

AN ABSTRACT OF THE DISSERTATION OF

John C. L. Erve for the degree of Doctor of Philosophy in Toxicology presented on April 26, 1995. Title: Alkylation of Peptides and Proteins by S-(2-chloroethyl) glutathione and Characterization of Adducts by Mass Spectrometry.

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Abstract approved: _____

Donald J. Reed, Ph.D.

Glutathione, a ubiquitous intracellular tripeptide, protects cells by reacting with electrophiles and radicals that can damage cellular macromolecules. However, glutathione conjugation is now recognized as one mechanism by which drugs and xenobiotics can also be converted to reactive intermediates having toxic consequences to cells. The halogenated alkane 1,2-dichloroethane forms S-(2-chloroethyl) glutathione (CEG) upon glutathione conjugation which can cyclize to an episulfonium ion. The episulfonium ion of CEG is a potent electrophile that can alkylate the N⁷ position of guanine in DNA. To investigate the possible role of protein alkylation by CEG as a determinant in the toxicity of 1,2-dichloroethane, it is necessary to better understand the alkylation chemistry of CEG toward proteins. Although covalent binding has been known to be correlated with the toxicity of many chemicals, it is now thought covalent binding is selective towards specific protein targets. My work has attempted to determine if the alkylation event is also selective for certain amino acids in a protein. To answer the specific question of where the adducts derived from the episulfonium ion of CEG have formed, a combination of mass spectrometric techniques, including tandem mass spectrometry, were employed. As models, I employed one peptide, oxytocin, and two proteins, human hemoglobin and *E. coli* thioredoxin. The results of my work show that for the episulfonium ion of CEG, alkylation is highly selective for cysteine over other amino acids with tyrosine being the next most reactive amino acid residue. However, the episulfonium ion is also selective amongst protein thiols as certain cysteine residues are not alkylated, possibly due to steric hindrance and/or charge effects.

**Alkylation of Peptides and Proteins by S-(2-chloroethyl)glutathione and
Characterization of Adducts by Mass Spectrometry**

by

John C. L. Erve

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Table of Contents

	<u>Page</u>
Chapter I	
Introduction	1
Chapter II	
Background	12
Covalent Binding	12
Dichloroethane	16
Biological Mass Spectrometry	20
Summary of Objectives	25
Chapter III	
Alkylation of Oxytocin by S-(2-chloroethyl)glutathione and Characterization of Adducts by Tandem Mass Spectrometry and Edman Degradation	26
Abstract	27
Introduction	28
Materials and Methods	30
Results	33
Discussion	43
Acknowledgments	47
Chapter IV	
Alkylation of <i>E. Coli</i> Thioredoxin by S-(2-chloroethyl)glutathione and Identification of the Adduct on the Active site Cys-32 by Mass Spectrometry	48
Abstract	49
Introduction	50
Materials and Methods	51
Results	55
Discussion	65
Acknowledgements	70

Table of Contents, continued

	<u>Page</u>
Chapter V	
Determination of Hemoglobin Sulfhydryl Status Following Alkylation by S-(2-chloroethyl)glutathione with Mass Spectrometry and Spectrophotometry . .	71
Abstract	72
Introduction	73
Materials and Methods	75
Results	82
Discussion	88
Acknowledgments	93
Chapter VI	
Conclusion	94
Summary	94
Future Work	95
Bibliography	97
APPENDICES	114
APPENDIX A	115
APPENDIX B	117

List of Figures

<u>Figure</u>	<u>Page</u>
1.1 Structure of glutathione showing the component amino acids.	1
1.2 Biosynthesis of GSH.	2
1.3 Mercapturic acid biosynthesis showing transformation of naphthalene.	4
1.4 Glutathione redox cycle.	6
1.5 Mechanism of formation of the episulfonium ion of CEG.	8
1.6 Possible mechanism of thioredoxin catalyzed reduction of protein disulfides.	11
1.7 The reactive metabolite of acetaminophen, NAPBQI, can be detoxified by sulfation, glucuronidation or GSH conjugation.	14
1.8 Metabolism of 1,2-dichloroethane showing two activation pathways.	19
1.9 Electrospray mass spectrum of <i>E. coli</i> thioredoxin obtained on a Sciex III plus triple quadrupole instrument.	22
1.10 Nomenclature for fragment ions obtained during CID.	24
2.1 Structures of compounds referred to in the text.	34
2.2 HPLC analysis of reduced oxytocin and its adducts for (A) an equimolar mixture of CEG:oxytocin and (B) a 5:1 molar mixture of CEG:oxytocin	35
2.3 Spectrum of daughter ions formed by CID of the $[M+2H]^{+2}$ parent (m/z 1342) of the mono-adduct-homo-dimer.	36
2.4 Spectrum of daughter ions formed by CID of the $[M+2H]^{+2}$ parent (m/z 672) of mono adduct Cys-1 (A), and mono adduct Cys-6 (B).	38
2.5 Spectrum of daughter ions formed by CID of the $[M+2H]^{+2}$ parent (m/z 838) of the bis-adduct of reduced oxytocin.	39
3.1. <i>E. coli</i> thioredoxin protein sequence showing tryptic peptides.	55
3.2. HPLC analysis of reduced thioredoxin and its adducts	56

