INACTIVATION OF GLUTATHIONE S TRANSFERASE ZETA BY DICHLOROACETIC ACID

By

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This document is dedicated to my parents, and my husband, for their love and support during these years.

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LIST OF ABBREVIATIONS.

1. DCA: Dichloroacetate.
2. DTT: Dithiothreotal.
3. δ ALA: delta aminolevulinate
4. CFA: Chlorofluoroacetate.
5. ECL: Enhanced chemiluminescence.
6. EDTA: Ethylene diamino tetraacetic acid.
7. EPA: Environment protection agency.
8. ESI: Electrospray ionization.
9. FA: Fumarylacetone.
10. FAA: Fumarylacetoacetate.
11. GC-MS: Gas chromatography – mass spectrometry.
12. GSH: Glutathione.
13. GST; Glutathione S transferase.
14. GSTz: Glutathione S transferase zeta.
15. HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
16. HPLC: High performance liquid chromatogrpahy.
17. LC-MS: Liquid chromatography coupled with mass spectrometry.
18. MAAI: Maleylacetoacetate isomerase.
19. MA: Maleylacetone.

20. MAA: Maleylacetoacetate.

21. MALDI: Matrix assisted laser desorption ionization.

22. MALDI-TOF: Matrix assisted laser desorption ionization-Time of flight.

23. NTBC: 2-(2-Nitro-4-trifluoromethylbenzoyyl)-cyclohexane-1, 3-dione.

24. SA: Succinyl Acetone

25. SDS-PAGE: Sodium dodecyl sulphate- polyacrylamide gel electrophoresis.

26. T-TBS: Tween tris buffered saline.

27. TOF: Time of flight

Abstract of Dissertation Presented to the Graduate School
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INACTIVATION OF GLUTATHIONE S TRANSFERASE ZETA BY DICHLOROACETIC ACID.

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The environmental contaminant dichloroacetate (DCA) is considered hazardous by the US environment protection agency (EPA) but is also an important drug in the clinical management of lactic acidosis. DCA has been shown to be carcinogenic in rats and causes peripheral neuropathy in humans. It is metabolized by glutathione S transferase zeta (GSTz) and inhibits its own metabolism by inactivating GSTz. This aspect of DCA metabolism is also important because GSTz also metabolizes endogenous substrates, namely maleylacetoacetate (MAA) and maleylacetone (MA), which are intermediates in tyrosine catabolism.

The objectives of this research were to study the time course of inactivation and recovery of GSTz following exposure to DCA at both clinical and environmentally relevant doses and to identify possibly adducts of DCA with GSTz. Research presented here also examined the in-vivo role of MA in inactivating GSTz. Experiments conducted showed that DCA completely inactivated GSTz after one

week of exposure and that enzyme recovery was not as rapid as inactivation. It took more than two weeks for the enzyme activity to return to control levels and enzyme expression remained below control levels even after eight weeks of withdrawing DCA treatment. These studies showed that enzyme recovery was slow and that the protein needed to be re-synthesized for activity to be restored. Another important finding was that environmental levels of DCA similar to those present in drinking water inactivated GSTz. Exposure of rats to DCA in drinking water at levels of 2.5 and 250 μg/kg/day significantly inhibited GSTz activity and decreased GSTz expression. Longer duration of treatment had a more prominent effect suggesting a cumulative effect. Adduct studies with recombinant hGSTz 1c-1c showed the presence of adducts of GSTz with glutathione (GSH), and glyoxalate which is the primary metabolite of DCA. Another important finding of this study was that the reactive endogenous substrate for GSTz, maleylacetone, did not inhibit GSTz activity or expression in-vivo. Previous studies with MA have shown that it inhibits GSTz in-vitro but this is the first study, to our knowledge, which showed that it was not an inhibitor of GSTz in-vivo.

CHAPTER 1
INTRODUCTION

Dichloroacetate (DCA) is a haloacetic acid formed in drinking water as a by-product of chlorination. Its concentration in chlorinated drinking water has been reported to be as high as 100μg/L in some samples (Gonzalez-Leon et.al. 1997). DCA is also found in ground water in polluted sites as it is a metabolite of industrial chemicals such as trichloroethylene. Table 1-1 shows the concentration of some haloacetic acids in ground water. DCA is a biotransformation product of some pharmaceuticals such as chloral hydrate (Henderson et.al. 1997). DCA derived from trichloroethylene was found in seminal fluid of human subjects (Forkert et.al. 2002). As well as being an environmental pollutant, it is also an investigational drug used in the treatment of several metabolic disorders. DCA is the only available treatment for congenital lactic acidosis, and lactic acidosis associated with conditions such as diabetes and malaria (Stacpoole et.al. 1989). Daily human exposure ranges from 4μg/kg in drinking water to about 50 mg/kg for therapeutic purpose. Reversible peripheral neuropathy has been observed after prolonged administration of DCA to humans. It also exhibits anxiolytic or sedative effects in adults given repeated doses of DCA (Stacpoole et.al. 1998**b**).

Studies in rodents have shown that DCA is a non-genotoxic carcinogen, (Chang et.al. 1992) and a peroxisome proliferator, (DeAngelo et.al. 1989). It also causes Ras-oncogene activation in B6C3F1 mice (Ferreira-Gonzalez et.al. 1995).

DCA is metabolized primarily in the liver by glutathione S transferase zeta (GSTz) to glyoxylate (James et.al. 1997,Tong et.al. 1998**b**). DCA inhibits its own metabolism after repeated administration due to inactivation of its metabolizing enzyme (James et.al. 1997, Cornett et.al. 1999). The mechanism of this inactivation is not clear, and understanding this mechanism may provide further information about DCA's metabolism.

Table 1-1 Concentration of haloacetic acids formed by chlorination of drinking water (Adapted from Boorman 1999)

Haloacetic acids	Concentration, µg/lt	
	Median	Maximum
Dichloroacetic acid	15	74
Trichloroacetic acid	11	85
Bromochloroacetic acid	3.2	49
Monochloroacetic acid	1.3	5.8
Dibromoacetic acid	<0.5	7.4
Monobromoacetic acid	<0.5	1.7

Pharmacokinetics of DCA

At therapeutic doses, DCA is rapidly and almost completely absorbed orally and peak plasma levels are achieved within 15-30 minutes of administration (Stacpoole et.al. 1998**a**). A very interesting feature of DCA kinetics is that repeated dosing of rats with clinical doses of DCA prolongs its half-life (James et.al. 1998). A similar effect was also demonstrated in human subjects (Curry et.al. 1991). An increase in plasma concentration of the drug and area under plasma concentration-time curve was observed after repeated dose of DCA. Studies also showed that the

total body clearance of DCA was reduced in rats pretreated with DCA (Gonzalez-Leon et.al. 1997). This increase in plasma t1/2 and decrease in clearance of DCA was due to impaired metabolism. Pharmacokinetics of DCA in large animals was found to be different than that in young animals. After two doses of DCA, 50mg/kg, peak plasma concentration in large rats was five fold higher than young animals, and the elimination half-life of DCA was slowed from 5.4 to 9.7 hrs (James et.al. 1998). Schultz et.al. (2002) showed that DCA treatment in drinking water had no effect on the elimination of the drug in 60-week old mice when compared to results obtained from aged matched controls. This report suggests DCA treatment caused no significant difference in the plasma t1/2 of the drug in aged animals whereas, in younger animals (10 weeks old) the t1/2 was significantly longer in animals treated with DCA. Another important finding of this study was that the capacity to excrete DCA in 60-week old control mice was 25 % of that of the 10-week old mice (Schultz et.al. 2002).

Metabolism of DCA

The glutathione conjugation of DCA is thought to proceed via a nucleophilic substitution reaction with the thiol group of glutathione acting as a nucleophile and the chlorine of the haloacid being the leaving group. The known metabolites of DCA (figure1-1) are glyoxylate, glycolate, oxalate, and carbon dioxide (CO2). Fischer 344 rats (180-240 g) given single oral doses of 28.2 or 282 mg of [^{14}C] DCA/kg excreted 25-35% of the dose as CO_2 and 12-35% in urine; 20-36% of the the radioactivity from DCA was recovered in rat tissues (Lin et.al. 1993). The rats that received 282 mg/kg excreted less of the ^{14}C as CO_2 and more in urine, and the percentage of unmetabolized DCA in urine ranged from 0.6% of the dose for the

28.2 mg/kg group to 20% of the dose for the 282 mg/kg group (Lin et.al. 1993). James et.al (1998) showed that 20-44% of the radioactivity from DCA (50mg/kg) was excreted as CO_2 in 24 hours.

Glyoxylate can be routed through several different pathways. It can be converted to oxalate or glycolate by lactate dehydrogenase. It can undergo transamination by aminotransferases to form glycine which can enter several pathways such as conjugation to exogenous carboxylic acids such as benzoic acid. Alternatively glyoxylate can be oxidised to CO_2 by α-ketoglutarate-glyoxylate carboligase. Radioactivity from C^{14} labeled DCA was found in glycine and serine residues of plasma proteins. Glycine conjugates like benzoyl glycine (hippuric acid) and phenylacetyl glycine were identified in the urine of DCA treated rats (James et.al. 1998).

Glutathione S Transferase zeta

GSTz belongs to a family of glutathione (GSH) conjugating phase 2 metabolism enzymes responsible for the detoxification of several xenobiotics. All GSTs exist as either homodimers or heterodimers. These enzymes metabolize electrophilic substrates by catalyzing the attack of sulfhydryl group of glutathione on the electrophilic center of the substrate. The sulfhydryl group of GSH is typically activated by either a serine or tyrosine residue in the GSH binding site of GSTs.

This residue binds to GSH by intermolecular hydrogen bonds and lowers its pKa from 9.3 to 6.5-7.4 (Nieslanik et.al. 1998). GSTz isomerizes maleylacetone (MA) to fumarylacetone (FA), through the nucleophilic attachment of GSH to the β-carbon of the cis double bond in MA.

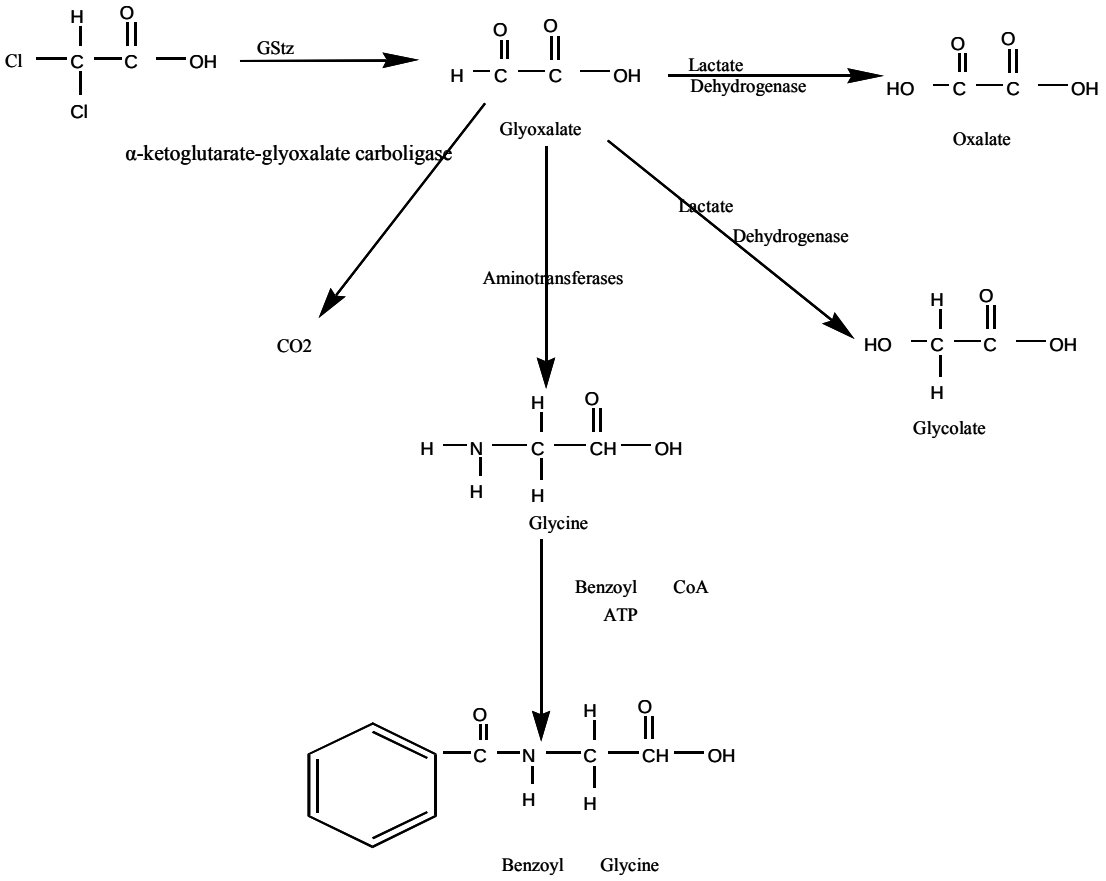


Fig 1-1 Metabolism of Dichloroacetate (James et.al. 1998)

Loss of GSH and reketonization results in the formation of the cis product. In alpha, mu, and pi classes tyrosine activates the GSH whereas in zeta and theta classes GSH is activated by serine. GSTz is a recently recognized addition to the GST family and shares very little similarity to other classes of GST's. It has activity against t-butyl and cumene hydroperoxides, but does not show activity against 1-chloro-2,4-dinitrobenzene, a model substrate used to measure GST activity (Harada et.al. 1987) Common substrates of GSTz1-1 include alpha-beta unsaturated carboxylic compounds such as MA, and alpha halo acids such as DCA and chlorofluoroacetate (CFA) (Table 1-2).

Table 1-2 Specific activities of some substrates of GSTz. Data are presented as means ± SD (n = 3). ND, not detectable. (Adapted from Tong et.al. 1998a)

Substrate	Activity nmoles/min/mg purified protein
bromochloroacetic acid	1411 ± 65
bromofluoroacetic acid	5028 ± 150
chlorofluoroacetic acid	3883 ± 43
dibromoacetic acid	155 ± 4
dichloroacetic acid	1038 ± 20
difluoroacetic acid	ND
fluoroacetic acid	72 ± 16

GSTz is a cytosolic enzyme found in a number of species ranging from Caenorhabditis elegans to rats and humans. GSTz, is identical to maleylacetoacetate isomerase (MAAI), an enzyme in the tyrosine catabolic pathway (Knox et.al. 1955). Its importance in this pathway is demonstrated by the fact that MAAI knock out mice that are given high protein diet have low survival rates (Fernandez-Canon et.al. 2002). These mice also showed renal and hepatic damage suggesting that absence of this enzyme in a natural habitat, where a diet with high protein content is likely, may lead to deleterious effects. Patients with tyrosinemia or other diseases of tyrosine catabolic pathway exhibit liver damage. This has been attributed to the accumulation of reactive tyrosine metabolites such as MA, fumarylacetone (FA) and fumaryacetoacetate (FAA).

MAAI is a dimer with a subunit molecular weight of 25 kDa. The active site in GSTz is more polar and smaller than that in other GSTs. The active site has an electropositive region surrounding it, which attracts negatively charged compounds like maleylacetoacetate (MAA) and DCA. The structure of the enzyme was recently elucidated by Polekhina and co-workers. The crystal structure shows the presence of a sulfate ion in the active site (Polekhina et.al. 2001). GSH and the sulfate ion are

located in a very deep crevice between the C-terminal and the N-terminal domain. The crystal structure also suggests that a carboxylate group in the substrate is essential. DCA is thus a good substrate and orients itself in the active site such that the carboxylate moiety forms a salt bridge with Arg 175 (Polekhina et.al. 2001). This salt bridge optimally orients DCA for GSH attack on the alpha carbon and loss of one of the chlorine atoms. However, beta haloacids are not substrates since these molecules cannot orient properly in the binding site for attack by GSH. Immunohistochemical analysis of tissues from rat showed that GSTz was expressed in hepatocytes and epithelial lining of gastrointestinal tract and other tissues (Lantum et.al. 2002a). Northern blot of human RNA from different tissues showed that GSTz mRNA was present in liver, heart, brain, skeletal muscle and kidneys (Fernandez-Canon et.al 1999).

Four polymorphic variants of human GSTz1-1 have been identified and are designated as 1a-1a, 1b-1b, 1c-1c, and 1d-1d (Blackburn et.al. 2001). The specific activity of each of these variants is different for each substrate. GSTz1a-1a has the highest activity towards DCA. It metabolizes DCA 3.6 times faster than other three polymorphs, but has the lowest Vmax for isomerisation of MA to fumarylacetone (FA) (Blackburn et.al. 2001). There was a 6-fold difference in the isomerase activity of the four isoforms with 1c having the highest isomerase activity. The rate (Vmax) of formation of FA was in the order: 1b-1b ~ 1c-1c > 1a-1a ~1d-1d (Lantum et.al. 2002b). Blackburn et.al (2001) reported that all the polymorphs had similar activity for chlorofluoroacetate (CFA), but Lantum et al (2002) reported that the Vmax of the

variants with CFA as substrate was different. GST1a-1a had the highest Vmax and Km for CFA followed by 1b-1b, 1c-1c and 1d-1d.

Possible Mechanisms of Dechlorination

Several theories exist for probable mechanisms of dechlorination of DCA. The proposed pathway (Anderson et.al. 2002) suggests that the first step involves conjugation with glutathione and loss of one chlorine to form a S-(α-chlorocarboxymethyl glutathione), Fig 1-2, **(1)**. This moiety may then lose the second chlorine to give a zwitterionic intermediate **(2)**, or it could react with the GSTz itself to form an inactive adduct. This glutathione intermediate **(1)** is reactive and may undergo an SN_2 type reaction in the presence of water to form glyoxylate. Another pathway suggests that the zwitterionic intermediate **(2)** itself could also react with the enzyme to form an inactive adduct similar to that suggested in the alternate pathway. The zwitterionic intermediate could also react with water in an SN1 type reaction to form the metabolite glyoxylate.

A study with deuterated DCA (Wempe et.al. 1999), showed that the deuterium label is retained in the glyoxylate formed from DCA. This study proposed a mechanism in which the zwiterrionic intermediate (2) is formed, which then reacts with water, loses the glutathione moiety to form glyoxylate.

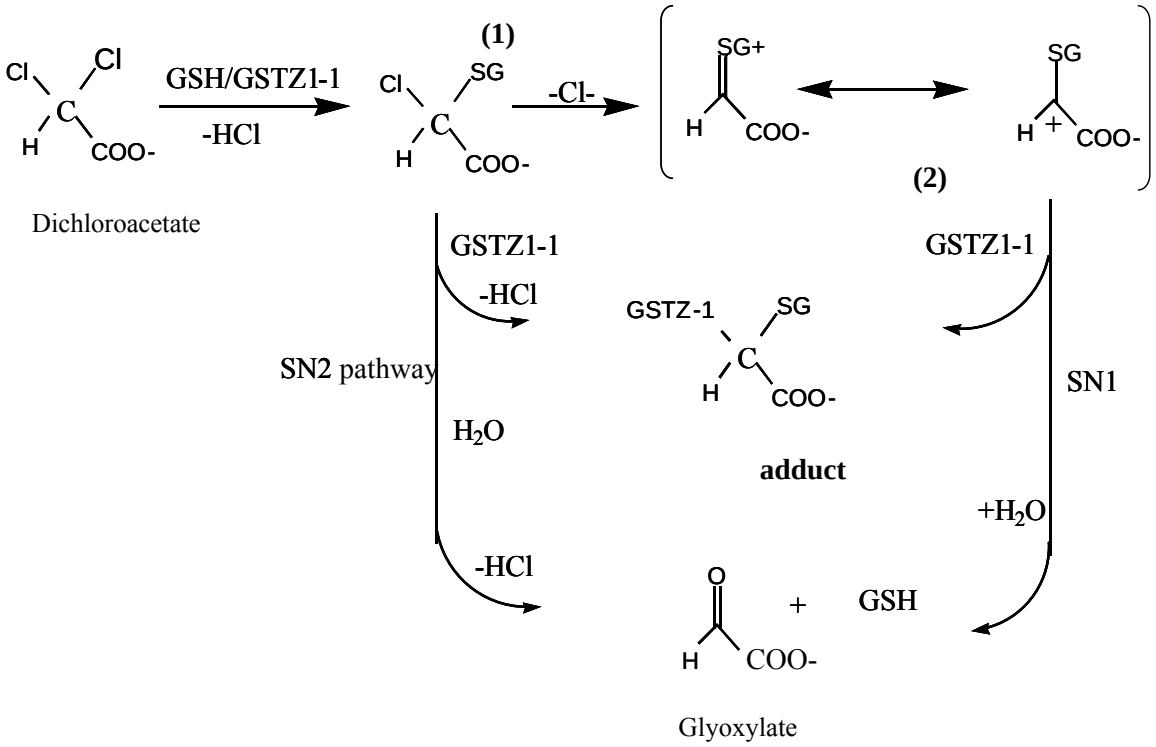


Fig 1-2 Proposed mechanism of dechlorination and inactivation (Adapted from Anderson et.al. 2002).

Inactivation of GSTz

The mechanism of GSTz inactivation by DCA is not entirely clear. Two hypotheses have been postulated for this inactivation. One is that DCA directly inactivates the enzyme by forming inactive adducts with it. The second hypothesis suggests that the enzyme is indirectly inactivated by its physiological substrates maleylacetoacetate or maleyl acetone which may get accumulated due to chronic DCA treatment. Since GSTz is identical to Maleylacetoacetate isomerase (MAAI), an enzyme of tyrosine catabolic pathway, DCA also perturbs tyrosine catabolism (Fig 1-3) by inhibiting this enzyme. MAAI isomerises MAA to fumarylacetoacetate (FAA) and maleylacetone (MA) to fumarylacetone (FA). Inhibition of this enzyme will lead to an accumulation of these physiological substrates, which are known alkylating agents (Seltzer,1973). Cytotoxic effects have been demonstrated for FAA, which

stimulates apoptosis in mouse hepatocytes and human HepG2 cells (Jorquera et.al. 1999). Accumulation of tyrosine metabolites could also be associated with toxic side-effects of DCA. Inhibition of MAAI may cause an accumulation of succinyl acetone (SA) which is an inhibitor of heme biosynthesis. A homozygous knock out mouse for MAAI showed elevated levels of SA in the urine (Lim et.al. 2004). The concentration of SA in the urine of these mice was 1.6 μmol/L and those that were treated with phenylalanine had 4.5 μmol/L. SA inhibits the formation of porphobilinogen from δ aminolevulinate (δ ALA), a key step in heme catabolism. This could lead to accumulation of δ ALA which is a neurotoxin.

