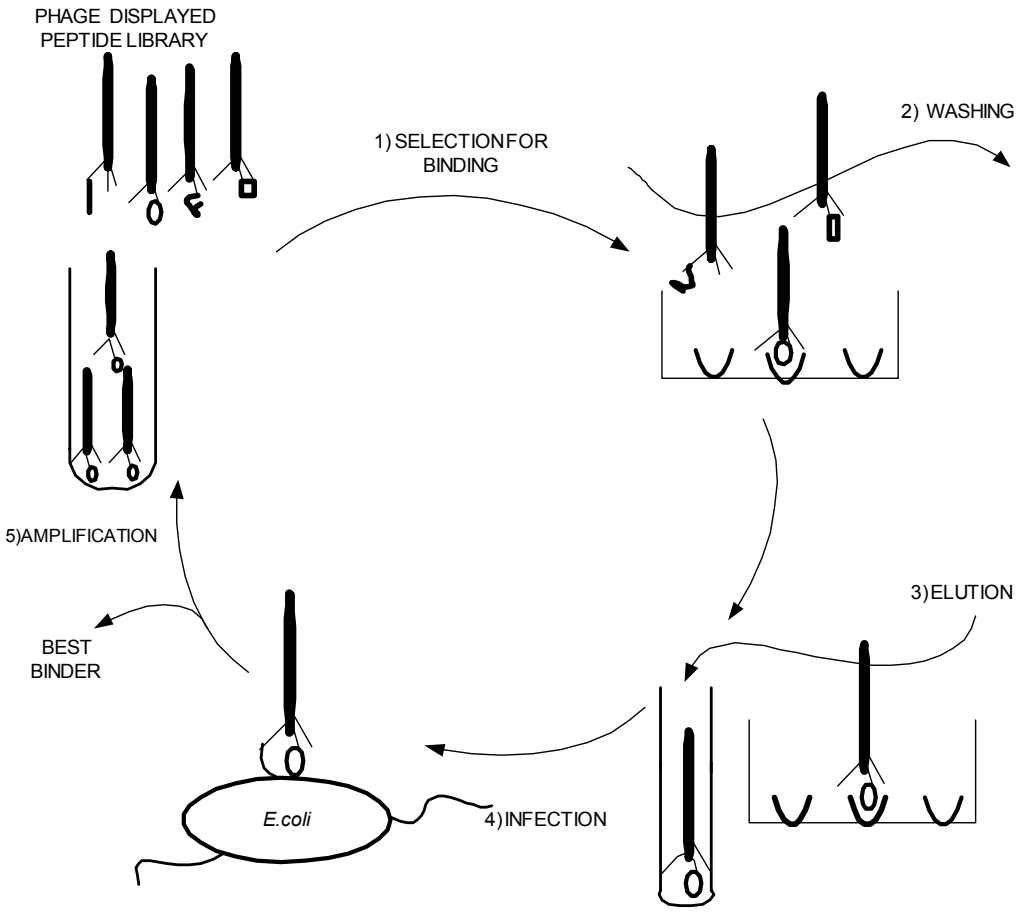

Figure 7. Principle for biopanning procedure.

One peptide (Pm1: GVVRGPSRG) was enriched after three rounds of panning, which indicates that GST M2-2 binds that particular peptide sequence. Competitive binding studies with the phage clone Pm1 and GST M2-2 confirmed the specific binding of GST M2-2 to the peptide sequence Pm1. GST P1-1 and GST A1-1 were also investigated for peptide binding properties. We found two different peptide sequences binding to GST A1-1 and GST P1-1 respectively (results not published). These results suggests that multiple GSTs display different binding specificity.

Interestingly, the peptide showed strong similarity to the C-terminal regions of c-Jun N-terminal kinases (JNKs) and Bcl3 (Table 2).

Table 2. Protein sequences with similarities to GST M2-2-binding peptides identified in the Swiss Prot data base

Phage clones	Query	Hits	Human protein	Recognized site (amino acid residues)
Pm1	GVVRG-PSR-GA	GVIRGQPSPLGA	JNK1	408 - 420
Pm1	GVVRG-PSRGA	GVVKDQPSDA	JNK2	408 - 417
Pm1	GVVRG-PSR-GA	GVVKGQPSPSGA	JNK3	408 – 419
Pm1	AGVVRGPSRGA	AGVLRGPGR	Bcl3	427-435

Activating protein 1 (AP-1) are dimeric transcription factors composed of various members of the Jun and Fos families. AP-1 has been shown to be important in regulating cell proliferation and apoptotic processes in response to cellular stress. AP-1 transcriptional activity is in part determined by phosphorylation of c-Jun by JNKs, which are preferentially activated by a number of environmental and cellular stimuli such as UV irradiation, cytokines, and chemicals, *e.g.* phorbol 12-myristate 13-acetate, PMA (Ip and Davis, 1998; Adler *et al*., 1992). JNKs exist as three isoforms, JNK1, JNK2 and JNK3. Interestingly, JNK3 and GST M2-2 are expressed in the same tissues (brain, heart and testis) (Gupta *et al.,* 1996; Rowe *et al.*, 1997), which suggests that the regulatory function of GST M2-2 could be highly tissue specific.