List of Figures, continued

3.3. MALDI/MS spectrum of (A) mono-alkylated reduced thioredoxin at m/z 12,004 and (B) bis-alkylated thioredoxin at m/z 12,340 following collection off the C8 HPLC column.	57
3.4. MALDI/MS spectrum of the tryptic digests of (A) mono adduct and (B) bis adduct.	58
3.5. Chromatographic separation of peptides produced following the tryptic digestion of (A) native thioredoxin and (B) thioredoxin mono-adduct.	59
3.6. (A) Ion-spray mass spectrum of alkylated T3 showing doubly and triply charged species.	62
3.7. Medium resolution FAB mass spectrum of isotopic pattern of T4 with m/z 2,141 obtained from a tryptic digest of oxidized thioredoxin alkylated with a 50% mixture of ^{15}N -labeled CEG	64
3.8. HPLC chromatogram of thioredoxin treated with IAA and CEG at pH 8.5	66
4.1. FAB mass spectrum of (A) crude CEG preparation showing CEG present as sodium adducts and (B) purified CEG.	76
4.2. Preparative HPLC chromatogram recorded during purification of CEG.	77
4.3. Calibration lines for cyanomethemoglobin standards at 540 nm.	79
4.4. Measurement of the molar extinction coefficient (ϵ) at 324 nm for (A) PDS and (B) hemoglobin.	81
4.5. HPLC chromatograms of hemoglobin treated with increasing amounts of CEG.	83
4.6. MALDI mass spectrum of (A) α -chain and three adducts and (B) β -chain and two adducts.	84
4.7. The ratio of sulfhydryl to hemoglobin concentration following treatment with 100X, 250X and 500X fold excess CEG.	85
4.8. Absorption spectra of hemoglobin and CEG-treated hemoglobin.	87
4.9. FAB/MS of a digest from a CEG-treated hemoglobin β chain.	89

List of Tables

<u>Table</u>	<u>Page</u>
1.1 Physical and Chemical Properties of 1,2-Dichloroethane	17
2.1 Edman degradation results for oxytocin and adducts.	42
2.2 Product ions for the bis-adduct, showing loss of glutamic acid and/or glycine from CEG.	45
3.1 Predicted and experimental masses of E. coli thioredoxin treated with CEG and digested with trypsin.	60
4.1 Calculated and observed V8 digest fragments of CEG alkylated hemoglobin by FAB mass spectrometry.	86
A.1 Samples used in the CEG assay	116

List of Abbreviations

GSH	glutathione
GSSG	glutathione disulfide
CEG	S-(2-chloroethyl)glutathione
HEG	S-(2-hydroxyethyl)glutathione
TCEP	tris-(2-carboxy)ethyl phosphine
CID	collisionally induced dissociation
FAB	fast atom bombardment
MALDI	matrix-assisted laser desorption-ionization
PITC	phenylisothiocyanate
DCE	1,2-dichloroethane
BCE	1-bromo-2-chloroethane
DHA	1,2-dihaloalkane
PDS	4,4'-dithiopyridine
GST	glutathione S-transferase
DTT	dithiothreitol
TFA	trifluoroacetic acid
IAA	iodoacetamide
TRX	thioredoxin
MIC	methylisocyanate
BSO	buthionine sulfoximine
NAPBQI	N-acetyl-p-benzoquinoneimine
γ -GT	γ -glutamyltransferase
γ -GCS	γ -glutamylcysteine synthetase
DBCP	1,2-dibromo-3-chloropropane
MS/MS	tandem mass spectrometry
PAH	polycyclic aromatic hydrocarbons
CA III	carbonic anhydrase III

Alkylation of Peptides and Proteins by S-(2-chloroethyl)glutathione and Characterization of Adducts by Mass Spectrometry

Chapter I

Introduction

The compound known as glutathione (GSH), first characterized chemically in 1929 by F. G. Hopkins, is present in animal cells and also in many plants and bacteria. It has been the subject of extensive investigation due to its importance in biology. Glutathione is a tripeptide composed of γ -glutamyl-L-cysteinylglycine; the cysteine thiol group has a pK_a of approximately 8.6 which is important for many of its functions (Reed, 1985). The bond between cysteine and glutamic acid is not a normal peptide bond, but has a γ -glutamyl link formed through the γ -carboxylate of glutamate with the α -amino group of cysteine (Figure 1.1); this serves to make it resistant to degradative peptidases. The cellular concentrations of reduced GSH are normally millimolar, ranging from 0.5 mM to 10 mM. It is now known that there are two distinct pools of GSH present in hepatocytes (Edwards and Westerfield, 1952; Riley and Lehninger, 1964). The cytosolic pool is the largest with approximately 85-90% of GSH; the remaining GSH is present in the mitochondria (Reed, 1985).

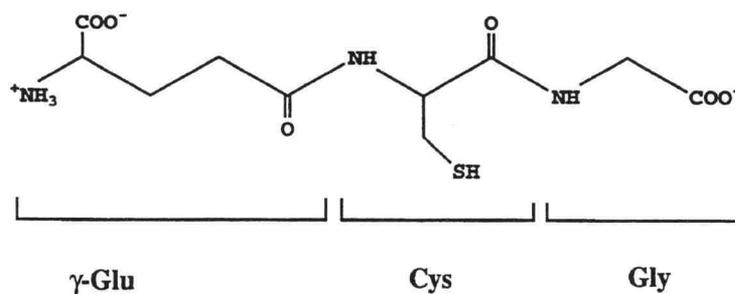


Figure 1.1 Structure of glutathione showing the component amino acids.