Previous in-vitro studies with maleyl acetone have shown that when MA was incubated with human hepatic cytosol (figure 1-4) it inhibited the amount of glyoxylate formed from DCA, in a dose dependent manner (Cornett et.al. 1999).

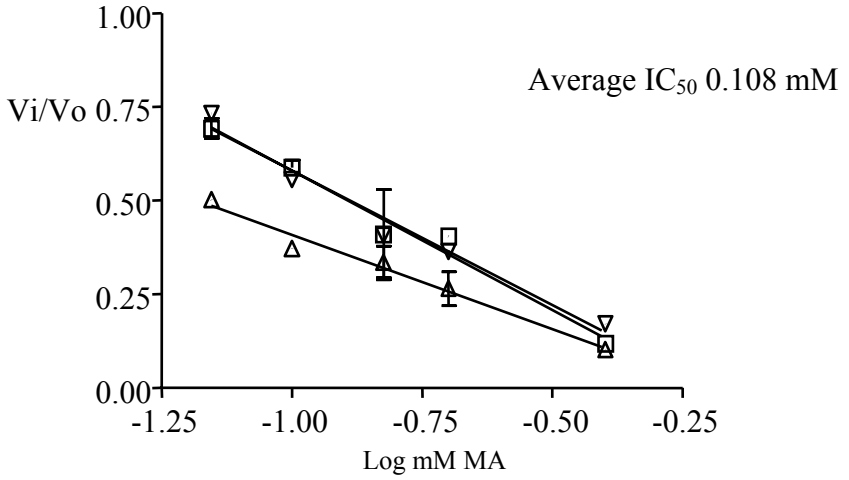


Fig 1-3 In-vitro inhibition of GSTz in human liver cytosol by maleylacetone

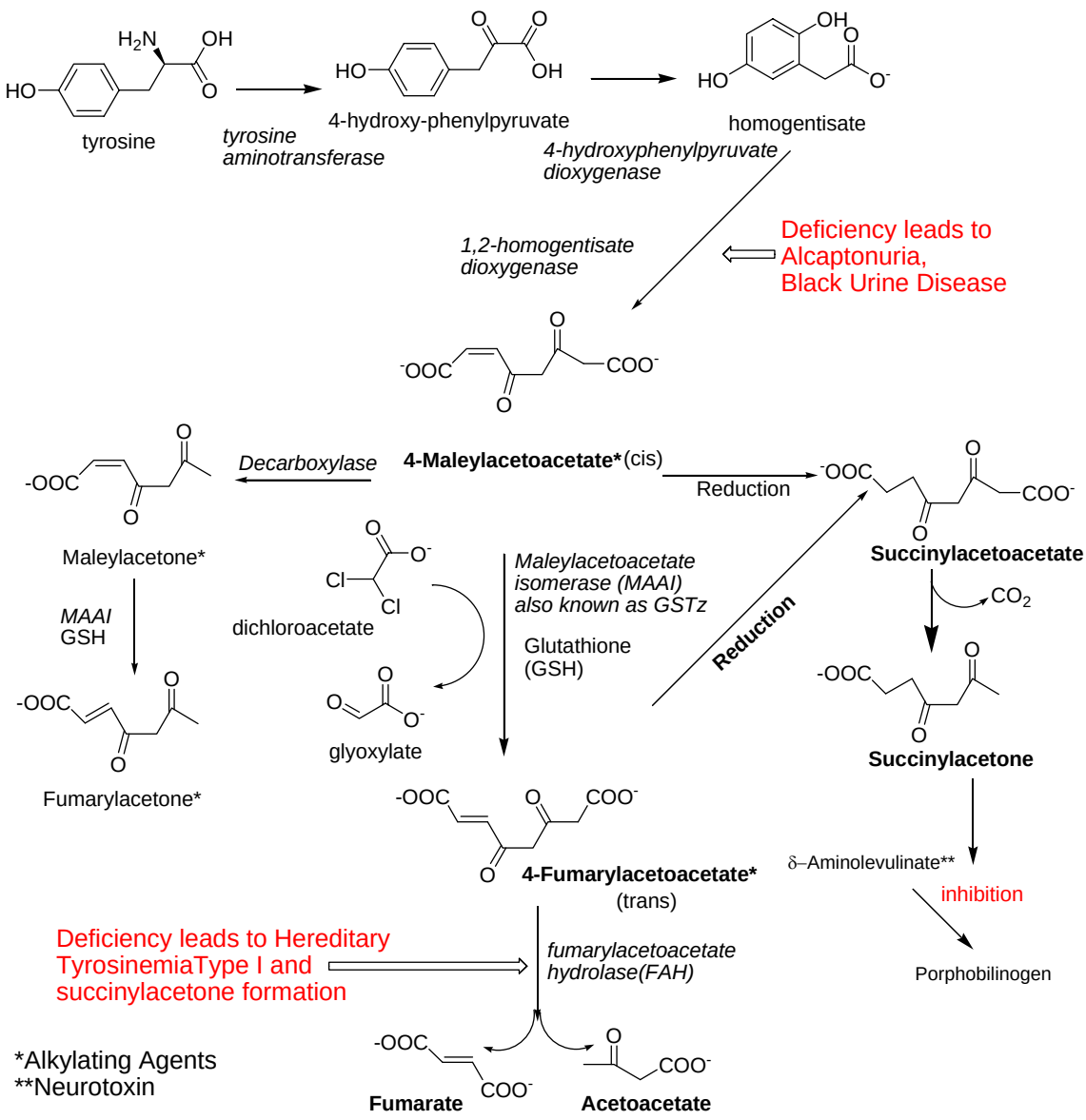
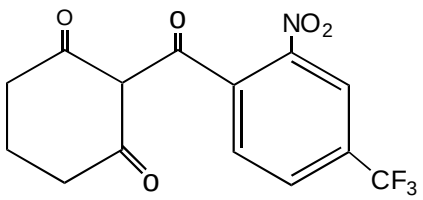


Fig 1-4 Tyrosine catabolic pathway

It was further shown by Ammini et.al. (2003) that activity of the enzyme was only partially restored after extensive dialysis of the protein suggesting reversible and irreversible components.

Using chlorofluoroacetate (CFA) as substrate, Lantum et.al. showed that MA and FA are mixed function inhibitors of GSTz1-1 activity. They further hypothesized that MA and FA are non- mechanism based inactivators of GSTz polymorphs. Mass spectral analysis of GSTz1-1 variants that had been previously incubated with MA and FA showed that MA and FA alkylated the Cys 16 and Cys 205 residues of the enzyme (Lantum et.al. 2002c). A similar experiment with succinylacetone (SA) however, showed that SA did not inhibit the enzyme. Thus an alpha beta unsaturated carbonyl in the substrate appeared to be the essential functionality for inhibition. Various haloacetates were examined for possible inactivation of GSTz. Administration of chlorofluoroacetic acid, bromofluoroacetic acid, 2- chloropropionic acid and difluoroacetic acid had no effect on GSTz activity.

To study the inhibition of GSTz by MAA and MA in-vivo we employed an inhibitor of 4 hydroxyphenyl pyruvate dioxygenase.



2-(2-Nitro-4-trifluoromethylbenzoyl)-cyclohexane-1, 3-dione

This compound 2-(2-Nitro-4-trifluoromethylbenzoyl)-cyclohexane-1, 3-dione (NTBC) prevents the formation and accumulation of tyrosine metabolites (Lock et.al.

1998). Thus pre-administering this compound to rats helped us determine the in-vivo role of MA and MAA in inactivation of GSTz. NTBC has been used in the treatment of hereditary tyrosinemia type I (HTT1). HTT1 is a severe inherited disease caused due to a deficiency of fumarylacetoacetate hydrolase. This deficiency leads to an accumulation of toxic metabolites, MAA, FAA, and SA. These compounds are thought to be the main cause of liver and kidney damage in HTT1 patients. Since NTBC inhibits 4-hydroxyphenyl pyruvate dioxygenase, it halts tyrosine catabolism in its second step and prevents formation or accumulation of the toxic metabolites thought to be responsible for liver disease.

DCA inhibited the activity and expression of its metabolizing enzyme and thus impaired its own metabolism.

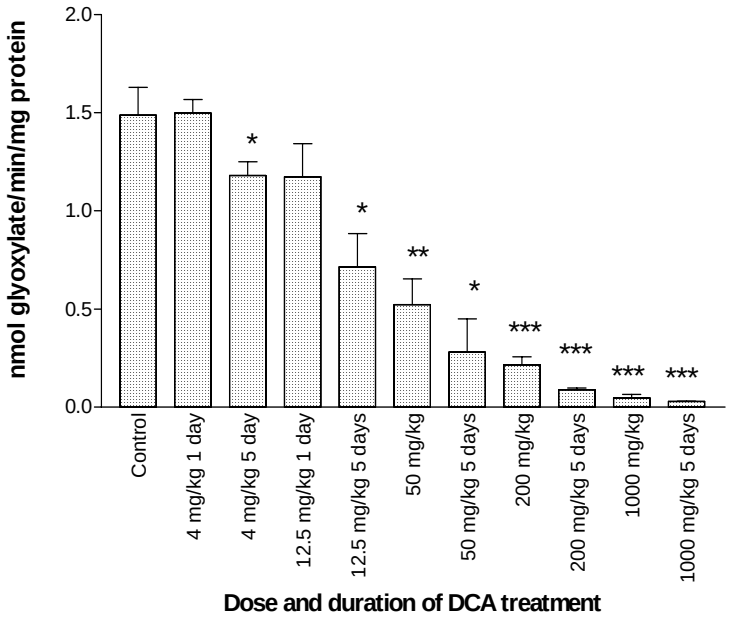


Fig 1-5. Dose dependent inhibition of GSTz specific activity by DCA. *, **, *** indicate that means are significantly different from the control (Reproduced with permission from Cornett et.al. 1999).

The loss in the specific activity of the enzyme was proportional to the dose and duration of treatment (figure 1-5). It was further shown that the amount of immunoreactive GSTz protein (Fig 1-6) in rat liver cytosol was reduced after DCA treatment. Cornett et.al. also studied the in-vitro inactivation of GSTz in rat and liver cytosol by DCA. DCA inactivated rat cytosolic GSTz but did not show the same effect on human cytosol. Later studies by Tzeng et.al. in 2000 showed that different polymorphic variants of the enzyme have different inactivation half lives. The longest half-life was exhibited by 1a-1a (23±1mins) and the other variants had similar half lives (9-10 mins).

Northern blots showed that the steady state levels of mRNA for the protein remained unaltered after a five-day treatment of DCA. Since message was not altered, it appears that DCA does not interfere with the transcription of GSTz. Above data indicate that GSTz was inhibited during its reaction with DCA and that adducts formed with DCA may lead to loss of immunoreactive protein.

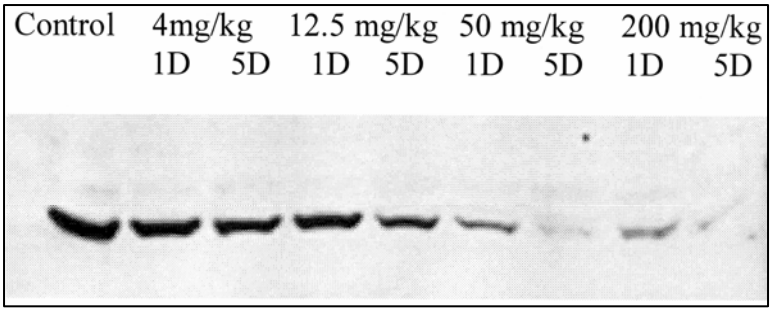


Fig 1-6 Western blot of GSTz protein from DCA treated rats showing a dose dependent decrease in amount of immunoreactive protein. (Reproduced with permission from Ammini et.al. 2003)

Recent mass spectral analysis of GSTz incubated with DCA in the presence of GSH (Anderson et.al. 2002), suggested that DCA,or a reaction intermediate reacts with a single nucleophilic site or residue on GSTz and thus inactivated the enzyme. It

was also proposed in this study that the nucleophilic site on the enzyme, which is covalently modified, is a cysteine-16 residue present in the catalytic site, and that this residue may form a disulphide bond with the thiol moiety in the reaction intermediate (Fig 1-2) . However, recombinant GSTz with a cys-16 deletion mutation was found to retain both, isomerase, and glutathione conjugating properties. This observation indicates that cys-16 residue may be present in the active site but it is not directly involved in catalyzing GSH conjugation. It was further shown (Lantum et.al. 2002c) that this cys-16 mutant showed some resistance to inactivation by MA. But this mutant was not completely immune to inactivation suggesting that there is another residue on the enzyme which may be involved in inactivation.

Table 1-3 Key residues involved in GSTz activity (Adapted from Board et.al. 2003)

Enzyme	Specific activity
	CFA (μmole/min/mg)
hGSTz1c-1c	0.91± 0.31
S14A	0.007± 0.001
S15A	0.025 ± 0.0004
R175A	0.216 ± 0.003
R175K	0.68 ± 0.04

Other residues are also implicated in inactivating the enzyme. Active site serines (position 14 and 15), arginine at 175 and also a cysteine at 205 may be involved in the reaction with DCA. These residues have reactive functional groups which may form adducts with DCA or a metabolite.

Table 1-3 stresses the importance of active site serines for GSTz activity. Mutating these residues dramatically decreased the activity of the enzyme. Replacing

the basic arginine with alanine caused a significant decrease in activity. This decrease was not as profound when arginine is replaced with another basic amino acid, lysine.

Anderson et.al. (2004) studied the reactivity of glyoxylate with nucleophilic amino acids. They found that when glyoxylate derived from the reaction of DCA with GSTz and GSH was allowed to react with nucleophilic residues, it formed adducts with these amino acids. Adducts containing one or two molecules of glyoxylate covalently bound to reactive groups on the amino acids were observed. Glyoxylate also reacted with N terminal amino group of the peptide to form an imine. This adduct had a mass shift of 74 Da from the unmodified peptide. Another peptide containing arginine, tryptophan, and cysteine formed an adduct with 2 molecules of glyoxylate. It was found that cysteine was not involved in the reaction. The exact chemical nature of modification could not be determined due to inadequate mass spectral information. These observations suggested that glyoxylate is a reactive metabolite which may react with amino acids in proteins. Its ability to react with GSTz was not reported.

Inhibition of GSTz by DCA is well studied but the time required for the enzyme to recover its activity after sub-chronic treatment, once the drug is withdrawn, is not known. Since DCA treatment destroys the protein, it is clear that re-synthesis of the enzyme is necessary to regain activity. In an experiment by Tzeng et.al. (2000) it was observed that the activity of recombinant hGSTz 1a-1a that was inactivated by DCA could not be restored by an overnight dialysis of the inactivated protein. This again indicates that synthesis of protein, not removal of the drug alone,

is necessary to recover activity. But the exact time course for its recovery hasn't been completely studied at both environmentally, as well as clinically relevant doses.

Specific Aims

1. **To study the role of maleylacetone in destroying GSTz.**

   Inhibition of GSTz by DCA may lead to accumulation of the endogenous substrates of the enzyme. These substrates, which are reactive, can alkylate the enzyme, thus destroying the protein. The effect of such accumulation on GSTz activity and expression needs to be elucidated. NTBC, an inhibitor of MA and MAA formation is used to study the detrimental effects of these substrates on GSTz. This compound when co-administered could prevent the formation and subsequent accumulation of MA and MAA. The effect of DCA on GSTz can be then studied in the absence of any interference from these substrates. This approach will provide an insight to the in-vivo effects of MAAI's endogenous substrates and their role in destroying the protein.

2. **To study the time course of loss and post-treatment recovery of GSTz following DCA administration.**

   Previous studies have proven that DCA administration results in the loss of activity and expression of GSTz. It is also important to determine the time required for the enzyme to regain its pre-treatment activity and expression after cessation of DCA treatment. This information could prove useful in the treatment and care of patients who are chronically administered the drug. In this work we have determined via in-vivo experiments, the time required for the immunoreactive protein to return to pre-treatment levels and the enzyme to return to pre-treatment activity.

3. **To test the hypothesis that environmental levels of DCA inactivate its metabolizing enzyme, GSTz.**

Dichloroacetate is considered an environmental contaminant by the EPA and is a by-product of chlorination and metabolite of industrial solvents. Since DCA is present in drinking water, it is a ubiquitous environmental pollutant affecting a large populace. Human exposure to DCA occurs chronically at low µg/kg levels. All studies done so far have been with clinical doses of DCA which are several times higher (mg/kg) than the environmental dose. It was important to determine the effect DCA has on GSTz activity at a dose to which majority of people are exposed.

4. **To identify and characterize, by mass spectrometry, the probable adduct(s) of DCA and its metabolites with GSTz.**

We hypothesize that dichloroacetate inhibits the enzyme by reacting with the protein and thus destroys it. The mechanism of inhibition was studied by identifying adducts of the protein with DCA or reaction intermediates. Amino acid residues involved in the reaction with DCA and GSH were identified by mass spectroscopic analysis of the enzyme after its reaction with DCA in the presence of GSH. This experiment helped establish a method to prepare and separate DCA modified GSTz and analyze the same by LC-MS and matrix assisted laser desorption ionisation (MALDI).

CHAPTER 2
MATERIALS AND METHODS

Chemicals

2-(2-Nitro-4-trifluoromethylbenzoyyl)-cyclohexane-1, 3-dione (NTBC) was obtained from Apoteket laboratories, Sweden. C^{14} radiolabeled DCA (specific activity 52mCi/mmole, 99% pure by thin layer chromatography) was purchased from American Radiolabeled Chemicals, St. Louis, MO. C^{14} DCA was converted to its sodium salt by the addition of equimolar sodium hydroxide and was diluted as necessary with unlabelled NaDCA for use in assays. Unlabelled DCA for dosing in rat studies was purchased from TCI America, Portland OR. Glutathione, HEPES, DTT, β-mercaptoethanol, unlabeled sodium dichloroacetate were all purchased from Sigma-Aldrich Chemical Company, St.Louis, MO. Zephyrhills mineral water was purchased from local stores. ECL chemiluminescence detection kit, Hyperfilm ECL, donkey anti-mouse and goat anti-rabbit secondary antibodies were purchased from Amersham Biosciences, Piscataway, NJ. HPLC grade trifluoroacetic acid was bought from Pierce. Methanol, acetonitrile, ammonium bicarbonate, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, potassium chloride, glycerol, sucrose, EDTA, hydrochloric acid, were purchased from Fisher Scientific. In Flow scintillant fluid was purchased from INUS, Tampa, Fl. Ecolume scintillation cocktail was bought from ICN, Costa Mesa CA. Tetrabutylammonium hydrogen sulfate (PICA®) was purchased from Waters Corporation, Franklin, MA.

Polyacrylamide Ready Gels (12 %), high and low range molecular weight markers, G250 coomassie Blue, tris-glycine SDS-PAGE buffers, Biorad mini Protean II Cell, and power supplies were purchased from Biorad, Hercules, CA. All other chemicals were of purest grade available and were bought from commercial suppliers.

Animal Dosing Protocol for NTBC Studies

Male Sprague Dawley rats, weighing between 180-200 gm were used in this study. They were divided into various groups and were weighed each day prior to dosing. Drugs were administered via oral gavage. They were housed in conventional cages, kept on a 12 hour light and dark cycle and given free access to food and water.

Since NTBC is insoluble in water and in ethanol, its solution was prepared by mixing NTBC with water and then adding ammonium hydroxide to the mixture to raise its pH to 10.5. NTBC quickly goes into solution at alkaline pH. The pH was then brought down to about 8.5 by adding hydrochloric acid. Solutions of 1mg/ml and 4mg/ml NTBC were prepared in this alkaline medium. Sodium DCA is readily soluble in water and 50mg/ml solution was made in deionized water.

Two preliminary studies were performed in which duration of NTBC treatment was varied. In the first study, 10 male rats were divided into 3 groups. One group (n=4) was given 50mg/kg DCA, 2nd group (n=3) was given NTBC (1mg/kg) for for 5 days and DCA (50mg/kg) along with NTBC for the last two days, 3rd group was given NTBC alone for 5 days. Animals were sacrificed after the last dose and livers were removed. Cytosol from liver was used to assay GSTz activity.

In the second preliminary study 24 male rats were divided into 6 groups with 4 animals in each group. Animals were treated with 50mg/kg NaDCA for either one or five days or were given 4mg/kg NTBC for 15 days. Some animals received NTBC for

15 days and NaDCA (50mg/kg) along with NTBC for the last one or five days of treatment. Study also included an untreated control group. Animals were kept in metabolic cages on the first and last day of treatment to collect urine.

Another study with 54 male animals was performed where a longer exposure to 2 different doses of NTBC was studied. Male Sprague Dawley rats were divided into 9 groups with 6 animals in each group. Animals were given 50mg/Kg NaDCA for either one or five days (N0D1 and N0D5 respectively). Some animals received 14 days of NTBC treatment (1mg/Kg or 4mg/Kg) and then DCA along with NTBC for either one or five days (N1D5 and N4D5). The study also included a no-treatment control (N0D0), 1mg/Kg NTBC control (N1D0) and 4mg/Kg NTBC control (N4D0).

Table 2-1 Dose and duration of NTBC and DCA treatment

	NTBC		
	(14 DAYS)		
DCA (50MG/KG)	0	1	4
0	N0D0	N1D0	N4D0
1 DAY	N0D1	N1D1	N4D1
5 DAYS	N0D5	N1D5	N4D5

Animals were kept in metabolic cages for the collection of urine (24 hours) on the 1st day of treatment, on the last day of NTBC treatment, on the 1st day of DCA treatment and on the final day of treatment. All animals were sacrificed on the last day of study by decapitation; their livers were removed for preparation of microsomes and cytosol.

Animal Dosing Protocol for GSTz Recovery and Low Dose Studies

Male Sprague Dawley rats were kept in individual cages, on a 12 hour light-dark cycle and given free access to food and water. Their initial weight was between

170-200 G. As the study progressed the weight of some animals increased up to 500 gm. Animals were kept in metabolic cages for 24 hours prior to and on the last day of both treatment and recovery, to collect urine. Urine was centrifuged to sediment food or fecal matter and the supernatant was stored at -80^{0}C until analysis.