Reporter gene assays were conducted in two different cell lines (human embryonic kidney 293 cell line and human embryonic retinoblast 911 cells) in order to show a biological effect of the interaction of GST M2-2 with JNKs. In both cell lines it was shown that the presence of GST M2-2 coactivates transcriptional activity of AP-1 upon stimulation with PMA.

Furthermore, the transcriptional consequence of a possible interaction between GST M2-2 and Bcl3 was also investigated. Bcl3 is a nuclear protein that was first discovered as a highly expressed protein in B-cell chronic lymphocytic leukemias (McKeithan *et al.*, 1997). Moreover, Bcl3 has been shown to coactivate AP-1 and retinoid X receptor, RXR (Na *et al.*, 1998; Na *et al.*, 1999). Our transcriptional

studies however, showed that GST M2-2 has no significant effect on Bcl3 coactivation of AP-1.

In summary, phage display is a powerful tool in functional genomics research by providing the potential to identify protein-protein interactions, such as those involving GSTs and JNKs. In paper IV using peptide phage display, a peptide sequence was identified to bind to GST M2-2. Furthermore, the identified peptide was shown to be highly similar to the C-terminal portion of JNKs indicating a regulatory function that agrees with earlier reports (Adler *et al.*, 1999; Cheng *et al.*, 2001; Cho *et al.*, 2001). However, it appears that GST M2-2 in contrast to GST P1-1, coactivates AP-1 transcription, which suggests that different GSTs display different regulatory functions.

Glutathione transferases and inflammation (Papers V and VI)

The majority of the medically related research on GSTs has been focused on cancer and chemotherapeutic drug resistance (2667 hits for "GST and Cancer" search in PubMed). However, the role of GSTs in inflammatory processes in general and in inflammatory bowel diseases (IBD) in particular has hardly been investigated at all (only 9 hits for "glutathione transferase and IBD" search in PubMed). Two papers presented here address the gastrointestinal distribution of different GST isoenzymes in an induced inflammation mouse model as well as in patients with Crohn's disease.

GSTs are generally considered as an important component of the cellular defense against oxidative stress. However, some GSTs display another interesting property, which is catalyzing the conjugation of GSH to prostaglandins (PG). PGs are postulated as natural ligands for the peroxisome proliferators-activated receptors (PPARs) (Forman *et al.*, 1995). PPARs are nuclear receptors that are believed to act in anti-inflammatory processes. One could speculate that some GSTs might be functioning in anti-inflammatory processes by being involved in synthesis of PG. Meanwhile, some other GSTs might be part of pro-inflammatroy processes by modifying the same molecule and compete with PPARs for their ligands.

Inflammatory Bowel Disease (IBD)

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders that collectively are called IBD. CD can be seen anywhere in the gastrointestinal tract. In contrast UC is restricted to the inflammation in the large intestine. There is probably no simple cause accounting for developing IBD. A combination of genetic susceptibility and environmental factors is most likely the cause of both UC and CD.

Although CD and UC are genetically related, the investigation on monozygotic twin pairs shows that the genetic factors are more important in Crohn's disease (Orholm *et al.*, 2000; Tysk *et al.*, 1988). In healthy intestine the mucosa is in an inflammatory state that is considered normal and which do not progress to disease. The immune system of intestinal mucosa is normally balancing the coexistence with the commensal intestinal bacterial flora. However, it is believed that in IBD patients an exaggerated T-cell response causes hyper-responsiveness to commensal bacteria (Sartor *et al.*, 1997). Therefore, alteration of the intestinal flora using antibiotics

together with probiotic treatment has been proposed as an alternative therapy (Shanahan, 2000; Marteau, 2001)

Smoking is a factor that affects the two forms of IBD differently. There is evidence that nicotine might be beneficial to patients with UC. However, it has an adverse effect on CD patients. The mechanism of the effect of smoking on IBD individuals remains unknown (Lindberg *et al.*, 1988). Knowing that GSTs play a central role in detoxication of harmful substances in tobacco smoke, the expression of GSTs might be crucial for smoking UC patients. However, if GSTs as possible pro-inflammatory enzymes, could be disadvantageous for IBD patients.