Synthesis of GSH takes place intracellularly and requires ATP and the sequential activity of two enzymes: γ -glutamylcysteine synthetase (γ -GCS) (EC 6.3.22) and GSH synthetase (EC 6.3.2.3) (Figure 1.2). Regulation of GSH synthesis can occur by feedback inhibition in which GSH controls γ -GCS activity. Recent work suggests that oxidative stress induced by quinones may increase GSH levels by a mechanism that elevates γ -GCS mRNA expression (Shi, et al., 1994). Inhibition of GSH synthesis has been studied due to its potential application in cancer medicine, whereby depleting cancer cells of GSH is hypothesized to sensitize them to chemotherapy or radiation. GSH synthesis can be decreased by buthionine sulfoximine (BSO), which inhibits γ -glutamylcysteine synthetase. However, when treating normal and transformed human lung fibroblast cells with BSO, normal cells were found more sensitive to the cytotoxic effects of BSO (Wan and St. Clair, 1993).

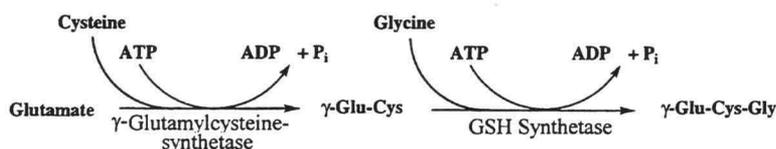


Figure 1.2 Biosynthesis of GSH.

GSH is an important part of the cell's defense mechanism towards xenobiotics because of its ability to detoxify reactive chemicals (Reed, 1985). The soft nucleophilic thiol is able to react non-enzymatically with soft electrophiles, such as, N-acetyl-p-benzoquinoneimine (NAPBQI) or 4-hydroxynonenal. Reactive hard electrophiles, such as, benzo[a]pyrene-7,8-diol-9,10-epoxide, require enzymatic conjugation by glutathione S-transferase (GST) (Ketterer, 1986). GSH conjugation, either enzymatic or non-enzymatic, serves two purposes: 1) eliminating the ability of a reactive electrophile to combine with critical cellular macromolecules by forming a chemically stable thioether, and, 2) creating a species with greater hydrophilic character that is more readily excreted from the body.

The GST enzymes catalyze GSH conjugation with drugs and chemicals having diverse structures and are therefore considered the most important enzymes for detoxification of electrophilic compounds (Armstrong, 1991). They were originally classified according to their isoelectric properties and substrate specificities, but this has been replaced in recent years in favor of a classification system based on genetics. The mammalian cytosolic enzymes are organized into four classes: α , μ , π and θ . Species differences do exist, however, with respect to the expression of certain enzymes in the liver. For example, enzymatic GSH conjugation is involved in detoxification of the putative carcinogenic metabolite of aflatoxin, AFB-8,9-exo-epoxide. Mice express high levels of the α -class GST isozyme responsible for this reaction which makes mice resistant to the carcinogenic effects of aflatoxin. In contrast, the constitutively expressed enzymes in humans have little activity towards reactive metabolites of aflatoxin B1, which suggests that humans should be quite sensitive to the genotoxic effects of aflatoxin (Eaton and Gallagher, 1994). In humans, enzyme polymorphisms exist and may increase or decrease a person's susceptibility to a chemical. Differential susceptibility is illustrated by the finding that a GST, belonging to the θ -class found in erythrocytes, capable of conjugating a number of halogenated methanes to the S-methylglutathione conjugate, is present only in approximately 60% of humans tested (Hallier, et al., 1993; Pemble, et al., 1994).

Many GSH-conjugates formed in the liver are metabolized to mercapturates by the sequential action of several enzymes, followed by excretion in the urine; this is the mercapturic acid biosynthetic pathway (Figure 1.3). The enzymes γ -glutamyl transferase (γ -GT) and cysteinylglycine dipeptidase are membrane bound enzymes that catalyze the extracellular cleavage of glutamyl and glycyl residues respectively, from the GSH-conjugate. N-acetyltransferase is a microsomal enzyme, found in both kidney and liver, that acetylates the amine functionality of the cysteine conjugate and requires the acetyl donor acetyl CoA. γ -GT and dipeptidases are found in highest concentration in the kidney and it was assumed for some time that mercapturic acid biosynthesis was exclusively an interorgan process whereby the GSH-conjugate synthesized in the liver was transported to the kidney for catabolism. Recent evidence