Since municipal drinking water contains µg/L levels of DCA, mineral water (Zephyrhills) was used as drinking water in low dose, control and post treatment i.e. recovery groups. This prevented the influence of any DCA other than the pre-determined dose. Animals receiving high dose (50mg/kg) of NaDCA were given municipal drinking water as µg/lt levels will not interfere at such high doses. Water consumption and body weight was monitored thrice a week and dose was adjusted based on changes in body weight and amount of water consumed by individual rat. This ensured accurate dosing which was essential, especially in the µg/kg dose groups.

To study time course of inhibition and recovery animals (n=6 per group) were given 50mg/kg/day NaDCA in drinking water for 1, 4, or 8 weeks. Animals were sacrificed at the end of treatment. Alternatively some groups were kept for an additional 1, 4, or 8 weeks after they were taken off the drug. These animals were sacrificed at the end of this recovery period. Each of the above groups had a corresponding untreated control for comparison.

To study the effect of low dose of NaDCA, male Sprague Dawley rats were given either 2.5µg/kg/day or 250µg/kg/day via drinking water for either 4, 8 or 12 weeks (n=6 per group). Enzyme recovery was studied after 8 weeks of treatment following which DCA was withdrawn for either 1 or 8 weeks. These animals were

then sacrificed and livers were used to prepare cytosol and microsomes. About 1-3G of liver from each rat was immediately frozen in liquid nitrogen and stored at -80^{0}C for future use.

Preparation of Cytosol and Microsomes from Livers

The animals were euthanized by placing them in carbon dioxide chambers. Once the animal showed no vital life signs such as breathing and heart beat, it was dissected open and blood was collected from the *vena cava* in a heparinized syringe. Blood samples were centrifuged to separate plasma. Red blood cells were washed with saline twice and stored at –80^{0}C. Livers were removed quickly and placed in ice cold homogenizing buffer (1.15% KCl buffered to pH 7.4 with potassium phosphate). The liver was rinsed twice, patted dry, weighed, and placed in a volume of ice cold buffer equal to 4 times the weight of the liver. It was then minced with scissors and transferred to a homogenizing vessel of Potter-Elvejhem type, and homogenized by motor driven Teflon pestle. The homogenate was poured in Sorvall centrifuge tubes and centrifuged at 13,300 g for 20 min. The supernatant containing cytosol and microsomes was transferred to polycarbonate ultracentrifuge tubes, and centrifuged at 170,000 g for 45 min. The supernatant was cytosol which was stored in aliquots at –80^{0}C. The protein concentrations of cytosol was determined by the method of Lowry et.al. 1951.

In-vitro GSTz Activity in Rat Liver Cytosol

Assays were conducted under saturating conditions of DCA and GSH as described in James et.al. 1997. Reaction mixture consisting of 0.1 M Hepes buffer, pH 7.6, 1 mM glutathione (freshly prepared), 1 mg of rat liver cytosol, and water to make a final volume of 0.25 ml was pre-incubated in a water bath at 37^{0}C for 2 min.

The reaction was started by adding 0.2mM C^{14} labeled NaDCA (11.86 mCi/mmole). The mixture was vortex mixed and incubated for further 10 minutes. The reaction was terminated by adding 0.5 ml of ice cold methanol. Blanks were samples in which reaction was stopped at zero time. The whole mixture was vortex mixed and centrifuged and supernatent was filtered through 0.45μm nylon centrifuge filter to remove particulate matter. The filtrate was then analyzed by HPLC.

HPLC Analysis

Samples from DCA assay were analyzed at room temperature by isocratic reversed phase HPLC (James et.al. 1997). An Isco (model 2350) pump with a manual injection port was used to inject samples into a 50 x 4.6mm Beckman octadecylsilane pre-column coupled to a 5μ, 80Å, 250 x 4.6mm Beckman octadecylsilane analytical column. The mobile phase used was 0.005M-tetrabutylammonium hydrogen sulfate (ion-pair reagent) in 30% methanol and the flow rate was 1ml/min. The eluent was analyzed first by UV detector (Dynamax, UV-1, Rainin instruments) at a wavelength of 220 nm, and then by radiochemical detector (Flo one Beta, Radiomatic instruments).

Western Blots

Liver cytosolic protein (40 μg) or recombinant hGSTz1c-1c (0.5-5 μg) was first denatured by heating at 90^0C for 5 minutes in a 2x SDS-PAGE sample buffer (0.5M tris-HCL, glycerol, 10%w/v SDS, 2-β mercaptoethanol, 0.05% bromophenol blue) and then loaded on 12% polyacrylamide gels. These gels were electrophoresed for 1hour at 200V in Mini Protean apparatus. Then the protein was transferred to a nitrocellulose membrane overnight at 4^0C at 30V.

The membrane was then blocked in 5% non-fat milk in 0.05% Tween-Tris Buffered Saline (T-TBS) at room temperature for 1 hour after which it was rinsed briefly twice with fresh changes of T-TBS, and then once for 15 minutes and twice for 5 minutes. Primary antibody (chicken anti-mouse GSTz) was diluted 1:30,000 in 5% non-fat milk in 0.05% T-TBS. The membrane was then incubated in the primary antibody for 2 hours at room temperature on a rotary shaker. It was then washed as in the first step. The secondary antibody (donkey anti-chicken) was diluted 1:50,000 in 5% non-fat milk in 0.05% T-TBS. The membrane was incubated in the secondary antibody for 1 hour at room temperature on a rotary shaker after which it was washed as before. The membrane was then incubated for 1 minute in the chemiluminescence solution. It was immediately exposed to X-ray film, which was then developed.

Western blots for quantitating GSTz in liver cytosol, had hGSTz 1c-1c as a control or standard for comparison. Same amount of the recombinant protein was loaded on each gel. The blots were developed, scanned and analysed using ScanAnalysis software. Area covered by cytosolic GSTz was compared with the standard hGSTz 1c-1c and the relative amount for each sample was calculated.

GC-MS Analysis of Urine

Tyrosine metabolites in urine from treated and control animals were analysed by A.L Shroads in Dr. Stacpoole's laboratory using a previous published method (Yan et.al. 1997). Briefly, urine samples were methylated by BF_3 (12%) in methanol, extracted into CH_2Cl_2 and analysed on a Hewlett Packard 5972 mass spectrometer. The column used was HP-WAX, 30mm x 0.25mm, 0.15 μm film thickness, helium was used as carrier gas and GC temperatures were 40^0C for 2 min, then to 100^0C at

5^0C per minute, then to 240^0C at 15^0C per minute, and held at 240^0C for 5 min. 2-oxohexanoic acid was used as an internal standard.

Recombinant hGSTz-1c

Recombinant 6X N-terminal His tagged GSTz-1c was expressed in E-coli cells and purified on a nickel affinity column (Qiagen, Valencia, CA) according to the manufacturers instructions. Construct for GSTz-1c and its expression was performed by Dr. Xu Guo in Dr Peter Stacpoole's laboratory. Protein concentration was measured by Biorad protein reagent. The purity of the expressed enzyme was determined by SDS-PAGE followed by western blot. Enzyme activity was determined using DCA as a substrate by the assay described earlier, but with 5 μg of protein.

Purification of Rabbit Anti-Human GSTz 1c-1c Antibody from Anti-Sera.

Rabbit anti-serum was obtained from Cocalico Biologicals (Reamstown, PA), batch # UF447 using recombinant hGSTz 1c-1c as antigen. This anti-serum was then purified using Pierce protein A antibody purification kit. Anti-Sera was diluted 1:1 with binding buffer (provided in the kit, pH 8.0, contains EDTA), applied to the protein A column and allowed to flow through. The column was then washed with the binding buffer at least 4 times or until the washings showed no absorbance at 280nm. The antibody was then eluted from the column with the elution buffer (pH 2.8 contains primary amine), fractions were collected and neutralized with 1M tris buffer pH 8.0. Fractions with the highest absorbance contained the IgG and were pooled. The antibody was then checked for cross reactivity with GSTz by using it as a primary antibody in western blots of liver cytosol or recombinant hGSTz 1c-1c.

Immunoprecipitation of GSTz from Rat Liver Cytosol

Seize® X protein A immunoprecipitation kit from Pierce was used for this procedure. Purified antibody from rabbit anti-sera was first desalted using Pierce desalting column (potassium phosphate buffer, pH 7.6) to remove primary amines which interfere with immunoprecipitation. The antibody was first bound to Protein A by mixing them and incubating for 30 minutes at room temperature. The bound antibody was then crosslinked with protein A using 25µl disuccinimidyl suberate (8mg reconstituted in 80 µl DMSO). After incubating the bound antibody with the cross-linking reagent for 30 minutes excess reagent was washed off thoroughly using wash buffer (0.14 M NaCl, 0.008 M $NaPO_4$, 0.002 M K_2PO_4, and 0.01M KCl, pH 7.4). Rat liver cytosol (2-3ml) was then incubated with the crosslinked antibody overnight at 4^0C. After this incubation protein A was washed to remove unbound antigen. Immunoprecipitated antigen is eluted by washing this protein A with elution buffer (pH 2.8 contains primary amine). The eluted antigen was then analyzed by western blotting.

DCA Modified GSTz-1c

Reaction mixtures consisting of 0.1 M ammonium bicarbonate buffer, pH 7.6, 1 mM glutathione (freshly prepared), 20µg-1mg of purified GSTz and 2-10mM DCA were incubated in eppendorf tubes for 2 hours. At the end of incubation the tubes were placed on ice to stop the reaction. In some experiments, incubation time was varied to increase the amount of modified GSTz formed but never exceeded 24 hours. Blanks were samples devoid of either GSTz, DCA or GSH. The reaction was terminated by placing the tubes on ice.

Binding of DCA to GSTz was studied by incubating 50µg GSTz-1c with 1.8mM C^{14} sodium DCA (12µci) and 1mM GSH in 0.1M ammonium bicarbonate for 24 hours. GSH was replenished once during the reaction. The reaction mixture was then placed in a 3000MW cut-off dialysis cassette (Pierce) and the protein was dialyzed overnight with 500 ml of 25mM ammonium bicarbonate. Dialysis buffer was analyzed by ion-pair HPLC (described before) to determine the amount of glyoxylate and unreacted DCA in the reaction.

Protein was denatured in SDS-PAGE sample buffer and electrophoresed on a 12% polyacrylamide gel for 1 hour at 200V. The gel was dried and autoradiographed overnight on Instant Imager.

To determine the amount of radioactivity bound to GSTz protein, 4µl or 2.5µg of the protein from incubate was placed in a scintillation vial. Ecolume scintillation cocktail was added and the radioactivity was measured by liquid scintillation spectrometry. The dialysis buffer was also counted on liquid scintillation counter to determine the radioactivity not bound to the protein. Background counts were deducted from the total dpm and the corrected values were then used to determine the moles of DCA bound per mole of GSTz and the amount of unbound radioactivity.

HPLC Analysis of Modified GSTz

Incubation mixture containing modified GSTz was analyzed by HPLC on a gradient reversed phase system. The samples were introduced by a manual injection port into a Jupitor 5µ, 300Å, 250 x 4.6mm C18 column. HPLC was controlled by Beckman Gold Nouveau software. The analysis was started at 100% Solvent A (40%acetonitrile with 0.1% trifluoroacetic acid) with a 40 minute linear gradient to

Solvent B (57% acetonitrile with 0.1% trifluoroacetic acid). The conditions were maintained at 100% solvent B for 10 minutes before returning to initial conditions. The eluate was first analyzed by a uv detector (Beckman instruments) at 214 nm and then by a fluorescence detector (Schimadzu) with excitation: 220nm and emission: 300nm. For some experiments fractions of modified and unmodified enzyme were collected. Fractions were then concentrated on a SpeedVac, and kept at 4^0C until further analysis.

Enzyme Digests of hGSTz-1c

GSTz-1c (100μg-1mg) was incubated with 2-10mM DCA, and 1mM GSH, in 0.1M ammonium bicarbonate for 24 hours at 37^0C. GSH was replenished once after 10 hours of incubation. In some reactions, GSTz-1c was incubated with 1mM GSH alone under similar conditions.

Reaction mixtures were then placed in a 3000 MW cut-off filter centrifuge tube (Microcon, Amersham Biosciences) and protein was recovered after 2 concentration dilution cycles using 25mM ammonium bicarbonate, pH 7.6. Protein was digested using trypsin, endoprotease lys-c, or Glu-c. Lyophillized sequencing grade trypsin, lys-c, and Glu-C (Roche molecular biochemicals, IN) were reconstituted in buffers according to manufacturers instructions. Enzyme to hGSTz1c-1c ratio was 1:25. Digestion was done at 37^0C for 18 hours. Digests of GSTz-1c which had not been incubated with GSH or DCA were also prepared. Lyophillized sequencing grade trypsin and lysine (Roche molecular biochemicals, IN) were reconstituted in buffers according to manufacturers instructions. Digests were concentrated with a SpeedVac concentrator and stored at 4^0C until analysis.

MALDI-TOF Analysis of Intact GSTz

Intact samples of unmodified GSTz and GSTz that had been previously incubated with 10mM DCA and 1mM GSH for 24 hours were analyzed by MALDI. Samples were first subjected to dialysis using a dialysis tube with 1kD cutoff membrane against deionized water at 4^0C for 24 hrs with 3 changes of water. These samples were then concentrated on a SpeedVac concentrator to about 5-10 pmol/µl. Samples were then spotted on the MALDI plate to which sinapinic acid (10mg/ml in 5% acetonitrile containing 0.1% trifluoroacetic acid) had been previously applied. After drying the matrix was re-applied.

MALDI-TOF analysis was done on Voyager-DE mass spectrometer (Applied Biosystems, Foster City, CA). MALDI-TOF mass spectroscopy was run in positive and linear mode. Ion extraction delay, grid voltage, and laser intensity were adjusted to achieve optimal resolution depending on sample mass.

Masses of modified and unmodified GSTz were measured to determine mass shift after reaction with DCA.

MALDI-TOF Analysis of Digests

Tryptic peptides derived from modified and unmodified GSTz, were first purified and desalted using C18 ZipTip® (Millipore, Bedford, MA). Samples were loaded on ZipTips that had been equilibrated according to manufacturer's instructions. The ZipTip was washed with 0.1% TFA to remove salts and the peptides were eluted with 50% acetonitrile with 0.1%TFA. Desalted samples were then applied to MALDI plate using α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.1%TFA as the matrix. Samples were analysed on a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). MALDI-TOF

spectrometry was run in positive, delayed extraction and reflectron mode. Ion extraction delay, grid voltage, and laser intensity were adjusted to achieve optimal resolution depending on sample mass.

LC/MS Analysis of Intact GSTz 1c-1c

50µg of GSTz that has been incubated with 10mM DCA, 1mM GSH for 24 hours, 50µg GSTz which had been incubated with 1mM GSH for 24 hours, and GSTz alone were first ultrafiltered as described before. These samples were injected into a Phenomenex Synergi 4µ Hydro-RP 80^0 A pore, 4 µm, 2x150mm column (Torrace, CA) equipped with a C18 guard column (2mmx4mm) which was coupled to a Thermofinnigan (San Jose, CA) LCQ with electrospray ionization (ESI). Proteins were eluted with a linear gradient at a flow rate of 0.15ml/min. The starting mobile phase was, 95% solvent A (0.5% formic acid and 2mM ammonium formate in water) and methanol was introduced in a linear gradient to 50% Solvent B (0.5% formic acid in Methanol) over 15 mins. The amount of methanol was then increased to 95% Solvent B over 80 minutes. Mobile phase was modified, post-column to assist ionization of the analytes. The modifying solution used was 1% formic acid in methanol (20µl/min) and was added via another pump. ESI-MS data was collected over 2 ranges: m/z 100-450 and m/z 440-2000.

LC-MS/MS of Digests.

Digests of GSTz1c-1c which had been incubated with DCA and/or GSH were prepared as described before. These samples were injected into a Phenomenex Synergi 4µ Hydro-RP 80^0A pore, 4 µm, 2x150mm column (Torrace, CA) equipped with a C18 guard column (2mmx4mm) which was coupled to a Thermofinnigan (San Jose, CA) LCQ with electrospray ionization (ESI). Proteins were eluted with a linear

gradient at a flow rate of 0.15ml/min. The gradient was started with 100% Solvent A (0.5% formic acid and 2mM ammonium formate in water) and a linear gradient to 95% Solvent B (0.5% formic acid in Methanol) was run in 80 mins. ESI-MS data was collected over 2 ranges: m/z 100-450 and m/z 440-2000. Data dependent MS/MS was done of the most intense ion in each scan. This data was analysed by Thermofinnigan Xcalibur software (version 1.2). Theoretical protein masses, peptide and MS/MS fragment ion masses were generated using Sequest Browser (Thermofinnigan San Jose, CA), Swiss-Prot and Protein Prospector (http://prospector.ucsf.edu).

CHAPTER 3
RESULTS OF NTBC STUDIES

Preliminary Studies

Tyrosine metabolism was blocked by NTBC to determine the effects of MA and MAA on GSTz activity and expression. Preliminary studies were conducted to determine appropriate dose of NTBC, duration of treatment, and to obtain preliminary information about the effects of NTBC on GSTz. etc.

Results of the short preliminary study described in chapter 2 showed that rats given NTBC for 3 days prior to DCA (2 days) had a higher GSTz activity than animals that received DCA alone (Fig 3-1). But this result was not consistent across the group. GSTz activity in one rat was significantly lower than other 3 in the group (n=4). Another rat had a very high GSTz activity compared to others in the group. Due to these outliers this study was not conclusive and required further investigation.

In the second preliminary study duration of NTBC pre-treatment was increased, to help understand in more detail the effects of NTBC on GSTz inactivation. As expected, GSTz activity of rats treated with DCA alone was significantly different than the untreated control (Fig 3-2). When NTBC was given prior to DCA, there was an increase in GSTz activity compared to its activity in rats given DCA alone. But this increase was not statistically significant due to high standard deviation in group given NTBC and DCA. This was a similar issue observed in the earlier study and hence data from the second study was not considered conclusive.

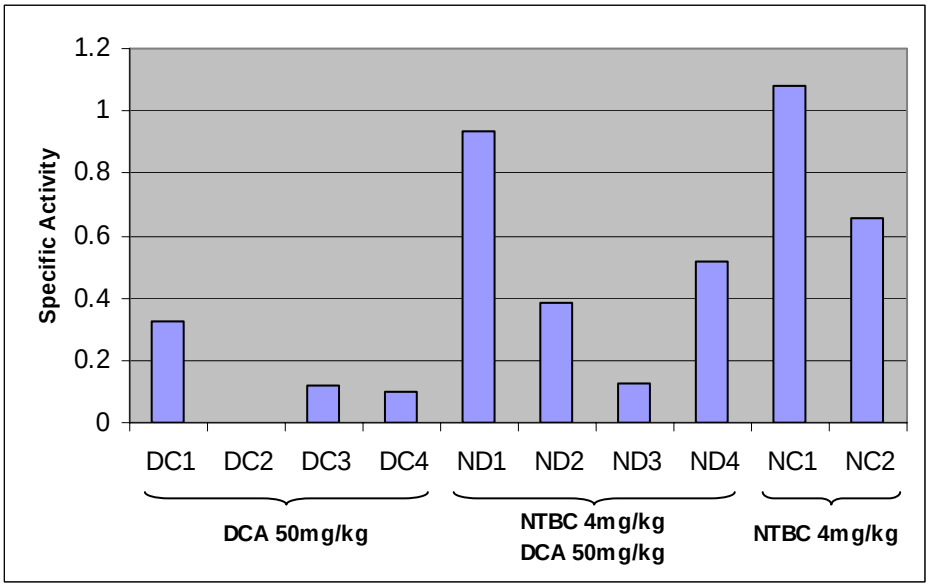


Fig 3-1 Results from first preliminary study. Preliminary study with 10 animals, given DCA (n=4), 50mg/kg shown as DC1-4, NTBC 4mg/kg and DCA (n=4), 50mg/kg shown as ND1-4, NTBC alone (n=2) 4mg/kg shown as NC1-2. Units of specific activity are nmoles/min/mg.

There was no difference between the GSTz activities of rats given DCA alone for one day and rats given NTBC prior to DCA (one day). Since these results did not completely coincide with those obtained from the 1st study, further research in this area was necessary.

Effect of NTBC on GSTz Activity and Expression

A final study was then performed to conclusively determine the effects of NTBC on GSTz activity and expression. In this study a low dose (1mg/kg) and a high dose (4mg/kg) of NTBC were given for 14 days prior to DCA administration. These doses were chosen to study dose dependency of response. GSTz activity was measured by determining the amount of glyoxylate formed during in-vitro metabolism of DCA by GSTz. The results are shown in fig 3-3.