Selective expression of detoxifying glutathione transferases in mice: effect of experimental colitis and the presence of bacteria (Paper V)

In paper V, the role of GSTs as an important part of cellular defense in inflammation was investigated. The expression of GST Alpha, GST Mu and GST Pi in general, and GST A4-4 specifically, was immunohistochemically studied in mice colon using specific antibodies raised against human GST A1-1, GST M2-2 and GST P1-1 as well as specific antibodies against mouse GST A4-4.

Dextran sulfate sodium (DSS) and carageenan (CAR) induce inflammation in the large intestine, which resembles chronic intestinal inflammation in human (Axelsson, 1999). DSS induced colitis as an experimental animal model was first reported 1990 (Okayasu et al., 1990). Today, it is frequently used to study inflammatory mechanisms (Axelsson *et al.*, 1996) and for IBD therapy (Spiik et al. 2002; Axelsson et al., 1996). CAR is a natural compound extracted from red seaweeds, which also induces colitis in animals. Both DSS and CAR are sulfated polysaccharides with different backbones. While DSS is made of glucose, CAR is a polygalactose. However, the sizes of the polysaccharides as well as the number sulfate groups are important for induction of colitis (Axelsson and Midtvedt, 1997). It should be noted that CAR has been shown to cause a more extensive inflammation in germ-free animals (Hirono *et al.*, 1981). Interestingly, CAR is used as a thickening and stabilizing agent in ice cream and toothpaste (Weiner, 1988; WHO 1984). So is there any danger to eat ice cream? The answer is that the CAR used in food is believed to be harmless because it has low grade of sulfation and high molecular weight. However, there are very few studies conducted on this subject.

In Paper V, the expression of GSTs in the colon of: 1) conventional and germ-free mice with and without experimental induced colitis; 2) germ-free mice exposed intestinal bacterial flora for 4, 8, 18 and 34 days; and 3) germ-free mice treated with a probiotic, *Lactobacillus* GG,

In agreement with an earlier report (Clapper *et al.*, 1999), the expression of GST Mu and GST Pi in epithelial cells of conventional mice was reduced in inflamed tissue sections (Table 3). In contrast to the investigation of Clapper and co-workers, we surprisingly found an induction of epithelial GST Alpha in DSS treated mice (Table 3). Moreover, the most striking expression feature was shown by epithelial mGST A4-4. The mGST A4-4 was almost undetectable in healthy conventional, as well as, germ-free mice. However, this enzyme was induced upon DSS treatment in conventional mice, and in germ-free mice with more extensive colitis induced by CAR (Table 3).

Table 3. The expression level of glutathione transferases in epithelial cells in colon of mice

Enzymes	Conventional mice	DSS-treated conventional mice	GF mice	DSS-treated GF mice	CAR-treated GF mice
GST Alpha	+	↑	+	↑	↑
GST A4-4	b	↑	b[1]	b[1]	↑
GST Mu	+	↓	+	↓	-
GST Pi	+	↓	+	-	↑

The expression of the GSTs in the biopsies was determined visually by light microscopy after immunostaining. b baseline expression.[1] The staining is less than base line.↑ Increasing expression compared to control animals.↓ Decreasing expression compared to control animals.- No change of expression compared control animals.

Another aspect of paper V was to investigate the influence of bacteria on the expression of GSTs in mice colon. When the germ-free-mice received normal flora, the epithelial GST expression pattern appears to change in a similar manner as colitis is developed (Tables 3 and 4). In other words, the expression of GST Mu and Pi was reduced. In contrast, mGSTA4-4 was induced after 8 days until 18 days of exposure to bacterial, suggesting that these enzymes could be involved in transformation of bacterial products. However, the expression of mGST A4-4 was back to base line level after 34 days, which could mean that the physiological balance is in order and

the enzyme is not needed anymore. Interestingly, all the GSTs studied in paper V were induced in the epithelium when the germ-free mice were associated with *Lactobacillus* strain GG (Table 4).

Table 4. The expression of GSTs in epithelial colon of germ-free mice associated with bacteria.