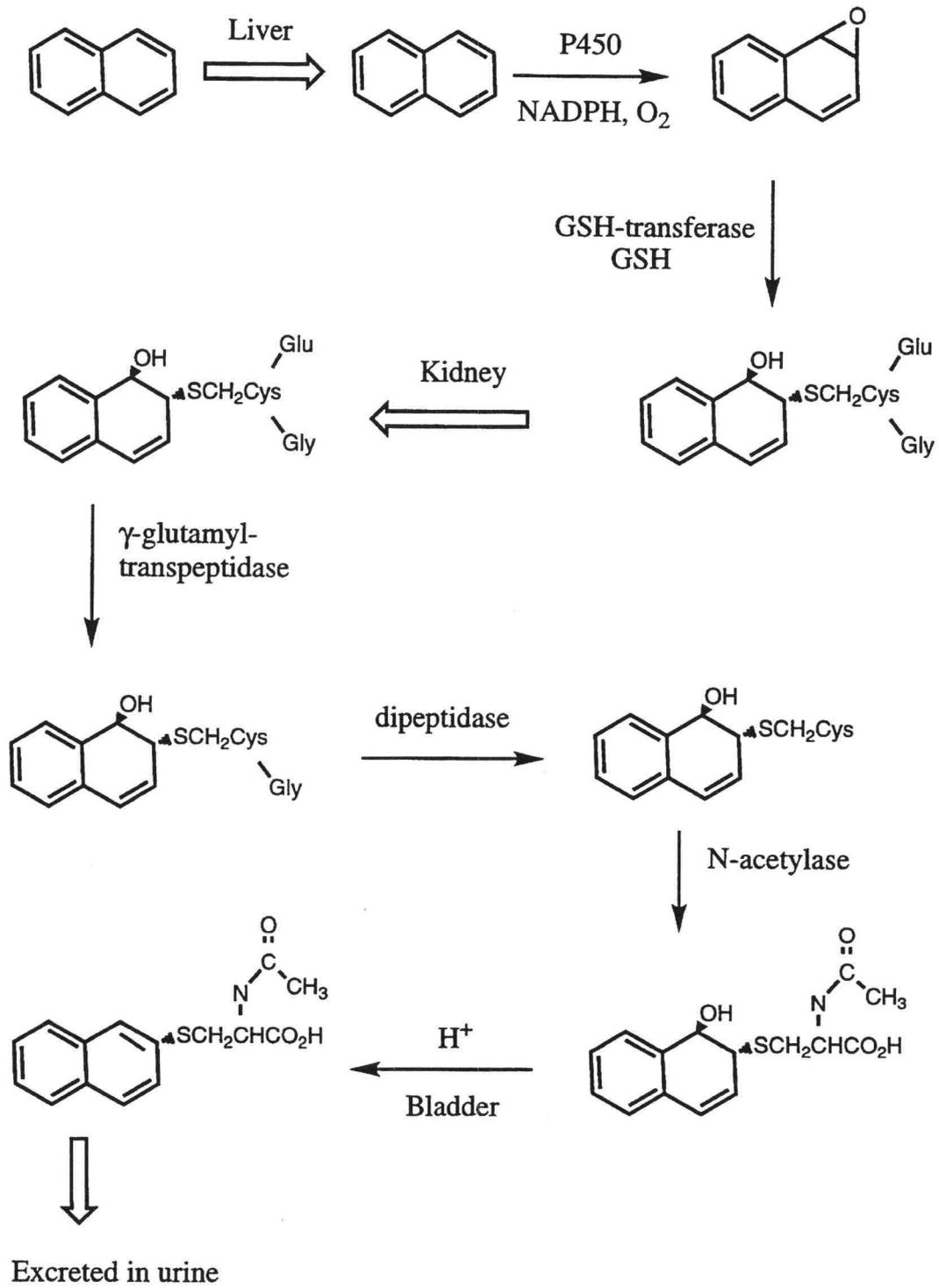


Figure 1.3 Mercapturic acid biosynthesis showing transformation of naphthalene.

indicates that xenobiotics may be converted to mercapturates exclusively in the liver via the intrahepatic γ -glutamyl cycle (Hinchman and Ballatori, 1994). In this process, GSH-conjugates formed in the liver are transported across the canicular membrane into the bile where they are broken down to cysteine conjugates. After reabsorption by the liver, the cysteine-conjugates are N-acetylated and reexcreted. Critical to either pathway is the transport of the GSH-conjugate out of the hepatocyte. Ishikawa has characterized an ATP-dependent GSH-conjugate export pump (Ishikawa, 1992). Removal of GSH-conjugates is important because their accumulation can decrease the efficiency of GST enzymes and thereby decrease detoxification reactions. For example, several GSH-conjugates of halogenated quinones have been shown to irreversibly inactivate GST (van Ommen, et al., 1991).

GSH is also important as a defense against reactive oxygen species generated as a consequence of aerobic metabolism (Cotgreave, et al., 1988). Reactive oxygen species include hydrogen peroxide (H_2O_2), superoxide anion radical, hydroxyl radical and singlet oxygen. GSH can serve as an intracellular reductant by functioning as a cosubstrate for selenium dependent GSH-peroxidase; this enzyme can convert H_2O_2 to H_2O with the simultaneous production of glutathione disulfide (GSSG). GSH readily donates a hydrogen atom to most carbon- and oxygen- radical species to yield the thiyl radical of GSH, two of which combine to form GSSG. Glutathione reductase has the primary role of catalyzing the reduction of GSSG to GSH in a reduced pyridine nucleotide (NADPH) mediated reaction; in conjunction with GSH peroxidase, this constitutes the GSH redox cycle (Figure 1.4).