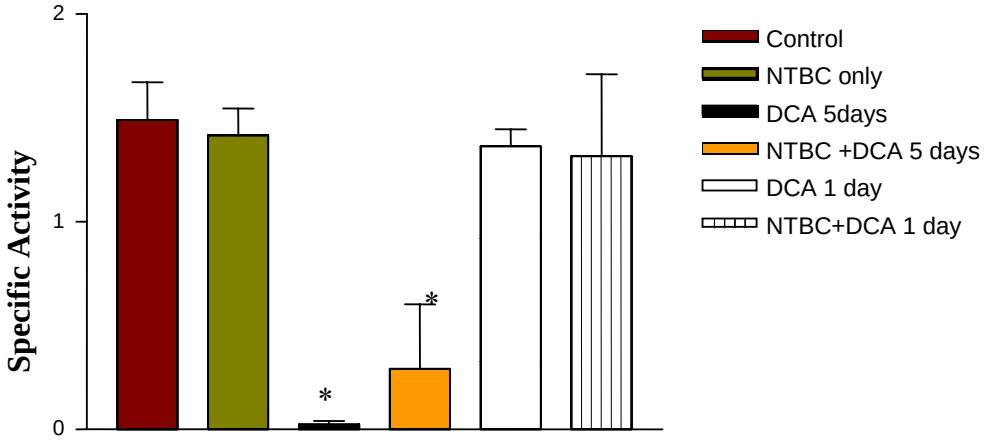


Fig 3-2 Results from second preliminary study. Animals were given 4mg/kg NTBC (15 days) and 50mg/kg DCA (1 or 5 days). n=4, error bars show standard deviation and units of specific activity are nmoles/min/mg. * indicates means are significantly different than controls ($p<0.001$)

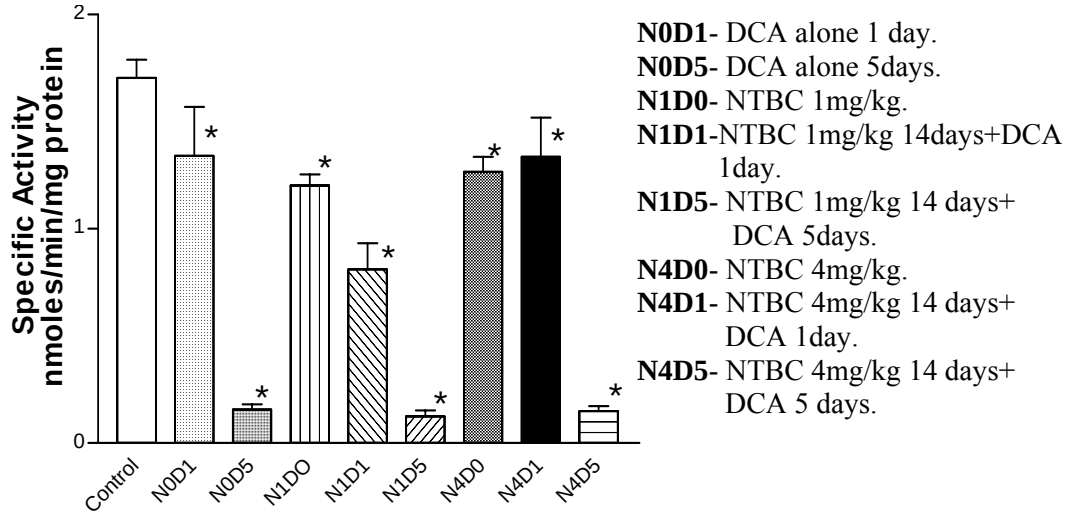


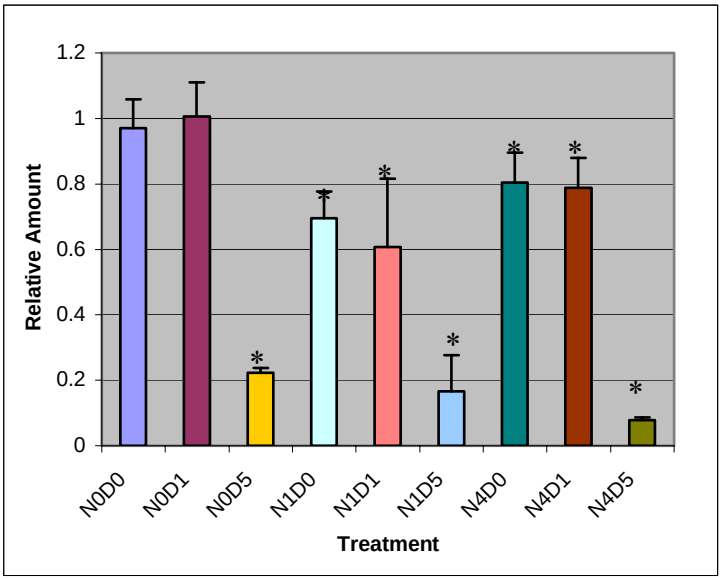
Fig 3-3 Comparison of GSTz activity in NTBC studies. Error bars show standard deviation. n=6, * indicates that means are significantly different from untreated control ($p<0.005$).

DCA treatment in the absence of NTBC i.e. groups N0D1 and N0D5 for 1 and 5 days respectively significantly reduces GSTz activity. Loss of activity was more pronounced after 5 days of treatment, which was consistent with previously published reports. NTBC treatment prior to administering DCA (groups N1D1, N4D1, N1D5

and N4D5) did not have any significant effect on GSTz activity. GSTz activity in N4D5 group was not significantly different than N0D5 group and enzyme activity in N1D5 did not differ from the activity in N0D5 group. But the enzyme showed significantly higher activity in N4D1 group than in N1D1 group suggesting that the higher dose of NTBC was probably the reason for increase in activity.

On the contrary when DCA treatment was increased from 1 day to 5 days there was no such change in activity i.e. N1D5 is not significantly different than N4D5. Interestingly, the NTBC controls (N4D0 and N1D0) showed a significantly lower activity than the no treatment control. Thus it appears that NTBC treatment itself is probably decreasing the activity. Western blots of hepatic cytosol showed that there is no change in the amount of GSTz expressed when NTBC is given prior to DCA treatment compared to GSTz in rats given DCA alone. DCA treatment however reduces the amount of protein expressed in all animals.

Western blots (Fig 3-4) also shows a significant reduction in GSTz expressed in animals treated with NTBC alone. This is in accordance with enzyme activity studies which showed a reduction in activity when rats were treated with NTBC alone. However, this decrease in enzyme expression was not dose related.



N0D0- Control
N0D1- DCA alone 1 day.
N0D5- DCA alone 5days.
N1D0- NTBC 1mg/kg.
N1D1-NTBC 1mg/kg 14days+DCA 1day.
N1D5- NTBC 1mg/kg 14 days+ DCA 5days.
N4D0- NTBC 4mg/kg.
N4D1- NTBC 4mg/kg 14 days+ DCA 1day.
N4D5- NTBC 4mg/kg 14 days+ DCA 5 days.

Fig 3-4 Relative quantification of GSTz from hepatic cytosol by western blot. n=6, error bars indicate standard deviation, * indicate means are significantly different than control i.e. N0D0 ($p<0.005$).

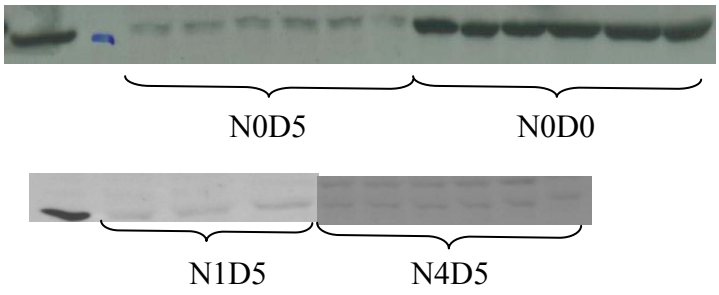


Fig 3-5 Representative western blot of rat liver cytosol. Labels represent 50mg/kg DCA for 5 days (N0D5), control (N0D0), NTBC 1mg/kg + DCA 50mg/kg (N1D5), NTBC 4mg/kg + DCA 50mg/kg (N4D5).

CHAPTER 4
RESULTS OF GSTZ RECOVERY AND LOW DOSE STUDIES

Inactivation and Recovery of Hepatic GSTz

Time course of inactivation and restoration of GSTz activity and expression were studied by analyzing cytosolic GSTz from animals treated sub-chronically with 50mg/kg/d DCA in drinking water.

Data from activity studies shows that GSTz activity is significantly ($p<0.0001$) lost after one week's treatment with 50mg/kg DCA. Over the 8 week treatment period DCA activity remained at very low levels which are significantly lower than corresponding controls. After 8 weeks of treatment animals were taken off the drug to study recovery. Enzyme activity was studied after 1, 2, 4 and 8 weeks of recovery. Figure 4-1 shows that although loss in activity was rapid, the recovery was slow. After 1 week of recovery the activity was significantly lower than the control. Only 54% of control activity was present after one week of withdrawing the drug. Even after 2 weeks of recovery the enzyme activity (84% of matched control) did not reach the same level and was significantly lower ($p<0.05$) than the corresponding control. Enzyme activity was restored to control levels only after 4 weeks of withdrawing the drug.

Western blots of cytosolic protein also showed a loss of immunoreactive protein expressed. Amount of immunoreactive protein from western blots was expressed as amounts relative to 0.01µg of GSTz1c-1c.

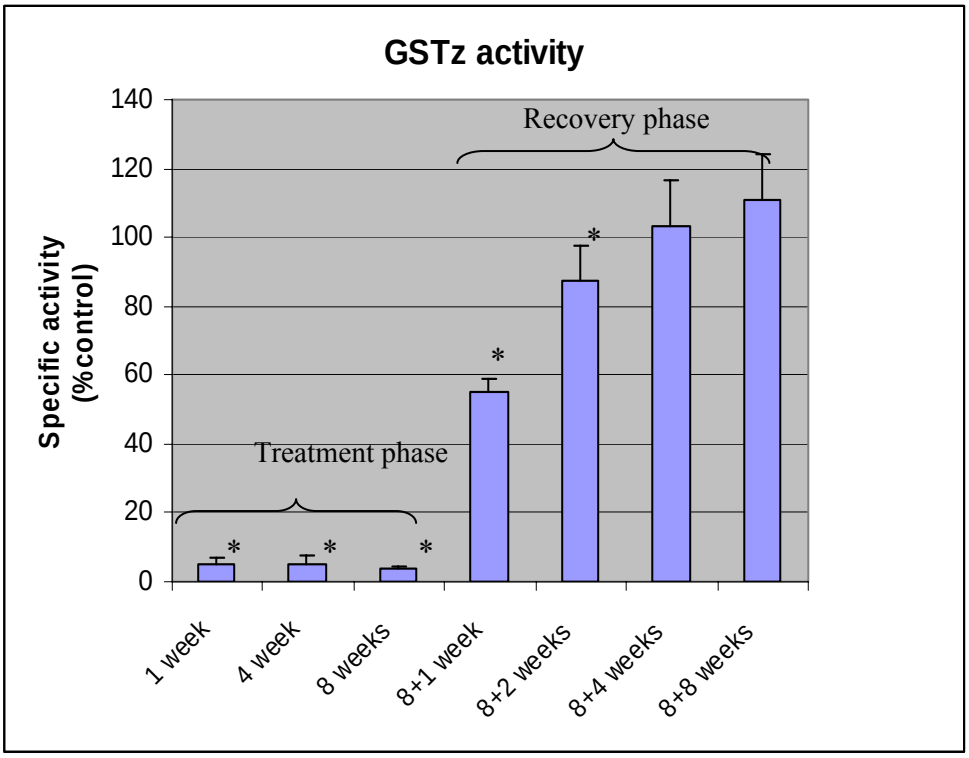


Fig 4-1 Inactivation and recovery of GSTz activity after treatment with 50mg/kg DCA in drinking water. Error bars indicate standard deviation, n=6. * indicate that means are significantly different than control.

One week of treatment with 50mg/kg/day of DCA resulted in 95% decrease in protein expression ($p<0.0001$) and this remained low over 8 week exposure (Fig 4-2). After DCA withdrawal, GSTz expression increased gradually, but unlike enzyme activity, it remained significantly lower than controls after 8 weeks of withdrawing the drug.

Drinking Water Consumption, Liver and Body Weight

Drinking water and body weight of each rat was monitored in order to adjust the amount of DCA given each day accordingly. Water consumption generally did not vary significantly within individuals or treatment groups depending on weight or dose. One exception was the 50mg/kg group treated for one week. This group

consumed 133.5±10 ml/kg/d and the matched control group drank 111.1±9 ml/kg/d. Individual animal was weighed thrice a week in order to maintain an accurate dose over the treatment period. There was no significant difference in the body weight between treatment groups during the course of study. Livers of each animal were weighed and liver to body weight ratio was calculated to determine the change in liver weight due to DCA. This ratio was not significantly changed in animals treated with low doses of the drug. The 50mg/kg/d DCA dose significantly increased the liver to body weight ratio after 8 weeks of treatment when compared to matched untreated control ($p<0.005$). This index returned to control levels 8 weeks after withdrawing the drug.

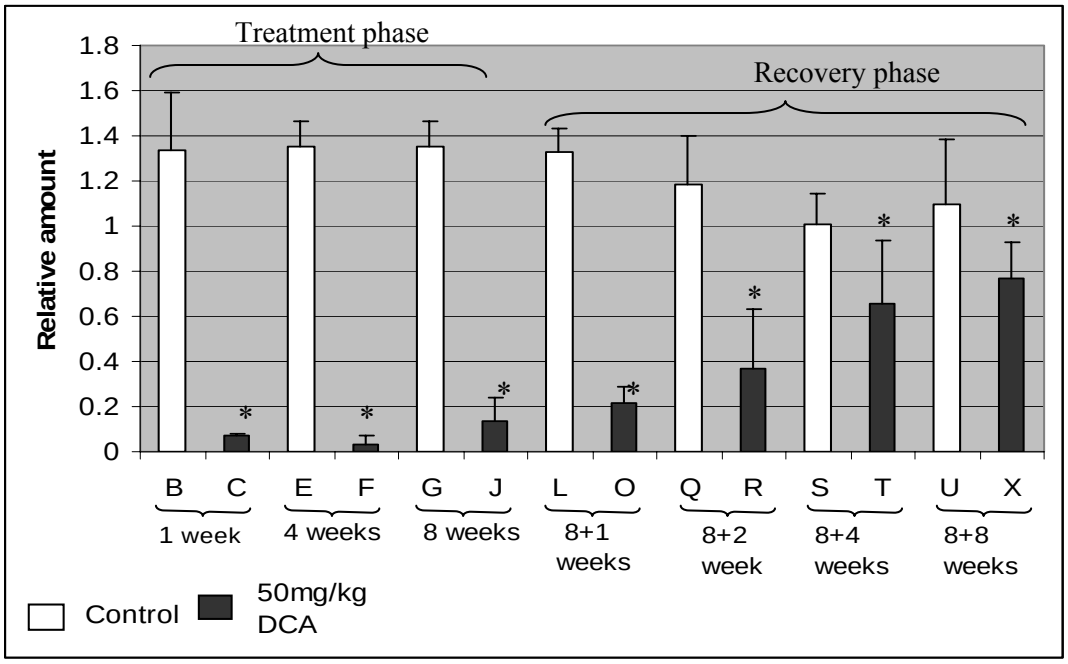


Fig 4-2 Loss and recovery of GSTz protein after treatment with 50mg/kg DCA. Values are expressed relative to 0.01µg of hGSTz 1c-1c. Error bars indicate standard deviation, n=6. * indicate that means are significantly different than control.

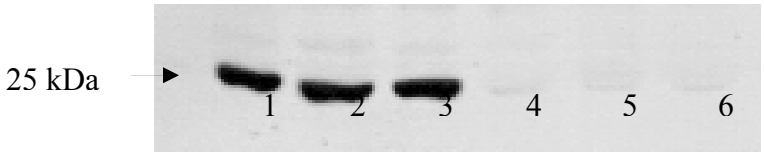


Fig 4-3 Representative Western blot of control and DCA treated (50mg/kg, 1 week) rat liver cytosol. Lanes 1-3 were loaded with control and lanes 4-6 were loaded with DCA treated liver cytosol.

Effect of Low Doses of DCA on Hepatic GSTz

In this study, animals were treated with 2.5 and 250 μg/kg/d of NaDCA for 4, 8 and 12 weeks. GSTz activity and expression was studied after this sub-chronic exposure to environmentally relevant levels of the drug. The specific activity (Fig 4-4) of the enzyme was significantly decreased after 8 weeks of exposure to both 2.5 and 250 μg/kg/d of DCA ($p<0.001$ in both groups). This effect was more pronounced in the 12 week treatment group where there was a suggestion of dose dependent effect. Recovery of activity was studied after the 8 week treatment. After 8 weeks of exposure to 2.5 and 250 μg/kg/d doses GSTz expression decreased 20% ($p<0.05$) and 30% ($p<0.01$) respectively (Fig 4-5). Exposure to these low doses of DCA for 12 weeks also significantly depleted GSTz protein. These observations show that DCA depletes GSTz activity and expression at environmentally relevant concentrations. Recovery of enzyme activity and expression were also studied after 8 weeks of treatment at these low doses. After one week of withdrawing the drug GSTz activity returned to control levels in both 2.5 and 250 μg/kg treatment groups (Fig 4-7). However enzyme expression (Fig 4-8) was not restored after one week of recovery. It returned to control levels in 2.5 μg/kg group after 8 weeks of recovery but remained significantly lower ($p<0.05$) than control in animals treated with 250 μg/kg of DCA.

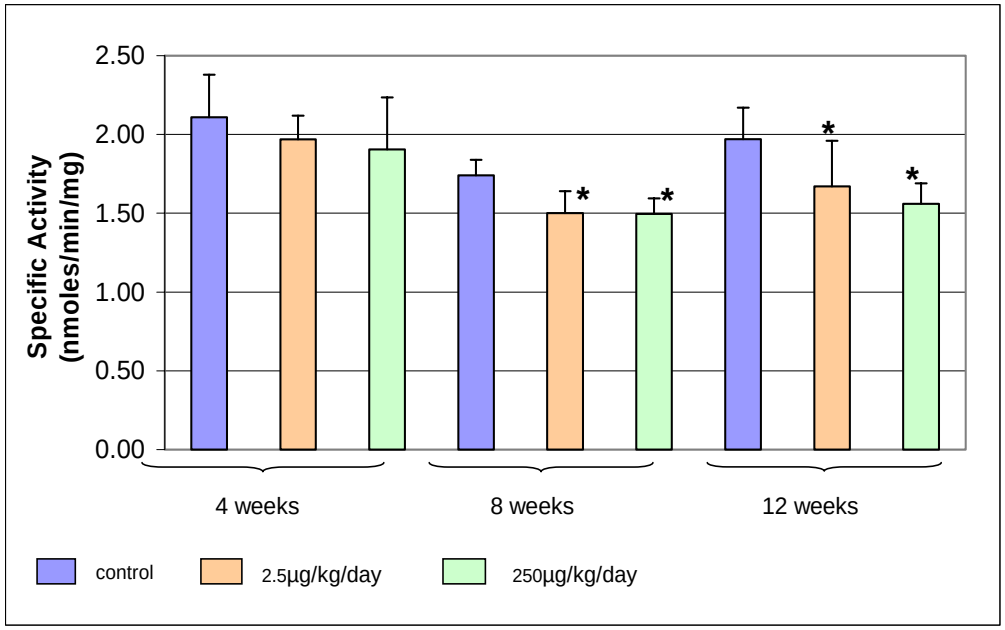


Fig 4-4 Specific activity of GSTz in rats treated with 2.5 and 250 µg/kg/d of DCA in drinking water. Error bars indicate standard deviation (n=6) and * indicate that means are significantly different than controls.

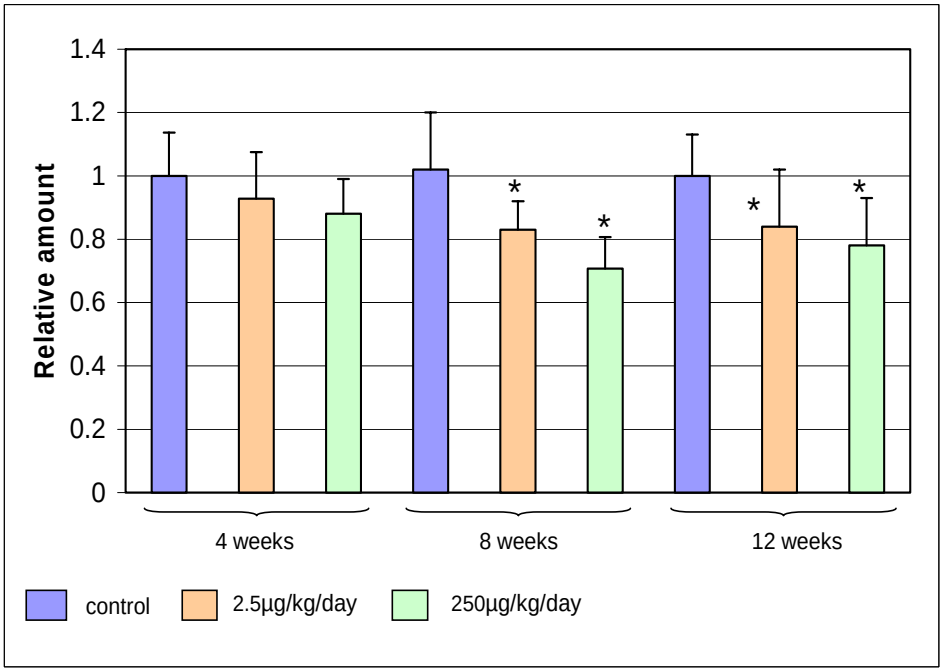


Fig 4-5 Expression of GSTz in rats treated with 2.5 and 250 µg/kg/d of DCA in drinking water. Values are expressed relative to 0.01µg of hGSTz 1c-1c. Error bars indicate standard deviation (n=6) and * indicate that means are significantly different than controls.

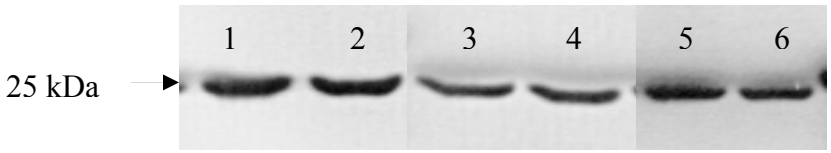


Fig 4-6 Representative western showing GSTz expression in control (lanes 1-2) 250µg/kg (lanes 3-4),and 2.5 µg/kg (lanes 5-6). Rats were exposed to these doses of DCA for 8 weeks.

Urinary Excretion of DCA and Maleylacetone

Rats excreted DCA in urine at the rate of 4.5 mg/kg/24h after one week of treatment with 50mg/kg DCA. This rate increased to 5.5 mg/kg/24 h after 8 weeks of treatment with the same dose. DCA excretion dropped dramatically (less than 10 µg/kg/24 h) after week of withdrawing the drug. No DCA was detected in urine of rats treated with 2.5 and 250 µg/kg/day of the drug. Urinary analysis showed that rats excreted MA at the rate of 60-75 µg/kg/24 h during 8 weeks of treatment with the high dose of DCA. MA could not be detected after one week of withdrawing the drug. MA was not detected in the urine of rats treated with both 2.5 and 250 µg/kg/day of DCA.