Enzymes	GF mice	GF mice + Normal bact. Flora (4 days)	GF mice + Normal Flora (8 days)	GF mice + Normal bact. Flora (18 days)	GF mice + Normal bact. Flora (34 days)	GF mice + Lactobacillus GG (4 days)
GST Alpha	+	↑	↑	↑	-	↑
GST A4-4	b[1]	b[1]	↑[2]	↑[2]	b[1]	↑
GST Mu	+	↓	↓[3]	↓[3]	-	↑
GST Pi	+	↓	↓[3]	↓[3]	-	↑

The expression GSTs in mice colon tissues was determined visually by light microscopy after immunostaining. ↑ Increasing expression compared to control GF mice.↓ Decreasing expression compared to control GF mice. [2] Increasing expression of mGST A4-4 with time compared with the base line expression. [3] Decreasing GST expression with time compared with the GST expression in GF healthy mice.

In conclusion, the expression of GST in lamina propia, epithelial, goblet, muscle and endothelial cells was investigated. Whether GSTs are acting as cellular defense against oxidative stress caused by inflammation or GSTs act as pro-inflammatory proteins needs further investigation. However, the results in paper V show a change in expression pattern of GSTs in experimental mouse model. Mu and Pi class GSTs seem to decrease, while the level of Alpha class GSTs appears to increase when the mice develop colitis. Moreover, mGST A4-4 is heavily induced upon inflammation, in the epithelium. The GST expression in germ-free mice changes upon exposure to commensal intestinal flora or *Lactobacillus* strain GG.

Distribution of glutathione transferases in the intestine of patients with Crohn's disease

In the final paper in this thesis, the expression of three major GST classes (Alpha, Mu and Pi) was investigated in the intestinal tissues of CD patients by immunohistological analysis. The presence of Alpha, Mu and Pi class GST was studied in lamina propia, epithelial, goblet, muscle, endothelial and macrophage-like cells.

All the three GST classes were expressed in the intestinal luminal border epithelium and macrophage-like cells. The luminal border epithelium needs highly efficient defense system to cope with the toxic compounds they come in contact with, from the food residues or even drugs. Therefore, it is not surprising that intestinal epithelium expresses GSTs. Macrophages were reported to produce increased amounts of free radicals in IBD patients (Mahida *et al.*, 1989; Keshavarzian *et al.*, 1992). In the biopsies, studied in paper VI, macrophage-like cells showed reactivity with anti GSTs antisera, which would be a logical cytoprotective reaction against free radicals.

Earlier report shows a decrease of GST activity with CDNB in colon of patients with UC (Bhaskar *et al.*, 1995). Our results indicate a reduction of Mu class GST and GSTA4-4 in lamina propia of inflamed tissues compared to non-inflamed tissues from the same patients.

A specific anti GST A4-4 antiserum was used in this study. While, goblet cells showed reactivity with anti GST A4-4 antiserum, GSTA1-1 antibody did not stain goblet cells. It should be noted that the observation, which was made in paper V regarding the induction of mGSTA4-4 in inflamed colon, could not be found in biopsies from CD patients.

Earlier reports show that the frequency of GSTM1*B was shown to be lower in CD patients. Since the catalytic activity of the products of GSTM1*B and GSTM1*A are the same, the finding could be interpreted that the GST M1-1 might play a non-enzymatic role, in which GSTM1*B and GSTM1*A products function differently (Duncan *et al.*, 1995). Furthermore, GSTT1 null is more frequent in total UC (Duncan *et al.*, 1995). However, in our investigation no significant difference in GST expression between inflamed and non-inflamed tissues was found. This might be due to the limited number of patients and the fact many patients were treated with different kind of medication, which may influence the expression of GSTs.

In summary, distribution of GSTs in different intestinal cell types in patients with Crohn's disease was mapped. A reduction of GST A4-4 and Mu class GST in lamina propia was observed. Alpha class GST (excluding GST A4-4) were absent in goblet cells in all non-inflamed tissues and almost all inflamed tissues. However, to draw any conclusions about the general expression levels of GSTs in CD patients, analysis on more individuals should be conducted.

CONCLUSIONS

Most of the scientific reports about GSTs address the catalytic function of these proteins. Considering the catalytic efficiency of GSTs, there are very few natural substrates (Baez *et al.*, 1995; Hubatsch *et al.*, 1998; Johansson and Mannervik, 2001). However, the expression level of GSTs (*e.g.* GST A1-1 represent 2-3% of the cytosolic protein in liver cells) is very high. Therefore, it is questionable if the enzymatic function is the only important task of GSTs. Why would nature, which normally produces only essential proteins, waste energy to express lazy enzymes instead of evolving more efficient catalysts? It could simply be that the structure of GSTs is fitted to accomplish many different tasks. Personally, I believe the role GSTs is a diverse and complicated matter, making these proteins an excellent example of why functional genomic research is so important.

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