Currently, much data has accumulated on the effects of GSH and its relation to the formation of senile cataracts. Cataracts is a condition of opacity in the lens and is commonly associated with the aging process and oxidative stress. When suckling mice were exposed to BSO, decreased GSH levels and electrolyte changes were observed preceding cataract formation (Calvin, et al., 1992). In humans, one study showed an inverse relationship between the amount of γ -glutamylcysteine synthetase activity and degree of subcapsular cataract (Rathbun, et al., 1993). Finally, administration of GSH isopropyl ester (YM737) to rats significantly delayed the x-ray induced development

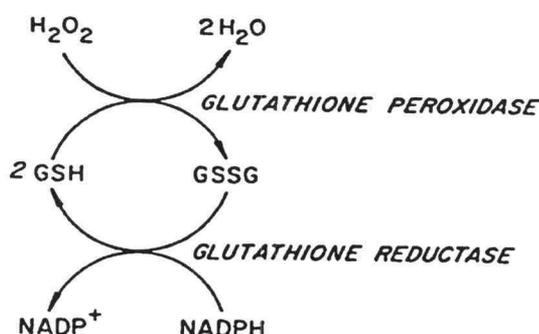


Figure 1.4 Glutathione redox cycle

of cataracts (Kobayashi, et al., 1993). Taken together, experimental evidence supports the hypothesis that chronic damage by oxidative stress produces cataracts and that GSH can have a beneficial role in preventing them.

Several endogenous compounds are converted to pharmacologically active compounds following GSH or cysteine conjugation. The best characterized to date are the leukotrienes, compounds which comprise the slow-reacting substance of anaphylaxis (Slauson and Cooper, 1990) and are potent smooth muscle spasmogens. Leukotriene C₄ (LTC₄) arises from GSH conjugation with the epoxide function of LTA₄ catalyzed by LTC₄ synthase. As a consequence of glutathione synthetase deficiency in afflicted individuals, LTC₄ is significantly reduced in monocytes and neutrophils which might be involved in the pathophysiology of glutathione synthetase deficiency disease (Mayatepek, et al., 1993). There has been a great deal of work focusing on the nitric oxide molecule and its numerous physiologic functions (Koshland, 1992). Recent work has suggested a role for GSH in mediating the bioactivity of NO through the formation of a GSH adduct of NO. Evidence suggests that this S-nitrosothiol might be the compound with biologic activity. It has been reported that S-nitrosothiols (RS-NO), in particular the GSH adduct, are present to varying degrees in different pathologic states in humans and are powerful relaxants of bronchiole muscle (Gaston, et al., 1993). In addition, i.v. administration of S-nitrosoglutathione reduced the blood pressure in dogs and monkeys to the same degree

and with the same rate as sodium nitroprusside (Park, et al., 1993), a drug used in the treatment of hypertension. More work remains to be performed before definite conclusions can be made regarding the connection between GSH and nitric oxide. Nevertheless, these examples suggest the possibility that other GSH conjugates yet to be discovered may have important pharmacologic functions.

Although GSH functions to protect cells from reactive intermediates, it has become increasingly clear that GSH can also enhance the toxicity of many chemicals (Monks and Lau, 1994). These are termed toxification reactions to distinguish them from detoxification reactions. An enzyme often associated with toxification reactions is cysteine conjugate β -lyase (β -lyase) which acts on cysteine conjugates formed by the mercapturic acid pathway. β -lyase contains pyridoxal phosphate and is present in the renal proximal tubule. It splits the bond between cysteine and the conjugate releasing a reactive species that can covalently bind to tissue macromolecules. Several aliphatic halogenated alkenes are potent nephrotoxicants as a result of the activity of β -lyase (Commandeur and Vermeulen, 1990). As an example, hexachloro-1,3-butadiene (HCBD) is nephrocarcinogenic when chronically administered to rats and mutagenic in bacteria. The mutagenicity was reduced in the presence of the aminoxyacetic acid, a β -lyase inhibitor used to differentiate pathways dependent on β -lyase, implying that this enzyme is required for activation of HCBD. Conjugation of certain quinones with GSH also results in the formation of selective nephrotoxicants. Bromobenzene is metabolized to 2-bromo-(di-glutathion-S-yl)hydroquinone and other isomers which may react with cellular macromolecules directly or generate reactive oxygen species (Monks and Lau, 1994).

An alternative mechanism of GSH-conjugate mediated toxicity occurs when the initial conjugation reaction is reversible (Baillie and Kassahun, 1994). Spontaneous reversibility of the GSH-conjugate bond appears to be limited to certain chemical classes, including isothiocyanates, isocyanates, α,β -unsaturated carbonyl compounds, formamides, alkyl carbamates and ureas (Baillie and Slatter, 1991). The significance of reversibility is that certain chemically reactive compounds can be transported as the GSH conjugate to distant target organs where the chemical is released to exert toxic

effects. Methylisocyanate (MIC), the chemical released during the Bhopal disaster, causes toxicity both to pulmonary and extra-pulmonary target organs (Lepkowski, 1994). Toxicity is thought due to carbamylation of critical macromolecules following release of MIC from GSH; this demonstrates the ability of GSH to carry MIC from the lungs to the kidney.