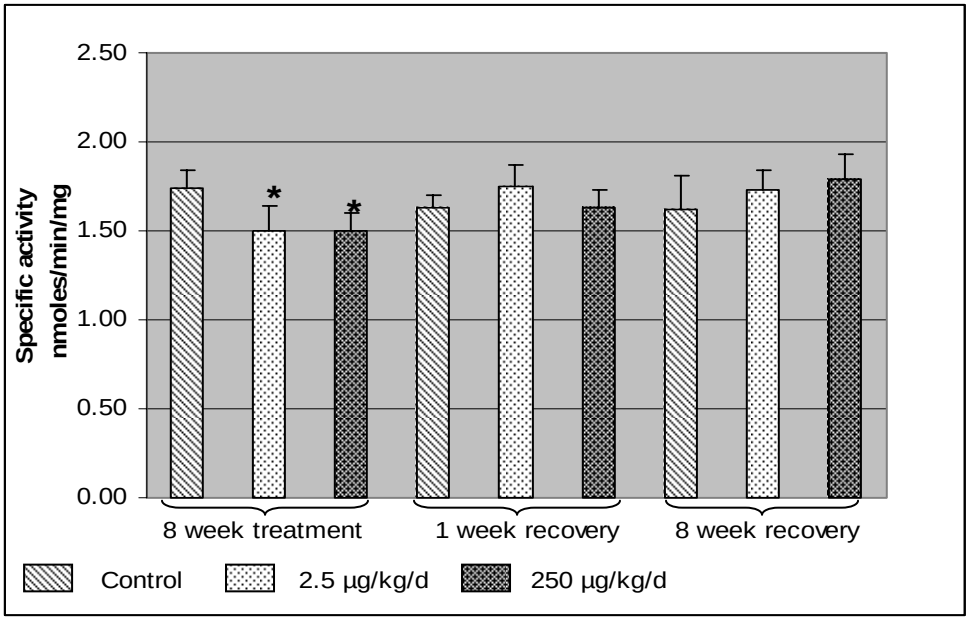


Fig 4-7 Recovery of GSTz activity in rats treated with low doses of DCA. n=6, error bars indicate standard deviation, * indicate that means are significantly different than control. Activity is restored after one week of withdrawing DCA.

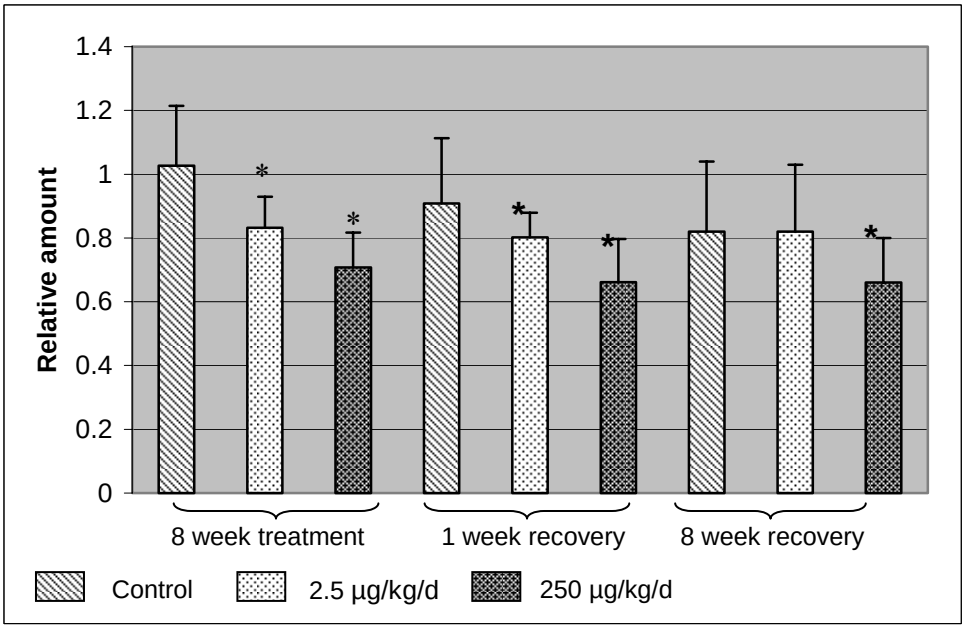


Fig 4-8 Restoration of GSTz protein in rats treated with low doses of DCA.Values are expressed relative to 0.01µg of hGSTz 1c-1c. n=6, error bars indicate standard deviation, * indicate that means are significantly different than control.

CHAPTER 5
ADDUCTS OF HUMAN GSTZ 1C-1C WITH DCA AND GSH.

HPLC Separation and Identification of Adduct

After its reaction with DCA in the presence of GSH, GSTz was analysed by reverse phase HPLC coupled with a fluorescence detector. Under the mobile phase conditions described, adduct of GSTz with DCA eluted between 30 and 33 minutes (Fig 5-1) and the parent unreacted GSTz eluted at about 35 minutes. This was confirmed by running purified and unreacted GSTz and blanks devoid of either GSTz, DCA or GSH. Purified enzyme eluted at about 35 minutes. The samples devoid of GSTz did not show any peaks in the spectra, and those devoid of either DCA or GSH showed a peak at 35 minutes which corresponded to the retention time of unreacted GSTz. This confirmed the identity of both peaks and showed that DCA formed an adduct with the enzyme only in the presence of GSH. It was seen that the amount of modified protein was dependent on the concentration of DCA. As the concentration was increased from 2 mM up to 10mM the area of the peak representing the modified protein increased and there was a corresponding decrease in the area of the peak representing unmodified GSTz.

For further mass spectral experiments it was important to have maximum amount of modified protein and as little contamination from the unmodified protein as possible. To achieve this, reaction was done with 10mM DCA and for 24 hours. After this incubation period about 85% of the protein was in its modified form.

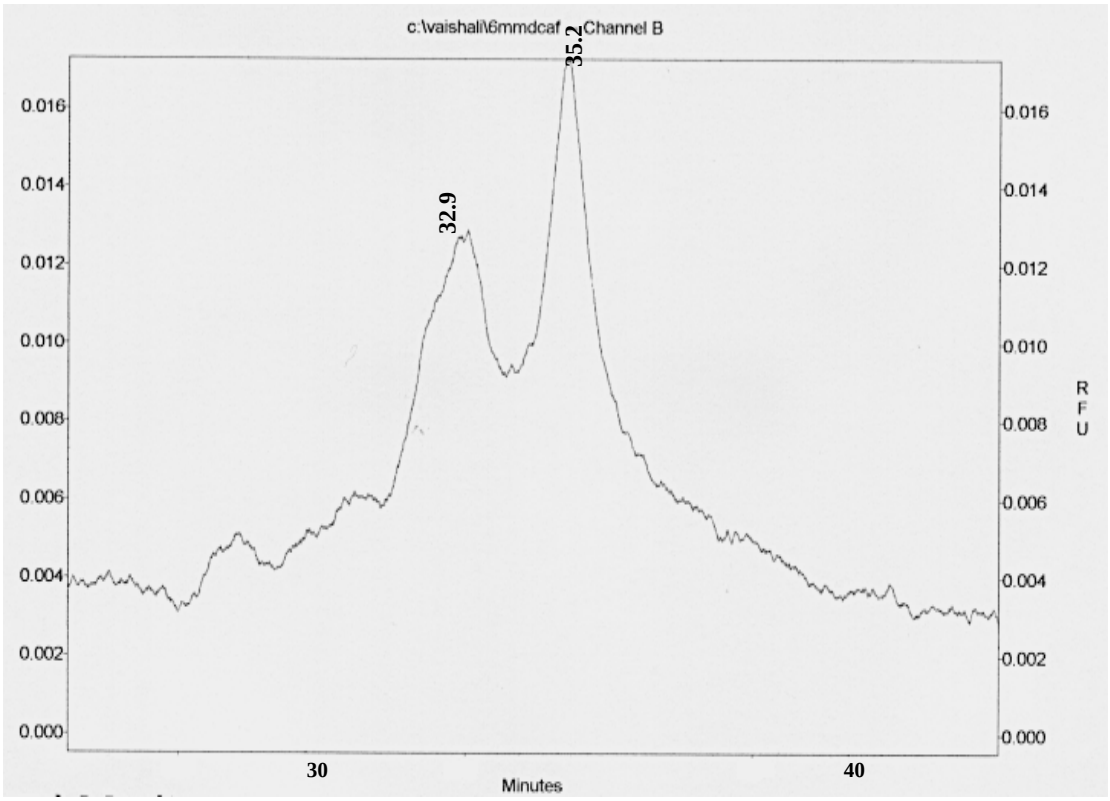


Fig 5-1 Separation of DCA modified GSTz from unmodified GSTz by gradient reversed phase HPLC. GSTz was incubated with 6mM DCA and 1mM GSH in 0.1M Hepes and analysed by gradient HPLC described in methods. Adduct has a retention time of 32.9 minutes and the unreacted GSTz 1c-1c eluted at 35.2 minutes.

Mass Spectral Analysis of Intact (undigested) Modified and Unmodified GSTz

To determine the mass of unmodified GSTz protein and that of the enzyme modified by both GSH and DCA the reaction incubates were purified by ultrafiltration or Ziptip and analyzed by matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI of unmodified and DCA modified protein showed a mass difference of about 278 mass units between the two proteins (Fig 5-2).

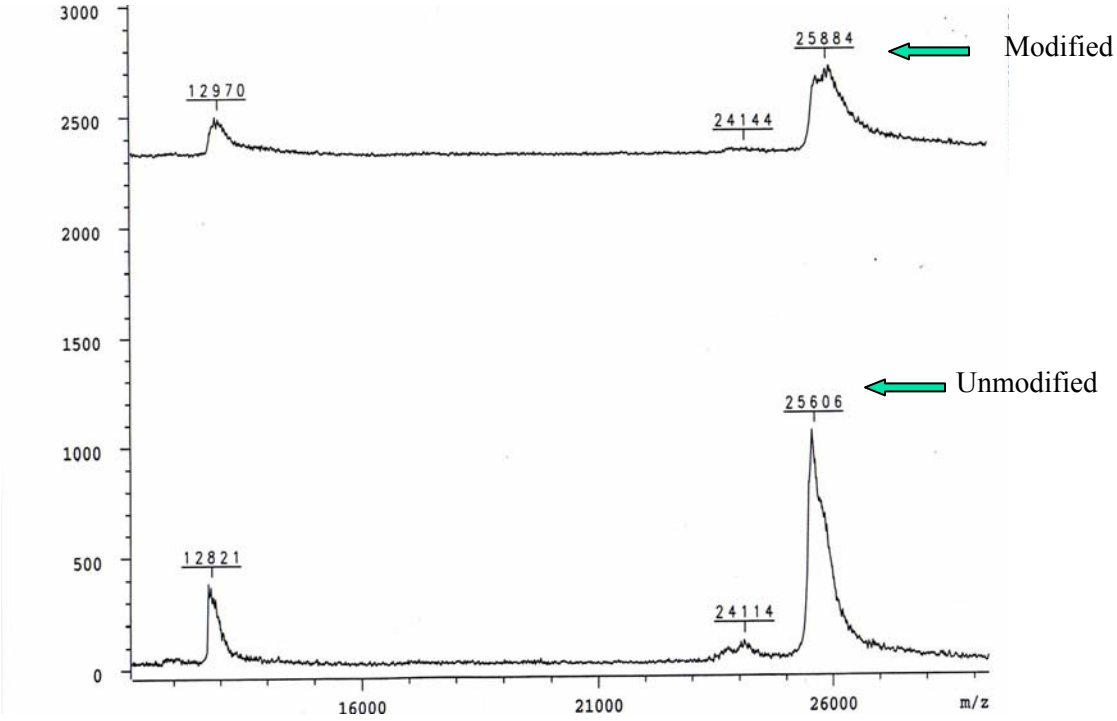


Fig 5-2 MALDI of undigested GSTz 1c-1c in unmodified and modified forms. GSTz was reacted with 10mM DCA and 1mM GSH for 24 hours. The samples were desalted and analysed by MALDI as desribed in Methods. Singly and doubly charged masses are shown.

Data from MALDI was however could not be conclusive, since at such high masses MALDI is not completely accurate. An expanded view of the MALDI spectra (data not shown) shows that the mass given by the software is merely the highest peak from several peaks typically observed in MALDI spectra of high molecular weight proteins. Thus this mass is an estimate rather than an exact number. But MALDI analysis proved that following reaction with DCA, GSTz1c-1c is converted to an adduct with a mass higher than the parent protein.

ESI of intact protein gave a mass spectrum from which mass of the unmodified protein was calculated as 25484 Kda. This is in close agreement to published mass and the mass obtained from MALDI. ESI of samples in which GSTz had been incubated with GSH alone yielded at least 3 possible proteins suggesting 3 possible

adducts with GSH. Calculated masses showed that these peptides had adducted up to 3 GSH molecules. They had masses of 25,789.6 +/- 8.1 u; addition of 1 GSH, 26,091.6 +/- 3.0 u; addition of 2 GSH's, and 26,398.5 +/- 4.0 u; addition of 3 GSH's.

ESI of DCA modified GSTz generated a mass spectrum so complex that it was not possible to calculate an accurate mass of the modified protein. It was concluded that digests of GSTz using endoproteinases would be the best possible method to identify modification of GSTz1c-1c by DCA.

Tryptic and Lys-c Digests of Unmodified GSTz 1c-1c

Trypsin and Lys-c are endoproteinases that cut a particular protein at specific internal residues. Trypsin cuts proteins at lysine (K) and arginine (R) residues whereas Lys-c makes cuts only at the residue lysine. Fragments formed from such digests can be identified from their masses.

For further confirmation of peptides, MS/MS of these fragments is performed which results in daughter ions. Energy applied during MS/MS typically fragments peptides along the backbone. Each residue of the peptide chain successively fragments off, both in the N to C terminal and C to N terminal direction (Fig 5-3). Depending on the location that the fragmentation occurs, and the nature of the ion remaining, MS/MS may result in, a, b, c (N terminal) and x, y, or z (C terminal) ions (Cole, 1997). y ion formation is the most likely to happen, and y ions are the ones most frequently seen. a and b ions are also observed, but large a ions are rarer than small ones. c and x ions are rarely seen and the existence of z ions is considered doubtful (Cole 1997).

Peptides that have been modified generate fragment ions which have a mass equal to the sum of masses of unmodified ion and the modification.

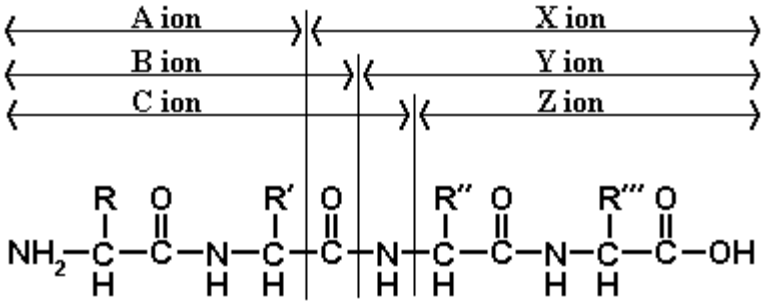


Fig 5-3 Fragmention pattern of peptides to give N and C terminal ions. Most common ions are y and b ions which are used for peptide identification.

Since each of these ions represents a certain amino acid residue, one can identify the site of modification by determining the mass of modified y and b ions.

Theoretical fragment ions can be generated using software programs such as Protein Prospector which are available on the internet. Using this program MS/MS ions of the active site peptide SSCSWR were generated (Table 5-1).

Table 5-1 Theoretical masses of y and b ions of SSCSWR using Protein Prospector.

b-H_2O ions	---	157.06	260.07	347.10	533.18	---	
b ions	---	175.07	278.08	365.11	551.19	---	
	1	**2**	**3**	**4**	**5**	**6**	
H -	**S**	**S**	**C**	**S**	**W**	**R**	**- O H**
	6	**5**	**4**	**3**	**2**	**1**	
							C-terminal ions
	---	638.27	551.24	448.23	361.20	175.12	y ions

Trypsin and Lys-c generated several peptide fragments of GSTz which were then identified by both MALDI and ESI as described in Methods. About 50-65% of the protein sequence was covered by these digests. Digests with endoproteinase Glu-C did not generate peptides that could be identified; hence this enzyme was not used for further experiments.

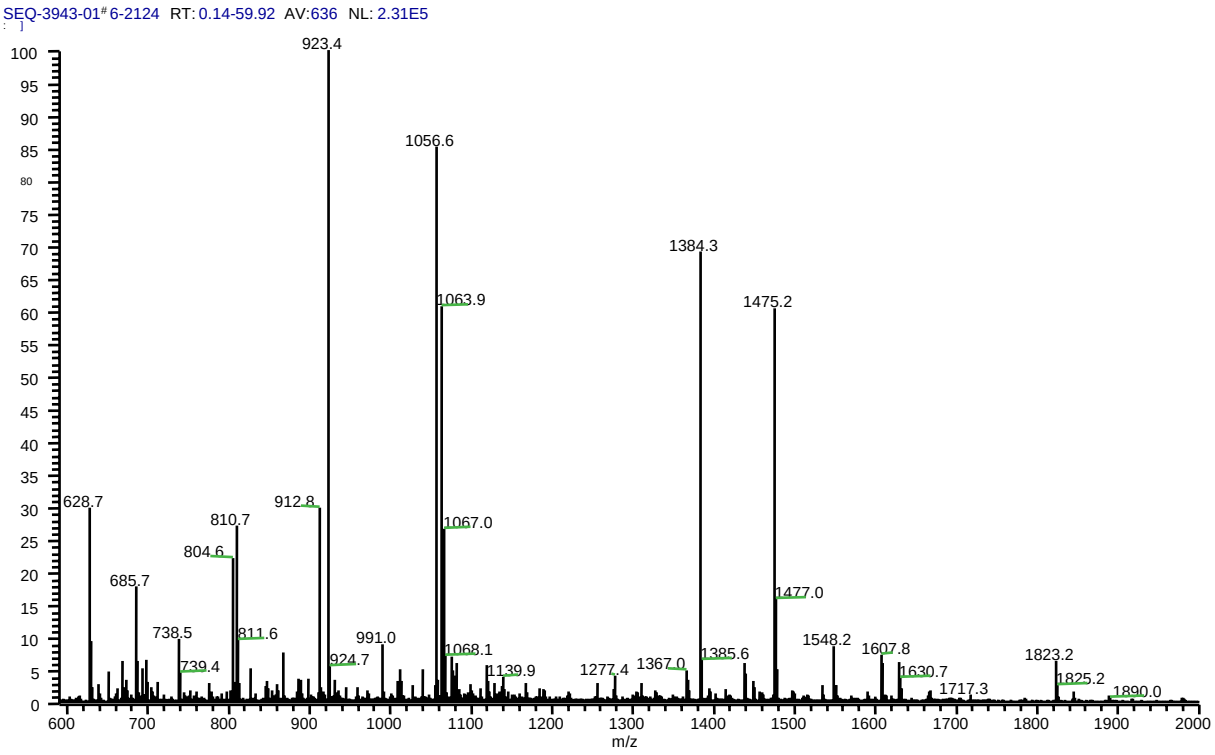


Fig 5-4 LC-MS/MS full scan of unmodified hGSTz1c-1c showing observed peptides. The sequence corresponding to each of the observed is shown in table 5-2

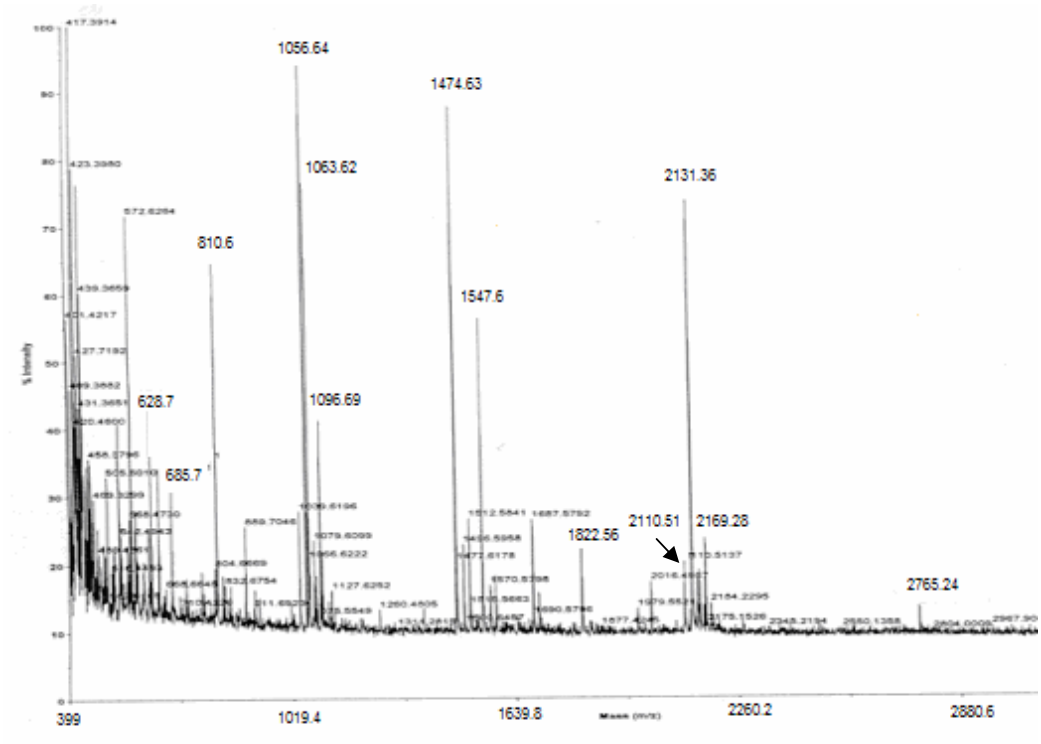


Figure 5-5 MALDI of unmodified GSTz showing tryptic peptides.

Details of the sequence of GSTz covered, and masses of the peptides by Trypsin and Lys-c are shown below:

Table 5-2 Peptides identified by Lys-c Digest. [a] indicates ions observed by ESI. [b] indicates doubly charged ions, [c] indicates triply charged ions.

Sequence covered	Theoretical mass	Observed ion	Amino acids
PILYSYFRSSCSWRVRIALALK	2628.43	2629[a,c]	6 -27
DFQALNPMK	1063.52	1063[a]	49-57
QVPTLK	685.42	685[a]	58-64
RASVRMISDLIAGGIQPLQNLSVLK	2678.72	2679[a,c]	96-120
FKVDLTPYPTISSINK	1823.42	1823[a,b]	176-191
RLLVLEAFQVSHPCRQPDTP TELRA	2875.5	2876[a,c]	192-216

Table 5-3 Peptides identified by tryptic digest in ESI and MALDI spectra. [a] observed by ESI, [b] indicates observed by MALDI, [c] indicates doubly charged ion.