The vicinal dihaloalkanes undergo a unique activation mechanism upon GSH conjugation. The first step in activation by GSH conjugation is the enzymatic displacement of a halogen ion by the thiol group of GSH catalyzed by GST. The conjugation product, S-(2-haloethyl)GSH, is known as a sulfur half-mustard. Rearrangement of the sulfur mustard can occur by intramolecular displacement of the remaining halogen (β -elimination) by sulfur (Figure 1.5). The resulting episulfonium ion, also known as a thiiranium ion, is an electrophile capable of reacting with

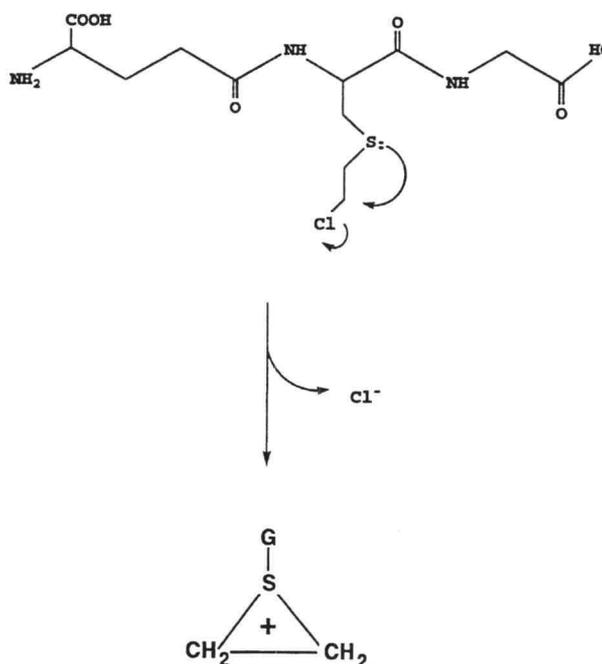


Figure 1.5 Mechanism of formation of the episulfonium ion of CEG.

nucleophilic sites on tissue macromolecules such as DNA and proteins (Dohn and Casida, 1987; Smit, et al., 1978). Although the half-life of S-(2-bromoethyl) glutathione is less than 10 seconds, it is stable enough to diffuse from hepatocytes where it is formed and adduct endogenously added DNA (Guengerich and Ozawa, 1983). The major adduct has been identified as S-[2-(N⁷-guanyl)-ethyl]glutathione. The formation of the episulfonium ion is highly dependant on suitable stereochemistry; this is demonstrated by the lack of mutagenicity of 1,3 and 1,4-dibromobutane, presumably, because increased chain length hinders intramolecular displacement of bromine by sulfur. The leaving group ability of the halogen substituent also is a determinant in mutagenicity. The higher mutagenicity of 1,2-dibromoethane (DBE) compared to 1,2-dichloroethane (DCE) is explained on the basis of better leaving ability of bromine as compared to chlorine when forming their respective episulfonium ions (van Bladeren, et al., 1981a). The agricultural chemical 1,2-dibromo-2-chloropropane (DBCP) can be conjugated with GSH to form an acutely toxic conjugate that causes renal necrosis and testicular atrophy (Kluwe, 1981); the conjugate is not, however, mutagenic in bacterial assays (Monks and Lau, 1994). The formation of two possible episulfonium ions allows for several different adducts to be formed with DNA as was found to be the case by Humphreys and coworkers. Reaction of synthetically prepared conjugate with calf thymus DNA resulted in the formation of three major N⁷-guanyl adducts, and one O⁶, N⁷-dialkylated adduct (Humphreys, et al., 1991). A bis-guanyl adduct was also identified illustrating the possibility that DBCP might serve as a cross-linking agent which might explain the toxic effect of DBCP in vivo.

S-thiolation is the general term denoting the reversible oxidation of protein thiols by disulfides and describes the combination of low molecular weight thiols with protein sulfhydryls (Ziegler, 1985). When reaction is with GSH, cysteine or cysteamine, for example, the process is known as S-glutathiolation, S-cystelation or S-cystamylation, respectively (Thomas, et al., 1994). It is known that increased oxidation of GSH to GSSG can lead to protein S-thiolation in vivo by protein/disulfide exchange (Reed, 1990). It is also possible that a radical initiated process may lead to

S-thiolation as well. Many proteins are known to undergo S-thiolation (Thomas, Chai and Jung, 1994) but the consequences of S-thiolation are known for only a few proteins. Evidence exists that support the hypothesis that protein S-thiolation is one mechanism by which the activity of certain enzymes may be modulated by changes in the cellular thiol/disulfide redox potential (Ziegler, 1985). For example, the activity of the glycolytic enzyme phosphofructokinase has been shown to be regulated by a reversible thiol/disulfide exchange with micromolar concentrations of GSSG and other biologically occurring thiols (Gilbert, 1982). The reverse reaction whereby the protein thiol is regenerated by a reduced thiol is known as dethiolation and can be catalyzed by certain redox active enzymes, such as, thioredoxin (Park and Thomas, 1989).