Sequence covered	Theoretical Mass	M+H ion (observed)	Amino acids
SSCSWR	725.15	725[a]	14-19
IALALK	628.43	628[a,b]	22-27
GIDYKTVPINLIK	1473.8	1475[a,b]	28-40
DGGQQFSK	866.4	866[a,b]	41-48
DFQALNPMK	1063.52	1063[a,b]	49-57
QVPTLKI	685.42	685[a,b]	58-64
IDGITIHQSLAIIEYLEETRPTPR	2765.48	2766[a,b] $(M+3H)^{3+}$	65-87
LLPQDPK	810.47	810.5[a,b]	88-94
MISDLIAGGIQPLQNLSVLK	2110.19	2110[a,b,c] $(M+H)^{2+}$	101-120
FKVDLTPYPTISSINKR	1823.42	1823[a,b]	176-192
LLVLEAFQVSHPCR	1611.86	1611[a,b]	193-206
QPDTPTELR	1056.53	1056[a,b]	207-215

The peptide containing the active site (SSCSWR) was identified by MALDI and LC-MS/MS of tryptic digests. The intensity of this peak was very low in MALDI, hence confirmation of this peptide was not possible by MALDI alone. But LC-MS gave a strong signal, and MS/MS identified y fragment ions of this peptide. Under the HPLC conditions described before this peptide eluted at 15.84 minutes. This peptide was not observed in the MALDI of DCA-modified GSTz1c-1c.

Table 5- 4 Theoretical and observed ions of SSCSWR.

Ion type $(M+H)^+$	Theoretical mass b ions	y ions	Observed mass b ions	y ions
	88	175.1	ND	ND
	175.1	361.2	ND	361.4
	278.1	448.2	ND	448.4
	365.1	551.2	ND	ND
	551.2	638.3	533.1	533.1
$(M+H)^+$, y4/b5-H_2O	533.2	533.2		
$M+H)^+$, y3-H_2O				430.22

Peptide containing the active site was also found in lys-c digests. But this peptide (PILYSYFRSSCSWRVRIALALK) was too large and hence detecting modifications of this peptide would be difficult. Hence further experiments focused on modifications of SSCSWR obtained by tryptic digests.

Both digests did not cover the region in the protein from residues 121-175. This region does not have a lysine or an arginine and hence could not be cut by either of the enzymes. The mass of this peptide is about 5942 Da which is too large to be identified as a singly or doubly charged peptide by ESI. It can only be observed if it is triply (or higher) charged. Hence this region of the protein was not identified by digests. The protein was then digested with endoproteinase Glu-C in order to obtain smaller fragments of this peptide. Digests with Glu-C did not yield peptides which could be identified by Sequest browser. Hence Glu-C digests were not investigated in more detail.

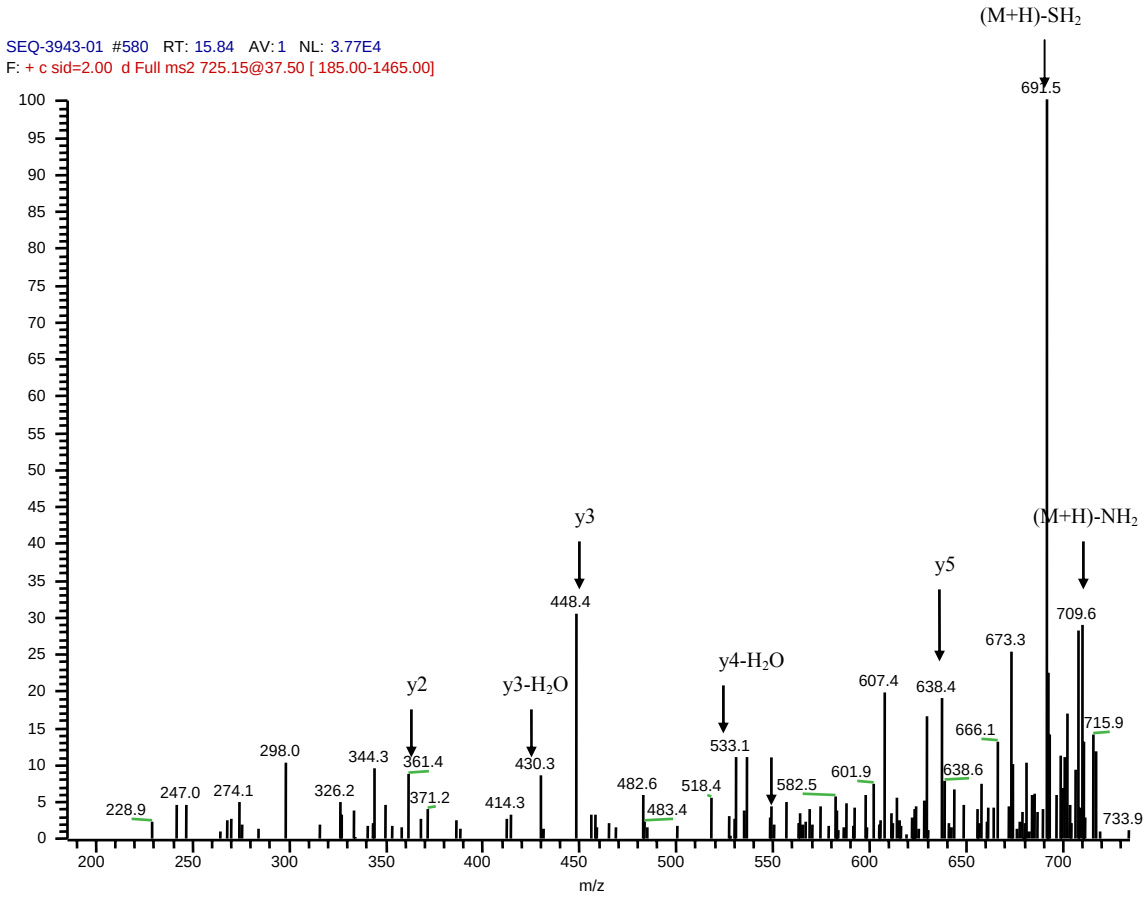


Fig 5-6 MS/MS of active site peptide (SSCSWR) showing daughter ions.

Tryptic Digest of GSH Modified GSTz 1c-1c.

GSTz 1c-1c that had been previously incubated with GSH was digested with trypsin and the resulting peptides were analysed by MALDI and LC/MS/MS. MALDI of tryptic digests identified a peptide whose mass was equal to the mass of GSH adducted peptide 193-206 (LLVLEAFQVSHPC^{205}R). Its mass was 1917 i.e. 1611+307-2H. Theoretical mass of this modified peptide is 1916.93. This peptide was not identified by ESI of tryptic digests. LC-MS of tryptic digests identified an adduct of GSH with the active site peptide (SSCSWR). The mass of this peptide is 1030 which is 305 mass units higher than the active site peptide. This peptide was not

observed in MALDI of digests A doubly charged ion of this peptide was also observed at 515.7 mass units. This peptide was eluted at 16.00 minutes which is similar to the retention time of unmodified SSCSWR. Most abundant ion in the spectra was 451.2 (doubly charged), resulting from a loss of γ-glutamate from the parent ion. Several y and b fragment ions (Fig 5-7) were identified which aided in assigning modified amino acid residues. Masses of modified y and b ions are shown in table 5-5.

The third adduct of GSH which was identified by ESI of GSH modified GSTz was not observed in the LC-MS or MALDI of tryptic or lys-c derived peptides. This adduct may be formed in the region from 121-175 amino acids. This region of the protein has cysteine residues which may form a mixed disulphide with the thiol of GSH. Since peptides from this region were not observed in the digests we were not able to identify and characterize this adduct.

Tryptic Digests of DCA Modified GSTz 1c-1c

hGSTz1c-1c that had been incubated with DCA in the presence of GSH was digested with trypsin and analysed by LC/MS. Spectra were searched for modifications of the active site and for other possible modifications of other peptides. Spectra showed the presence of active site peptide, but its abundance and hence the intensity of signal was low. Since the abundance of this peptide was low the instrument which was set for data dependent MS/MS did not perform MS/MS of this ion. This is an important observation, since it suggests that this peptide has undergone a reaction and has been modified.

Tryptic digest of DCA modified GSTz showed a peptide with a mass of 1104.5 which corresponds to the mass of active site peptide+glutathione+glyoxylate. This

peptide eluted at 16.24 minutes which is similar to the elution time of unmodified active site peptide.

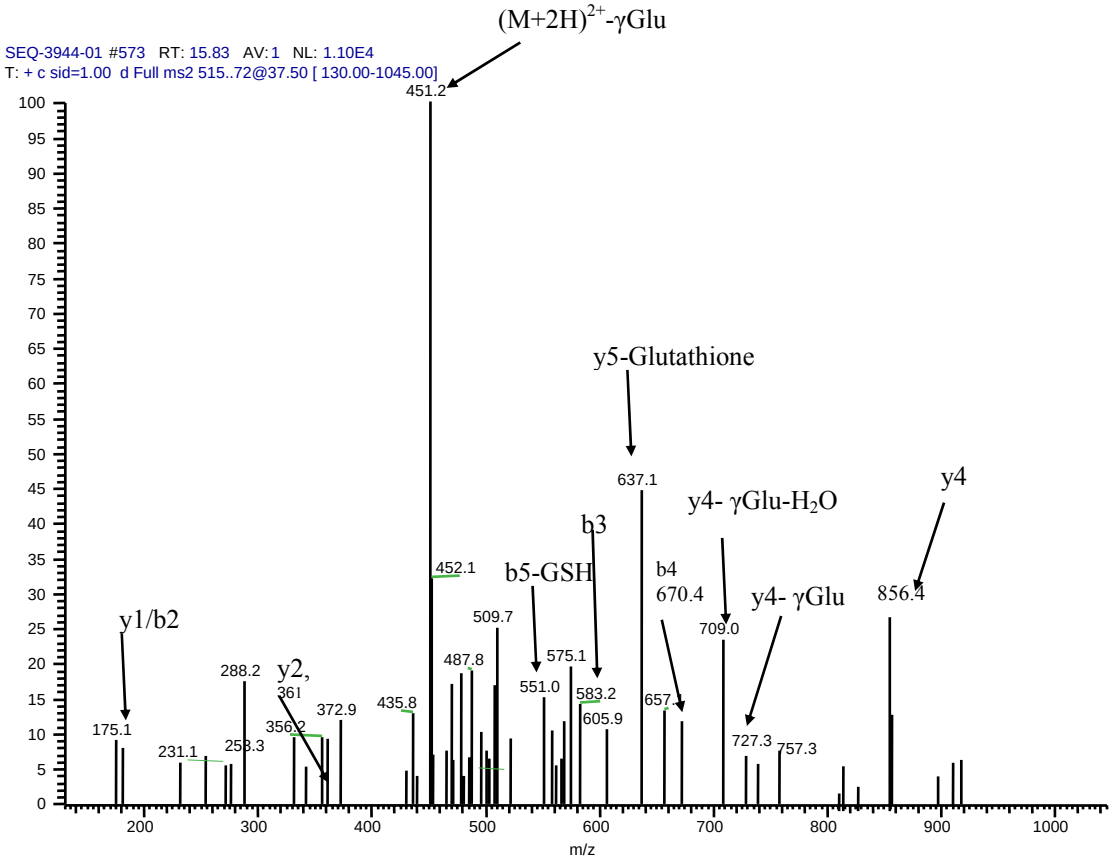


Fig 5-7 MS/MS of glutathione modified active site peptide (SSCSWR). y and b ions refer to fragment ions observed in the modified peptide. This peptide was observed in MALDI and ESI spectra of GSTz 1c-1c that had been incubated with GSH.

Masses of unmodified y and b ions of SSCSWR are shown in table 5-3 and those of modified ions are shown in table 5-6. These ions have been modified by 74 (glyoxylate) and/or GSH to give corresponding modified ions.

MS/MS fragment ions (fig 5-8, table 5-7) show glyoxylate modified b2 and b3 ions suggesting that the one of the first three residues of the peptide (**SSC**SWR) have been modified. b2 is modified by 74 i.e. glyoxylate suggesting that the serine at position 14 or 15 (**SS**CSWR) may be covalently modified by glyoxylate.

Table 5-5 Theoretical and observed mass of y and b fragment ions in GSH modified SSCSWR. (active site peptide) M+H ion has a mass of 1030.4 Doubly charged ion had a mass of 515.7.

Ion type	Theoretical mass	Actual mass
y1	175.1	175.1
y2	361.2	361
y3	448.2	448.4
y4	856.2	856.4
y5	943.2	ND
b2	175.1	175.1
b3	583.1	583.2
b4	670.1	670.4
b5	856.1	856.4
y4-γ Glu	727.2	727.3
y4-γ Glu-H_2O	709.3	709.1
$(M+H)^{2+}$-γ Glu	451.2	451.2

Table 5-6 y and b ions observed in the MS/MS of GSH and glyoxylate modified SSCSWR. M+H ion has a mass of 1104.5. This ion was observed in DCA modified GSTz samples.

Ion type	Theoretical mass	Actual mass
y1	175.1	ND
y2	361.2	361.2
y3	448.2	448.4
y4	856.2	856.4
y5	943.2	943.3
b2	249.1	249.5
b3	657.1	657.4
b4	744.1	ND
b5	930.2	930.3
y4-H_2O	838.2	838.3
b4-NH_3	727.1	727.3
b5-H_2O	912.2	912.3

Further analysis of ions showed that y1, y2, y3 are not modified but y4, y5 and y6 are adducted. Modified y4 ions has a mass of 856 which the mass of unmodified y4 (551) + mass of GSH (307)-2H. Previous results show that the cys16 is covalently modified by GSH which is further confirmed in this mass spectrum. Adducted y5 ion has a mass of 943 which corresponds to the mass of unmodified y5 (638) + mass of GSH (307)-2H.

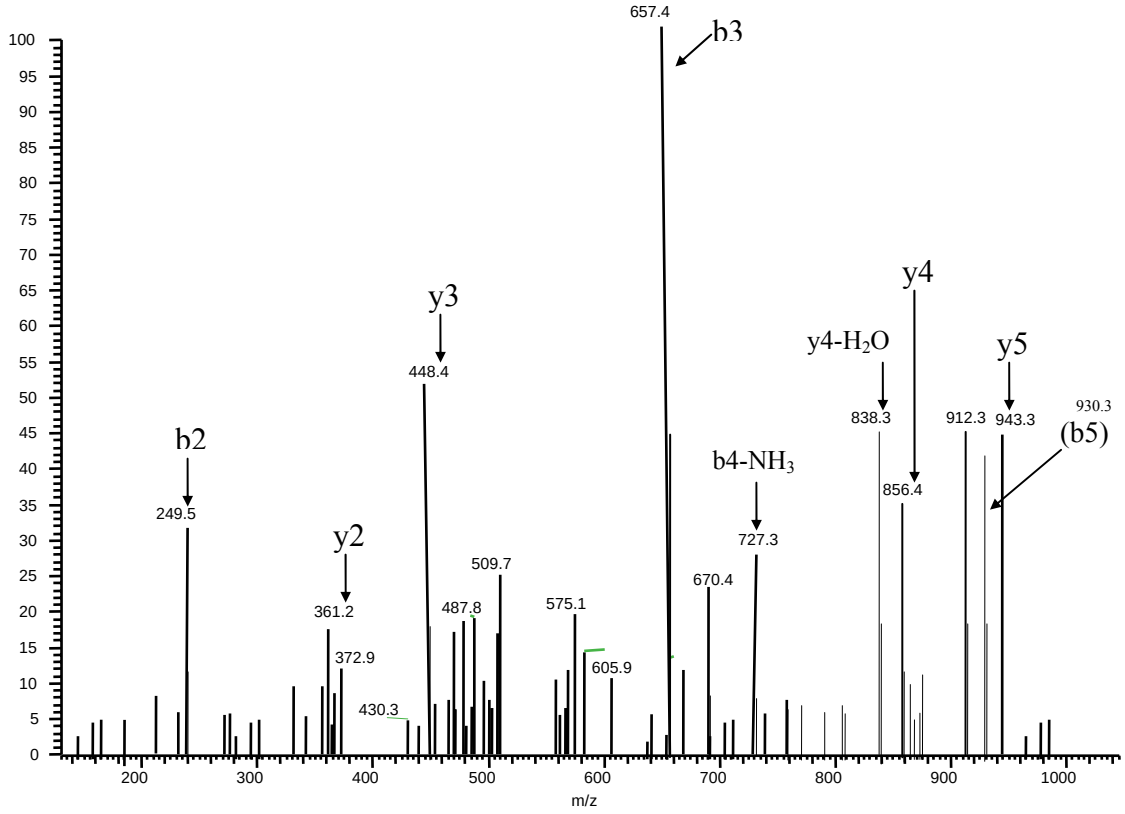


Fig 5-8 MS/MS of GSH and glyoxylate modified active site peptide (SSCSWR).y and b fragment ions are shown.

Another important fragment ion, b3 is modified by 379, which corresponds to the mass of glyoxylate + GSH. The final C terminal i.e. y6 (^{14}SSCSWR^{19}) shows modification by both GSH and glyoxylate, thus suggesting that serine 14 has been modified by glyoxylate. Above data indicate that serine 14 in the GSH binding site

has been covalently modified by glyoxylate and the glutathione forms a disulphide bond with the sulfhydryl of cysteine 16. The hydroxyl of the serine may act as a nucleophile (Fig 6-1) and attack the carbonyl carbon of glyoxylate and thus form an adduct with the active site.

Glyoxylate modified SSCSWR was also observed in the spectra of DCA modified GSTz. This peptide had a mass of 799.6 (fig 5-9, table 5-7) which is equivalent to mass of the active site peptide (725) + glyoxylate (74).

Table 5-7 y and b ions in glyoxylate modified SSCSWR. M+H ion has a mass of 799.6.

Ion type	Theoretical mass	Actual mass
y1	175.1	175.1
y2	361.2	361.4
y3	448.2	448.7
y4	551.2	551
y5	638.2	638.4
b2	249.1	249.3
b3	352.1	352.1
b4	439.1	439.8
b5	625.2	625.1
(M+H)-H_2O	781.3	781.6

Modification of either one of the serines at position 14 or 15 was determined by b2 ion. Unmodified b2 ion has a mass of 175 which when modified by glyoxylate (74) gives a mass of 249. Other fragment ions also are diagnostic for modification of serine residue. Since modification is near the N terminal of the peptide all the b ions are modified. All the y ions of the peptide are I unadducted form except y6 ie. (^{14}SSCSWR^{19}).This again suggested that serine 14 is modified by glyoxylate.

ESI of trypsin digest of the DCA modified GSTz yielded other peptides which are different from the peptides observed in GSH modified GSTz and unmodified GSTz. The peptide with the sequence DGGQQFFSK was modified by 56 mass units. the mass of unmodified peptide was 866 and that of the modified was 922.4 (Fig 5-10).

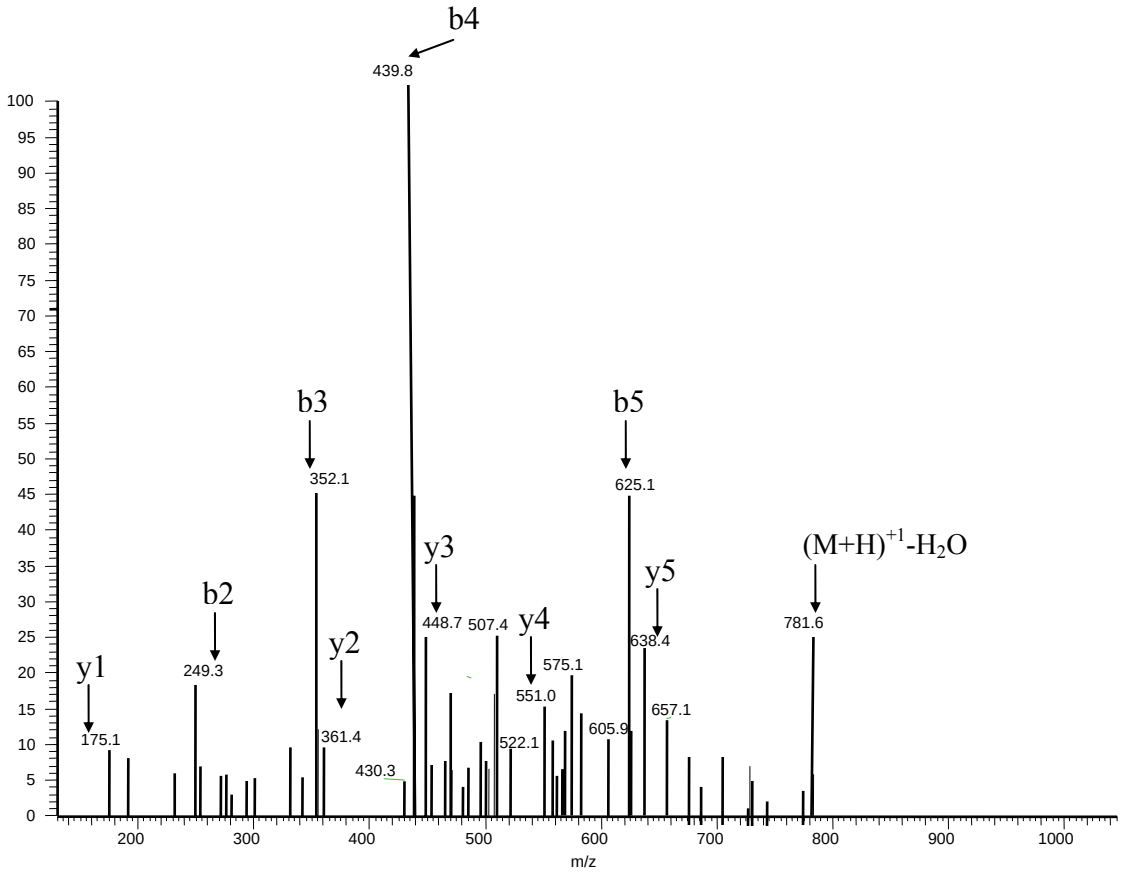


Fig 5-9 MS/MS of glyoxylate modified active site peptide. GSTz1c-1c was incubated with 1mM GSH and 10mM DCA, digested with trypsin and analyzed by LC/MS as described in methods.