Chemicals that generate reactive oxygen species may result in extensive S-thiolation of cellular proteins and may be a common response to oxidative stress (Chai, et al., 1994). Protein thiol depletion is considered as a possible mechanism of toxic cell death as it has been observed that loss of protein thiols often proceeds loss of cell integrity (Snyder, 1990). In hepatocytes treated with diquat, t-butyl hydroperoxide or menadione, the sulfhydryl enzyme carbonic anhydrase III (CA III) formed three different S-thiolated species as determined by isoelectric focusing. The biological significance, however, of CA III S-thiolation is unclear as alkylation of the two reactive sulfhydryls at cys-183 and cys-188 had no effect on enzymatic activity. It has been suggested that S-thiolation of CA III may serve to protect other more critical enzymes during oxidative stress conditions and prevent the formation of irreversibly oxidized enzymes (Chai, Hendrich and Thomas, 1994). It is also conceivable that CA III can trap reactive electrophilic chemicals and thereby protect critical thiol groups.

Protein disulfides formed as a result of oxidative stress can inactivate vicinal dithiol containing enzymes and cannot be reduced by physiologic concentrations of GSH (Gitler, et al., 1994). The explanation for the inability of GSH to reduce protein disulfides is that GSH initially forms a mixed disulfide with the enzyme (protein - (SH)-SSG). The partially reduced protein has a thiol in close proximity to the mixed

disulfide resulting in a high effective thiol concentration. Thus, the most likely reaction is not with GSH but with the nearby protein sulfhydryl resulting in the reformation of protein disulfide. Protein disulfide reductases can fulfil the important function of restoring protein thiols. The reductive functions of thioredoxin are due to the low pK_a of the active site thiol on cys-32, which makes this thiol extremely reactive; the low pK_a of the active site thiol is thought due to interaction with basic residues around the active site (Holmgren, 1985). Thioredoxin is a component of the thioredoxin system which along with thioredoxin reductase and NADPH, can reduce oxidized proteins (Holmgren, 1989) some ten thousand times more effectively than DTT (Gitler, Mogyoros and Kalef, 1994). A "ping-pong" mechanism is thought to be involved in the reductive process, as illustrated in Figure 1.6. It has also been reported that thioredoxin can completely regenerate glyceraldehyde 3-phosphate dehydrogenase (G3PDH) that has been oxidized by hydrogen peroxide. Oxidation of G3PDH is thought to result in a sulphenic acid rather than disulfide formation so that it appears that thioredoxin can also reduce monothiol oxidized proteins containing the cysteine sulfhydryl as sulphenic acid (Fernando, et al., 1992). In contrast, glutaredoxin, also known as thioltransferase, is more efficient than thioredoxin at reducing enzymes containing mixed disulfides than enzymes inactivated by mono thiol oxidation to sulfenic or sulfinic acid (Yoshitake, et al., 1994).

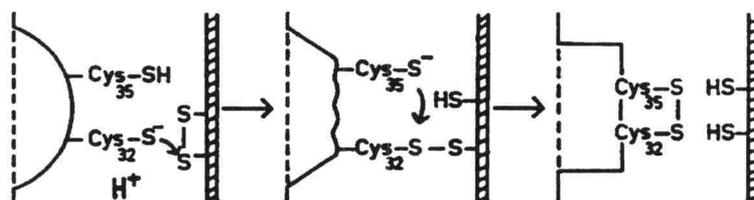


Figure 1.6 Possible mechanism of thioredoxin catalyzed reduction of protein disulfides. Adapted from Holmgren.

Chapter II

Background

Covalent Binding

Xenobiotics can interact with proteins in a number of ways. Drug binding to a specific receptor which elicits a particular cellular response is a basic tenet of pharmacology. In general, drugs bind reversibly so that the drug action is transient. In the field of toxicology, an important area of study is the action of enzymes on xenobiotic substrates to transform them chemically. In contrast, many xenobiotic chemicals can, either directly or following metabolic activation, irreversibly bind to macromolecules and cause toxicity. Metabolic transformations can be classified into Phase I and Phase II metabolism. Phase I metabolism, by the P450s and flavin monooxygenases, generally utilizes molecular oxygen to oxidize the xenobiotic thereby introducing functional groups such as, hydroxyl or carbonyl functionalities. Phase II metabolism achieves the formation of a more water soluble compound by glucuronidation or sulfation of the product produced by Phase I metabolism. Many chemicals, however, are converted to electrophilic metabolites, electron deficient species that react readily with electron rich centers. Reaction of electrophilic metabolites with nucleophilic sites in macromolecules is central to many different types of toxicity including carcinogenesis, mutagenesis, cellular necrosis, hypersensitivity reactions, methemoglobinemia, hemolytic anemia, blood dyscrasias and fetotoxicities (Gillette, et al., 1974). Although much information exists regarding the reactions that produce toxic metabolites and the chemical disposition of these metabolites, less is known about how they target cellular constituents and how these interactions cause cell injury (Nelson and Pearson, 1990).