MS/MS shows all the y ions intact suggesting that the modification has occurred at the N-terminal end of the peptide. All the b ions of the peptide have been modified by 56 mass units. This suggests that the residue modified is the N-terminal end residue, “D” i.e. aspartic acid. Literature search for a shift in 56 mass units did

not offer any reasonable explanation. This modification could be a result of a reaction with glyoxylic acid (Fig 6-1).The free carboxylic group in the aspartic acid could form an anhydride with the glyoxylate. The mass of this adduct would be 56 mass units greater than the unmodified peptide.

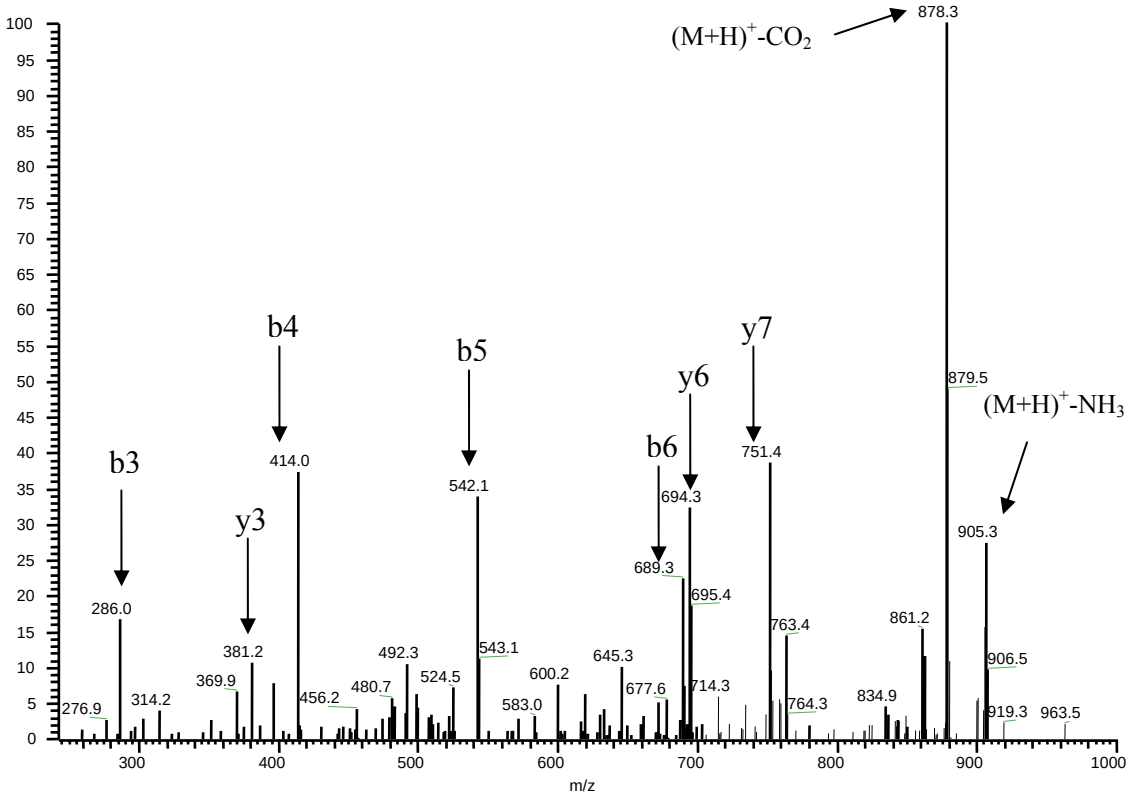


Fig 5-10 MS/MS of glyoxylate modified DGGQQFFSK. $b2^{ND}$, b3:286.0 (230.08), b4:414.0 (358.14), b5:542.1 (486.19), b6:689.3 (633.26), b7: 776^{ND} (720.3), $y1^{ND}$, y2: 234.15^{ND}, y3:381.2 (381.2), y4: (509.27), $y5^{ND}$, y6: 694.3 (694.4), y7:751.4 (751.37), ND indicate not detected. Masses in parentheses are those of theoretical, unmodified ions.

A second peptide with the sequence, DFQALNPMK (1063.5) was also similarly modified by 56 mass units to yield a peptide with the mass 1119.3 (Fig 5-11), and its doubly charged fragment with mass 560.66 (Fig 5-12). All the b fragment ions have been modified but none of the y ions have been modified. It can thus be concluded that a residue at the N terminal of the peptide has been modified. This

peptide also has an aspartic acid residue at its N terminal end which is covalently modified by glyoxylate.

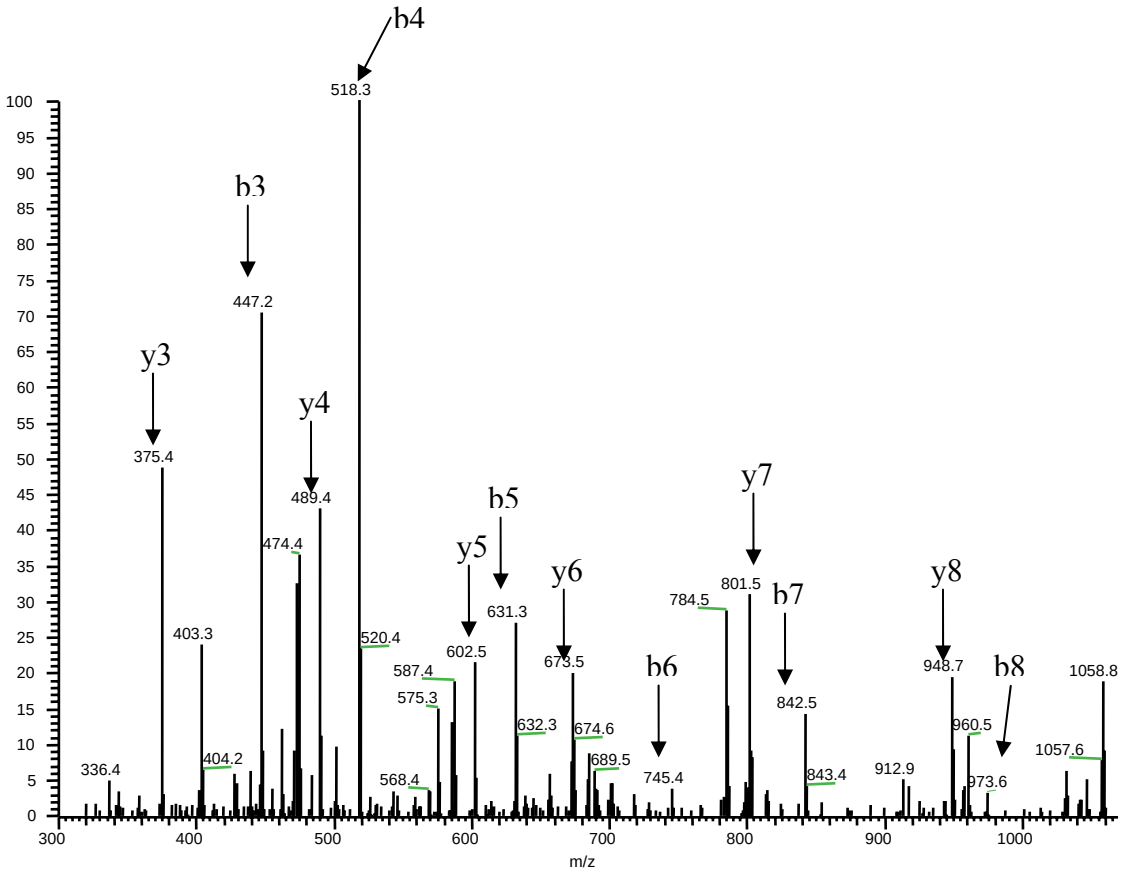


Fig 5-11 MS/MS of glyoxylate modified DFQALNPMK. Mass of this peptide is 1119.3 which is 56 Da higher than parent peptide. Modification is at N terminal aspartic acid. $b2^{ND}$, b3:447.2 (391.16), b4:518.3 (462.2), b5:631.2 (575.28), b6:745.4 (689.33), b7:842.5 (786.38), b8:973.6 (917.42), $y1^{ND}$, $y2^{ND}$, y3:375.4 (375.21), y4:489.4 (489.25), y5:602.5 (602.33), y6:673.5 (673.37), y7:801.5 (801.43), y8:948.7 (948.5). ND indicates not detected and masses in parentheses are those for unmodified theoretical ions.

A third peptide which was modified had the sequence, MISDLIAGGIQPLQNLSVLK. The monoisotopic mass of the unmodified peptide is 2110. A doubly charged peptide with mass 1054.6 was observed in the LC/MS of unmodified GSTz which corresponds to the mass of the doubly charged unmodified peptide. LC/MS of DCA modified GSTz shows that this peptide was modified by 56

mass units. A doubly charged modified peptide with the mass 1084.2 (Fig 5-15) was observed.

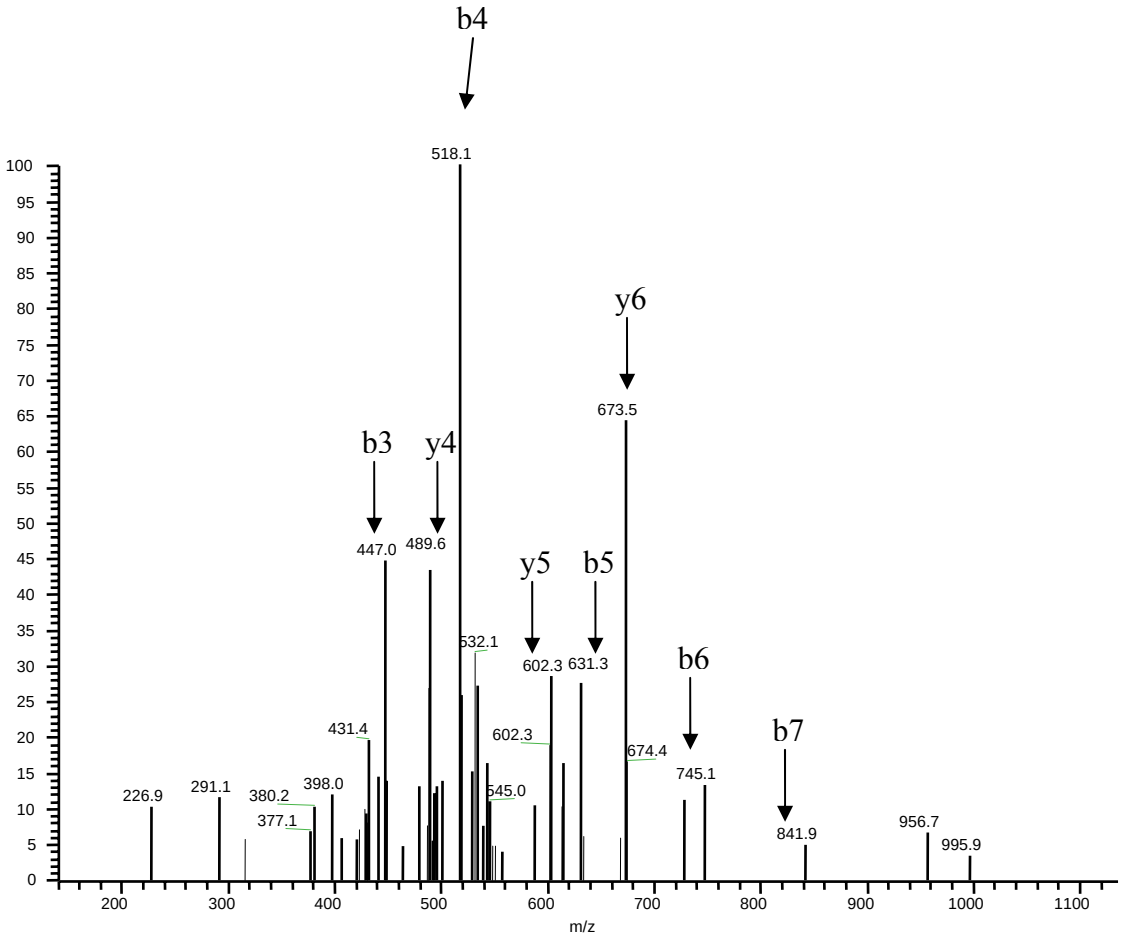


Fig 5-12 MS/MS of doubly charged glyoxylate modified DFQALNPMK. y and b ions same as those present in Fig 5-11 are observed

MS/MS of the peptide showed ions which were diagnostic for the identification of the modified residue. Modified b4 ion (503.2) suggested that the modification is at or before the 4th (N terminal) residue in the peptide MIS**D**LIAGGIQPLQNLSVLK. This residue (aspartic acid) was found to be covalently modified in other peptides discussed earlier. It was thus concluded that the aspartic acid was covalently modified by glyoxylate by 56 Da.

147.11	y_1	560.28	b_5	971.52	b_{10}	1366.81	y_{13}	1779.04	y_{17}
245.13	b_2	673.36	b_6	1011.62	y_9	1437.78	b_{14}	1851.00	b_{18}
260.20	y_2	673.42	y_6	1099.58	b_{11}	1437.84	y_{14}	1866.07	y_{18}
332.16	b_3	744.40	b_7	1139.68	y_{10}	1550.93	y_{15}	1964.09	b_{19}
359.27	y_3	801.42	b_8	1196.63	b_{12}	1551.82	b_{15}	1979.15	y_{19}
446.30	y_4	801.48	y_7	1252.76	y_{11}	1664.01	y_{16}		
447.19	b_4	858.44	b_9	1309.72	b_{13}	1664.90	b_{16}		
559.38	y_5	914.57	y_8	1309.78	y_{12}	1751.94	b_{17}		

Fig 5-13 Theoretical y and b ions of MISDLIAGGIQPLQNLSVLK generated by Protein Prospector

Binding of C^{14} Labeled DCA to GSTz1c-1c

GSTz1c-1c was reacted with C^{14} DCA in the presence and absence of GSH analyzed by SDS-PAGE and autoradiography. GSTz1c-1c bound C^{14} DCA in GSH dependent manner as seen in Fig 5-13.

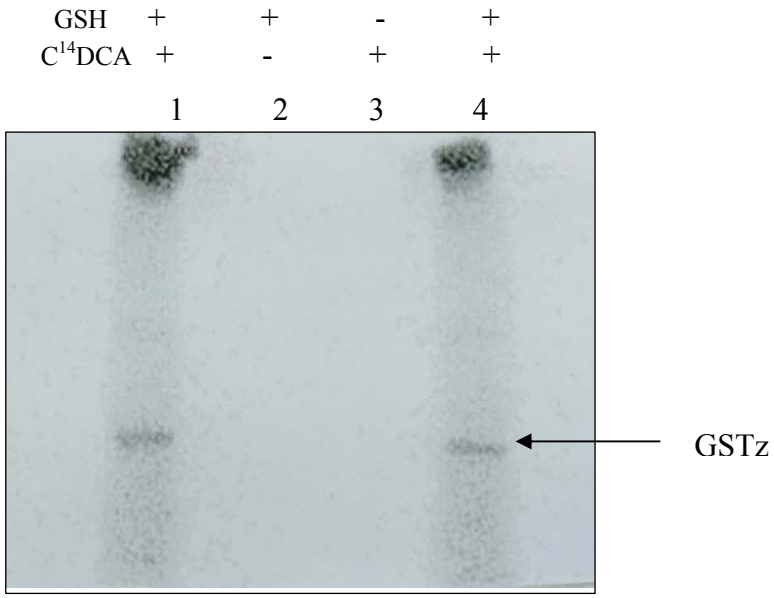


Fig 5-14 Binding of C^{14} DCA to GSTz1c-1c. Autoradiography of dried SDS PAGE gels

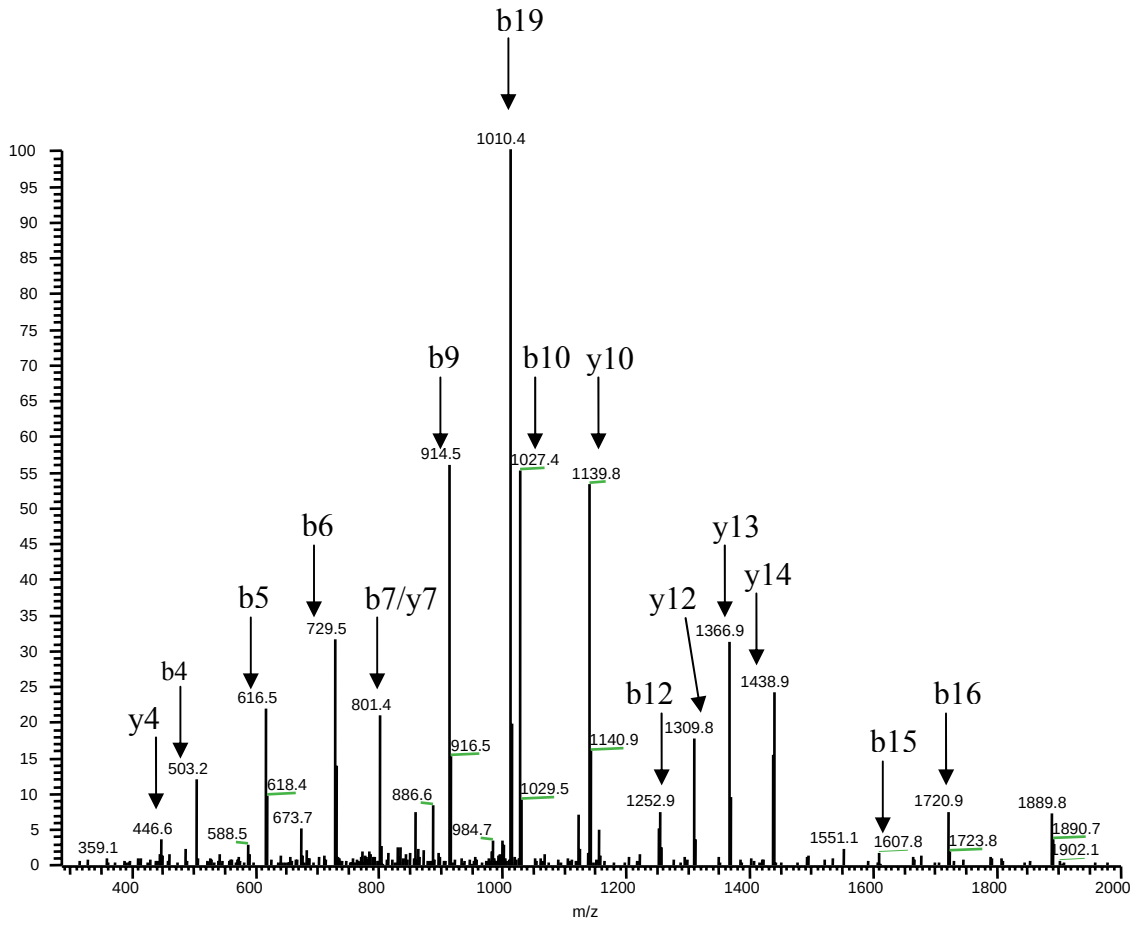


Fig 5-15 MS/MS of doubly charged, glyoxylate modified MISDLIAGGIQPLQNLSVLK. Mass of this peptide is 1084.2 Modification of 56 Da is observed at aspartic acid. The following modified ions were detected $b2^{ND}$, $b3^{ND}$, b4:503.2, b5:616.5, b6: 729.5, b7: 801.4, $b8^{ND}$, b9: 914.5, b10:1027.4, $b11^{ND}$, b12: 1252.9, $b13^{ND}$, $b14^{ND}$, b15:1607.8, b16:1720.9, $b17^{ND}$, $b18^{ND}$, b19:1011.4 $(M+2H)^{2+\cdot}$.ND indicates not detected. Unmodified y and b ions are shown in Figure 5-13.

Figure 5-14 shows that the enzyme is labeled with radioactivity from DCA only in the presence of GSH. GSTz1c-1c binds C^{14} DCA in GSH dependent manner confirming the fact that DCA is a mechanism based inhibitor of GSTz. The autoradiograph also shows a band at high molecular weight near the wells where protein was loaded. It is not known if this band is that of a high molecular weight protein or adduct or simply an artifact of the reaction.

Binding of DCA to GSTz was determined by liquid scintillation counting. It was found that 5.68% of the radioactivity remains bound to the enzyme after overnight dialysis of the protein to remove any unbound substrate or product. After converting the dpm bound to the protein to moles, 5.44 moles of DCA were bound per mole of enzyme.

Dialysis buffer was analysed by HPLC to determine the amount of unreacted DCA in the unbound fraction. HPLC showed that 85% of DCA is converted to glyoxylate after 24 hours of incubation with the enzyme and 15% was unreacted DCA.

Purification of Rabbit Anti-hGSTz 1c-1c Antibody.

As described in Chapter 2, serum from rabbits which had been given GSTz 1c-1c as an antigen was purified by protein A. This purified antibody was then used as a primary antibody against cytosolic as well as recombinant GSTz to determine its purity and cross reactivity. Western blots of recombinant GSTz 1c-1c showed that this antibody shows strong cross-reactivity with the protein. It is a very sensitive antibody and can detect as low as 10 ng of the recombinant purified protein (Fig 5-16). It could detect cytosolic GSTz even in 5 ug of cytosolic protein (Fig 5-17).

This antibody was used to immunoprecipitate GSTz from liver cytosol. If successful the protein thus obtained could then be used in MS experiments. The same technique could also be used to isolate adducts of GSTz with DCA. These in vivo adducts when analysed by MS could provide important information about the mechanism of DCA's metabolism and inactivation of GSTz.

Attempts to immunoprecipitate GSTz from cytosol using the method described in chapter two were not successful. The eluate from the protein A column cross-

linked to the antibody, when analysed by western blotting did not show the presence of GSTz. The elution buffer used had a low pH (2.8) which may have destroyed the protein. A gentle elution buffer, which does not have a low acidic pH, from Pierce was used to ensure that GSTz was not destroyed during elution. But even with this elution system no immunoprecipitated GSTz was recovered. This could be due to inefficient binding of the antibody to GSTz or even due to too strong antigen-antibody binding.

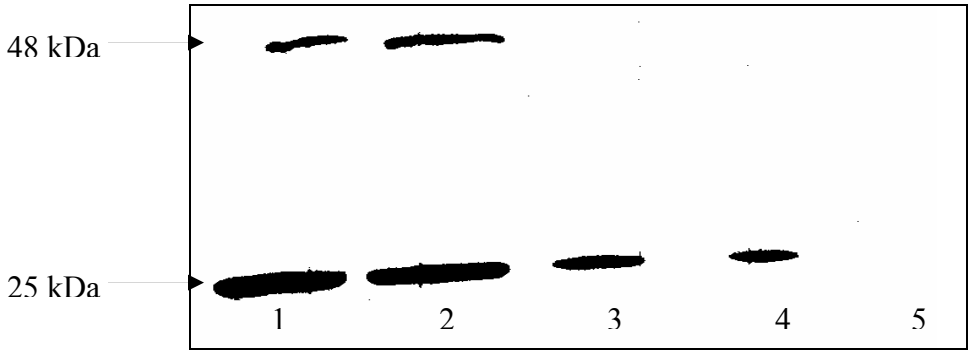


Fig 5-16 Western blot of rat liver cytosol using purified rabbit-anti human GSTz. Lanes 1 to 5 were loaded with 40, 20, 10, 5 and 1 µg of cytosolic protein. Antibody cross reacted with GSTz in all except the 5th lane. In lanes 1 and 2 a high molecular weight band can be seen. This could be another protein or a dimer of GSTz.

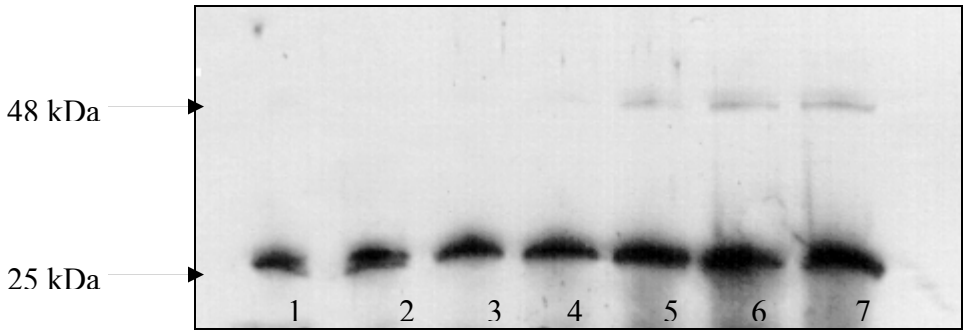


Fig 5-17 Western blot of recombinant GSTz 1c-1c using purified rabbit anti-human GSTz as primary antibody. Lanes 1 to 7 were loaded with 10, 15, 20, 25, 50, 80, and 100 ng of GSTz 1c-1c. The antibody can detect as low as 10 ng of the protein. In lanes 5-7 another high molecular weight band is observed which may be the dimer of the protein.

CHAPTER 6
DISCUSSION

Dichloroacetate is an important environmental chemical which is used clinically for the treatment of metabolic disorders such as congenital lactic acidosis. It is also used to treat acquired lactic acidosis due to malaria and other diseases. DCA facilitates lactate removal by activating pyruvate dehydrogenase complex (PDH) by inhibiting PDH-kinase (Reviewed in Stacpoole et.al 1998a,b). DCA is found in drinking water as a by-product of chlorination and is a metabolite of important industrial solvents such as trichloroethylene and tetrachloroethylene. Haloacetates are ubiquitous in the environment and have been found in fog, rain, ponds, lakes etc (Rompe et.al. 2001, Karlsson et.al. 2000). DCA treatment (0.2-2.0 g/l in drinking water for 2 weeks) reduced serum insulin levels (Lingohr et.al. 2001). The decrease persisted for at least 8 weeks. Repeated administration with DCA prolongs its plasma half-life in humans and rodents several fold. This change in pharmacokinetic properties is due to inhibition of its metabolism as shown by James et.al 1997, 1998, Ammini et.al. 2003 and in this work. DCA inhibits GSTz, its metabolizing enzyme *in vitro* and *in vivo* in a dose dependent manner. Loss of activity is accompanied with a loss of immunoreactive protein.

GSTz is an important enzyme in tyrosine catabolism and its inhibition may lead to accumulation of tyrosine metabolites such as MAA and FAA. These are reactive metabolites which may react with the enzyme leading to further inactivation. To

examine the role of these metabolites in GSTz inactivation, we blocked tyrosine catabolism by NTBC, prior to MAA formation. NTBC inhibits the second enzyme in tyrosine catabolism pathway (Fig 1-3); 4 hydroxyphenyl pyruvate dehydrogenase. This in turn inhibits the formation of reactive tyrosine metabolites such as MAA and MA downstream in the pathway. Inhibiting the formation of these metabolites may help protect GSTz. MAAI knock out mice develop hepatic and kidney toxicity when given high tyrosine diet (Fernandez-Canon et.al. 2002). This phenotype was reversed by administering NTBC demonstrating the potentially harmful effects of tyrosine metabolites.

In the present study NTBC was administered prior to DCA and enzyme activity and expression were studied. But in this study no protective effect was observed at the dose and treatment period studied. We conclude that effect of DCA on GSTz is the predominant factor in inactivation and subsequent destruction of the enzyme. The in-vivo role of MA in inhibiting the enzyme might be small or perhaps MA does not accumulate to a concentration at which it can have serious effects on the enzyme. This is in accordance with other reports which show that incubation of cytosolic or recombinant GSTz with DCA in vitro inhibits the enzyme. Furthermore recent studies have shown that in-vitro inhibition of cytosolic GSTz by MA is partially reversed by GSH (Ammini et. al. 2003). Inactivation of polymorphic variants of GSTz by MA and FA was studied by Lantum and co-workers. They found that all the variants were inhibited by both MA and FA in the absence of GSH. But when similar experiments were done in the presence of saturating concentrations of GSH (1mM) the enzymes retained their activities, which

suggested that GSH plays a protective role in this interaction. The mechanism for this protective action is not known.

This is an important factor in the present context since the cellular concentration of GSH (5mM) is saturating for GSTz because of its low Km for GSH (0.075mM, James et al. 1997) Thus inhibition of GSTz by MA and FA in the body is only possible if the cellular levels of GSH fall below the Km of GSH. Certain diseases, such as hereditary tyrosinemia type-I, are associated with low hepatic glutathione concentrations (Lloyd et.al. 1995). Many xenobiotics form glutathione-conjugates and, thereby, reduce cellular glutathione concentrations (Lambert et.al 1976). But even in these cases the possibility of the GSH concentrations falling below the Km is rare. This information supports the conclusion that inhibition of GSTz by MA does not play a major role in inactivating the enzyme and inhibition is related DCA.

It was also observed in this study that GSTz activity and protein levels were lower in rats treated with NTBC alone compared to control. Thus NTBC itself appeared to have an effect on the enzyme. This was unusual since NTBC itself is not known to inhibit GSTz. We postulate that this depletion of GSTz may be related to depletion of MAA and MA, the endogenous substrates of the enzyme. Reduced availability of its biological substrates may lead to decreased expression of this enzyme. This may be due to either decreased transcription of message or a decrease in translation of the message. Future studies could address this issue by performing northern blots on liver mRNA from rats treated with NTBC. These experiments may provide information on GSTz message levels in NTBC treated and control animals.

Although inactivation of GSTz by DCA has been studied before, recovery of enzyme activity and protein after long term administration has not been investigated. Determining the time required to restore GSTz activity after chronic dose is important for understanding DCA metabolism and pharmacokinetics. To study the recovery of activity and GSTz protein we used male Sprague Dawley rats as models. These animals were exposed sub-chronically to clinically relevant dose of DCA (50mg/kg/day) and restoration of GSTz was studied. Treatment with DCA decreased enzyme activity and expression after one week of exposure and remained suppressed during the 8 week treatment period. Once the drug was withdrawn enzyme activity increased gradually. Activity remained below control levels after 2 weeks of withdrawing the drug. It took up to 4 weeks for the enzyme activity to return to control levels. The loss of activity of GSTz due to DCA treatment correlated with the loss of immuno-reactive protein observed in the westerns. This suggested that inactivated GSTz was degraded by proteolysis. Recovery of activity and protein followed different patterns. Protein expression did not return to control levels after 4 weeks of recovery. In fact amount of immunoreactive protein remained significantly lower than control even after 8 weeks of withdrawing DCA. Partial restoration (approximately 65% of controls) of DCA elimination capacity and hepatic GST-zeta activity occurred after 48 h recovery from 14 d 2.0 g/l DCA drinking water treatments in B6C3F1 mice (Schultz et.al. 2002). Pharmacokinetic studies in humans have shown that a wash-out period of several weeks is necessary before the kinetics return to pre-treatment levels (Stacpoole et.al. 1998). This indicates a species dependent difference in the rate of protein re-synthesis. Anderson et. al. (1999) studied the turnover of GSTz protein after a single dose of DCA

in Fisher rats. They reported the t1/2 of protein recovery to be 3 days. According to this study GSTz activity and expression returned to pre-treatment levels 10-12 days after the exposure. However our studies show that activity does not return to intial levels even after 2 weeks of withdrawing the drug. We see a return to control levels only after 4 weeks of recovery. The protein expression does not return to pre-exposure levels even after 8 weeks of recovery. The report by Anderson and co-workers also studied the recovery of GSTz protein which paralleled the recovery of GSTz activity. This discrepancy in our data and the data published by Anderson et.al. may be a function of the prolonged exposure period we used in this study. After 8 weeks of exposure the enzyme activity and expression are at very low levels and hence a longer recovery period is necessary for the enzyme to regain its activity. Schultz et. al. (2002) reported that interrupting protein re-synthesis by actinomycin-D blocked the recovery of GSTz activity. These studies show that, not just removal of the inhibitor but re-synthesis of protein is essential to regain the activity of the protein. This study further showed that effects of DCA are long lasting and that re-synthesis of the enzyme is gradual. The reasons for this slow synthesis of protein are currently unknown. Since DCA is excreted unchanged in the urine, it is unlikely that it lingers in the body and continues to inactivate the enzyme. It is possible that DCA or its metabolites may interfere with pathways of protein synthesis.

DCA treatment resulted in decreased maternal weight gain and increase in liver weight in pregnant rats (Smith et.al. 1992). This increase in liver weight after DCA exposure was attributed to possible hyperplasia or hypertrophy. Our studies show an increase in liver to body weight ratio in rats administered 50mg/kg DCA. But this ratio

returned to control levels once DCA was withdrawn. It is not known whether increase in liver weight was due to hypertrophy related to DCA exposure. Histopathology on liver tissue from these studies may be useful in determining whether DCA treatment led to hypertrophy.

High doses of DCA (50mg/kg) led to accumulation of MA in the liver which was excreted in the urine. Cornett et. al. (1999) had also shown a similar excretion of MA in the urine of rats treated with 200mg/kg DCA. This indicated DCA treatment perturbed tyrosine and led to accumulation of tyrosine metabolites. However MA was not detected in the urine after one week of withdrawing the drug. This is an interesting observation since GSTz which metabolizes MA was not completely recovered after one week of recovery. About 55% of GSTz activity and only 30% of GSTz protein recovered after one week of withdrawing DCA. This suggests that even low levels of GSTz are perhaps sufficient to metabolize endogenous MA and MAA resulting from tyrosine catabolism. Another reason for not detecting MA in the urine samples is probably the stability of this compound. We have observed that synthetic MA is not a stable compound and can isomerize to FA under aqueous conditions or degrade to other products. Thus it is possible that urinary MA may have degraded and hence its concentration was below the detection limit of our method. Urinary DCA levels increased during the 8 week treatment period. This increase in DCA levels showed that loss in GSTz activity inhibited the metabolism of DCA. The levels of monochloroacetate (MCA) were very low suggesting that conversion to MCA was not the predominant pathway of metabolism and that dechlorination to glyoxylate is the major route of biotransformation.

Exposure to DCA is mainly due to its presence in municipal drinking water or by ground water contamination, as observed at certain superfund sites. Daily exposure to DCA in non clinical setting is typically around 4μg/kg but may be exceeded at some sites. Previous studies with DCA have predominantly used a clinical dose of the drug (25mg/kg to 50mg/kg), which is several times higher than its concentration in environment. Since human exposure to DCA is widespread at μg/kg/day dose, it is important to determine the effects of DCA at this environmental dose. The present study has addressed these important aspects of DCA metabolism. In these studies exposure to DCA in the environment was mimicked by exposing rats to DCA in drinking water. Commercially available mineral water (Zephyrhills) was used to prevent the influence of DCA present in municipal drinking water. Analysis of this water showed that DCA concentrations in the water were below our method detection limits (25-1000 pg/ml, Jia et.al. 2003). It was observed that low doses of DCA significantly inhibited GSTz activity and expression. This effect was more pronounced in rats treated for longer duration suggesting a cumulative effect of exposure to DCA. This finding suggests that DCA concentrations found in some municipal drinking water alters hepatic GSTz. This is a significant discovery since it has important implications on human health. GSTz is an important enzyme involved in tyrosine catabolism and its inhibition may lead to accumulation of reactive tyrosine metabolites and toxicity related to such metabolites.

Finally we also looked at identifying possible adducts of DCA with recombinant hGSTz1c-1c which was expressed in E-coli. Adduct of the enzyme with DCA was identified and separated from unadducted enzyme by HPLC. This adduct was further characterized using various mass spectrometry methods. At least 3 adducts of the protein

with GSH were identified and two of them were further characterized by MS/MS. In both cases thiol of GSH formed a covalent disulphide bond with the thiol of a cysteine residue. Enzyme modeling studies showed that the cysteine 16 residue in the active site is located 2.8 Å from GSH (Polekhina et.al. 2001). Although this distance is not close enough to form a disulphide linkage we demonstrate the formation of a disulphide adduct of the thiol of GSH with the cysteinyl sulphur of cysteine 16. Anderson et. al. (2002) also showed the presence of similar adduct. A disulphide adduct of GSH was also identified with Cys 205 by MALDI-TOF. Cys 205 has been shown to react with MA and FA and cause inactivation of the enzyme (Lantum et.al. 2002b). This cysteine was also shown to bind to DTT when this compound was modeled in the crystal structure (Polekhina et.al. 2001). Catalytic significance of these adducts is unknown. Glutathione adducts have physiological importance since GSH may bind to the enzyme under cellular conditions. Similar adducts of GSH with GSTO1, GSTM4 and PmGSTB1 has also been reported (Board et.al. 2000, Rossjohn et.al. 1998, Cheng et.al. 2001)

Another interesting adduct was that of glyoxylate with the active site serine. The mechanism of this reaction may involve the hydroxyl of the serine which may act as a weak nucleophile and attack the carbonyl carbon of the acid. The resulting adduct has a hemiacetal like structure. GST proteins have a serine or a tyrosine residue in their active sites. These residues bind GSH and thus activate the thiol for reaction with xenobiotic substrates. Any modification of this residue may lead to inactivation and subsequent degradation of the protein. This adduct is also important because it demonstrates the ability of glyoxylate to react with protein nucleophiles. Reaction of glyoxylate with cellular proteins could possibly lead to toxicity.

It was surprising that glyoxylate did not react with cysteine since the cysteinyl thiol has a lower pKa than the hydroxyl group and is a stronger nucleophile. This result was consistent with a previous report in which glyoxylate was reacted with cysteine containing peptides. Glyoxylate did not react with the cysteine but reacted with other amino acid nucleophiles (Anderson et.al. 2004). Active sites of GST are characterized by the presence of serine and cysteine residues. The crystal structure of GSTz showed that serine at position 15 is oriented more favorably for interaction with GSH and xenobiotic substrates and that the hydroxyl of serine 14 points away from the active site (Polekhina et.al. 2001). But site directed mutagenesis showed that both the serines are essential for isomerase and GSH conjugation activities (Board et.al. 2003). Alignment of the hGSTZ1 sequence with other GSTs suggested that Ser-14 aligned with the active-site Ser-11 in the Theta-class GSTs and Ser-9 in the insect Delta class (Board et.al. 1997). The active site of GSTz isolated from *Arabidopsis thaliana* shows serines at positions 17 and 18 and a cysteine at position 19 (Thom et.al. 2001). The mutation of Ser-17 in the A. thaliana Zeta-class GST (equivalent to Ser-14 in hGSTZ1) reduces its activity to <2% and <6% of the wild-type activity with DCA and MA respectively. Above information suggests that zeta class of GSTs have a conserved SSC motif in their active site. All these residues play a role in either binding GSH or stabilizing the thiolate of GSH. Thus any modification of these residues, chemical or genetic may lead to inactivation of the enzyme.

Serine residues present in active sites have been known to form hemiacetal like tetrahedral adducts with enzyme inhibitors. Peptide aldehydes which are substrates for serine proteases react with the hydroxyl group of active site serine to form hemiacetals

(Kahayaoglu et.al.1997). Aldehyde type peptide inhibitors react with serine residues in pseudomonas carboxyl proteinase and hemiacetal type linkages and thus inactivate the enzyme (Oyama et.al. 2002). In our study also we find a similar reaction between the aldehyde of glyoxylate and the hydroxyl of serine to form a hemiacetal. Patil et.al (1989) demonstrated the formation of glyoxylate adducts with the active site of threonine dehydratase. It was proposed that this covalent interaction with the active site residues inactivates the enzyme.

Glyoxylic acid and its esters used in organic chemistry are stored as their hydrates and hemiacetal like compounds to avoid the rapid polymerization of the aldehyde. Glyoxylates are susceptible to nucleophilic attack by heteronucleophiles which results in the formation of the corresponding acetal like compounds (Meester et.al. 2003).

Other adducts of the enzyme with reaction intermediates were also observed. But these were not formed with the active site residues. Reactions occurred with aspartic acid residues of the protein. This may have been a reaction between glyoxylate and the carboxylic acid moiety of the aspartic acid. The two carboxylic acid groups may form an anhydride which will result in the mass increase of 57 Da. These findings suggest that glyoxylate is a reactive metabolite and is involved in covalent interactions with the enzyme. It is also clear that there are several sites on the enzyme which can be modified. Importance of these modifications in inactivating the enzyme is unknown. These adducts may target the enzyme for proteolysis.

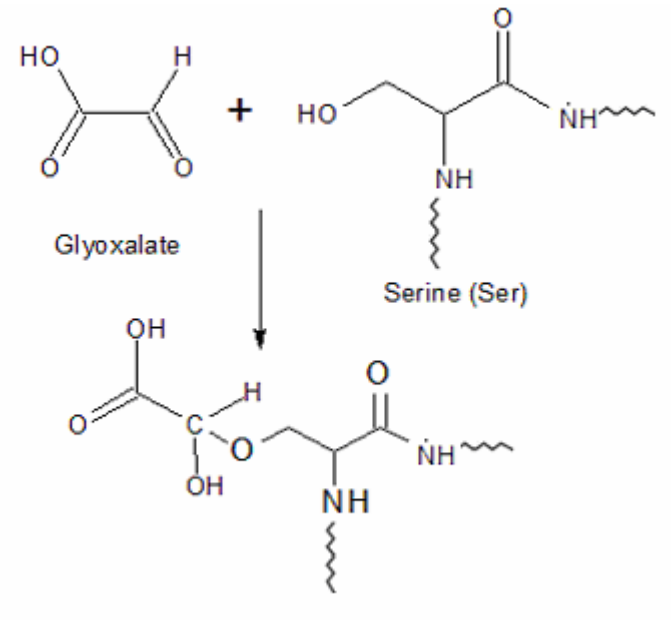


Fig 6-1 Proposed reaction between Serine and Glyoxylate. This reaction results in addition of 74 Da to the protein

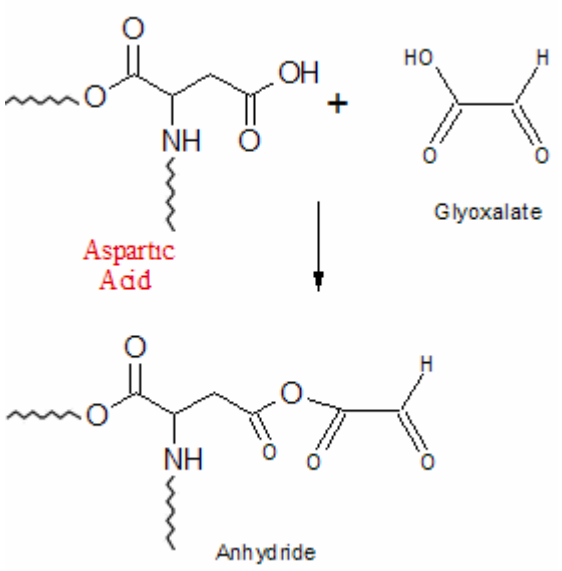


Fig 6-2 Reaction of glyoxylate and aspartic acid to form anhydride. This covalent interaction leads to mass increase of 56 Da

This study did not however identify any adducts of the active site peptide with DCA itself or with a reaction intermediate. Anderson et.al. (2002) identified an adduct of Cys 16 in the active site with S (chlorocarboxymethyl glutathione) which they

suggested may be the reason for inactivation of the enzyme. This study did not observe this adduct. It either may be a low abundance adduct or an unstable intermediate due to which is difficult to identify by the methods used in these studies. Future research could address isolating and purifying this enzyme and any adducts with DCA or its metabolites from cytosol of control and treated rats. This protein could then be characterized by mass spectroscopy. This will help identify the exact mechanisms of DCA's inhibition of GSTz.

Biological significance of adducts of glyoxylate with amino acids is not known. The toxicity of ethylene glycol has been attributed to its metabolite, glyoxylate (Richardson 1973). Glyoxylate caused partial inactivation glucose 6 phosphate dehydrogenase (Anderson et.al.2004). Aldehyde containing xenobiotics are known to play a role in toxicity. Croton aldehyde derived from crotyl alcohol was found to contribute to toxicity by forming adducts with cellular biomolecules (Fontaine et.al. 2002). A range of rat liver cytosolic proteins showed C^{14} label derived from C^{14}DCA (Anderson et.al. 2004).This raises the possibility that metabolism of DCA by GSTz may generate a bioactive metabolites which may react with biomolecules.

Toxicology and inactivation of GSTz by DCA is a complex mechanism which warrants further research. The involvement of MAAI in DCA toxicity is still unclear. Exposure to DCA and subsequent inactivation of MAAI may trigger other pathways leading to toxicity.

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BIOGRAPHICAL SKETCH

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