Historically, the concept of electrophilic metabolites was developed by Elizabeth and James Miller in their studies to elucidate the mechanism of chemical

carcinogenesis (Miller and Miller, 1966). Although they initially studied binding of a metabolite of the chemical dye, N,N-dimethyl-4-aminoazobenzene, to hepatic protein which was found to precede induction of tumors (Miller and Miller, 1947), attention turned to DNA after discovery of its helical duplex structure in 1953. (Watson and Crick, 1953). Interaction of electrophiles with DNA is a common property of carcinogens (Miller and Miller, 1969). A chemical is called a "procarcinogen" prior to metabolism and is converted to a "proximate" and finally, to an "ultimate" carcinogen which is electrophilic (Miller and Miller, 1981). Metabolism to electrophiles is the common property that explains how structurally diverse chemicals, such as the classic chemical carcinogens, 2-acetylaminofluorene, 4-aminobiphenyl and 2-naphthylamine, can react with DNA. Exceptions are the direct acting alkylating agents that form electrophiles without metabolic activation, such as, the nitrogen mustard mechlorethamine. The structural characteristics of many DNA-reactive metabolite adducts have been determined; this information has allowed progress to be made in answering the question of how alteration in structure initiates carcinogenesis. In contrast to DNA and carcinogenesis, much less is known regarding the role that protein covalent modification plays in producing acute lethal cell injury. Reactive drug metabolites and protein covalent binding has been extensively studied with respect to a role in causing the toxicity of certain drugs (Hinson and Roberts, 1992; Nelson and Pearson, 1990). This line of research was initiated in the late 1960's and early 1970's by Brodie, Gillette and coworkers at the National Institutes of Health (Gillette, Mitchell and Brodie, 1974). Protein binding of carcinogens has also been investigated extensively. The focus of these studies either investigates basic mechanisms of carcinogen interactions or, to develop methods of biomonitoring exposure to carcinogens in the work place (Ehrenberg, et al., 1974).

It is believed that there are two general mechanisms by which protein binding can lead to toxicity: 1) inactivation of a critical regulatory protein(s) or enzymes; and, 2) formation of a neoantigen that can induce an immune hypersensitivity reaction. One extensively studied drug illustrating the first possibility is the analgesic acetaminophen. P450 metabolism of acetaminophen forms a toxic metabolite, N-

acetyl-p-benzoquinoneimine (NAPBQI). Once formed, NAPBQI may be detoxified by GSH conjugation, sulfation or glucuronidation (Figure 1.7). However, when these

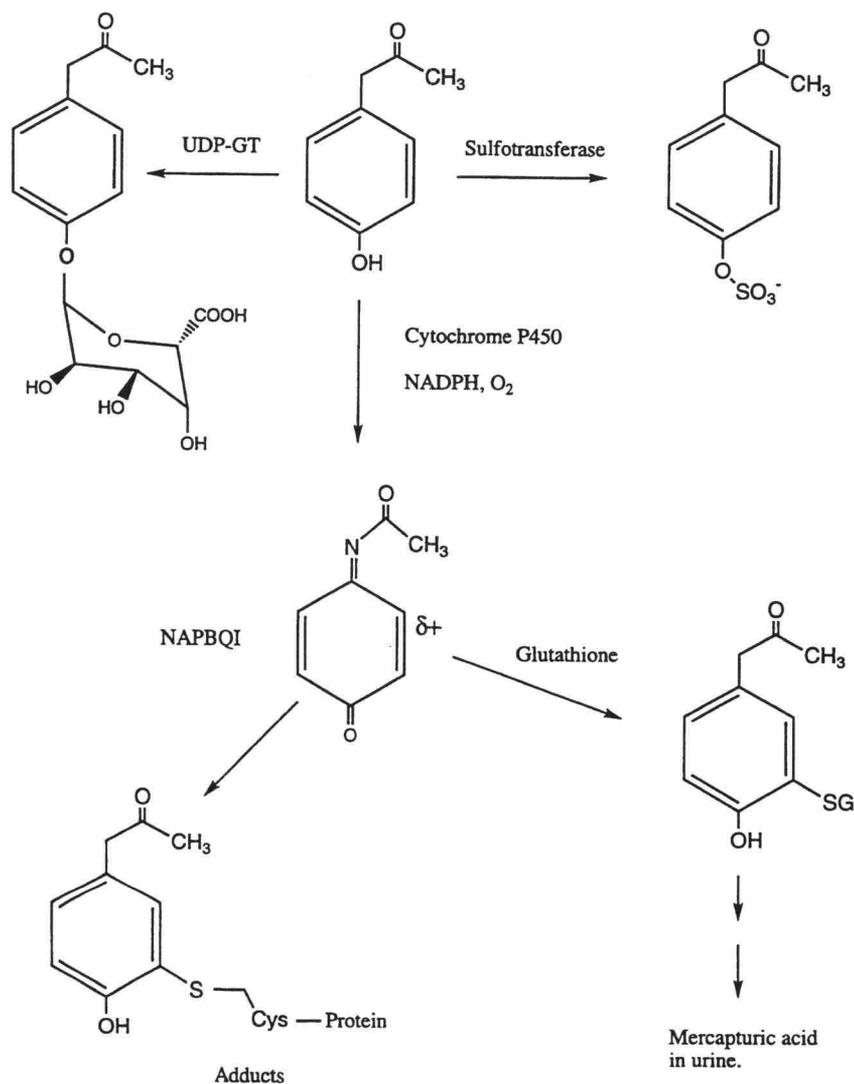


Figure 1.7. The reactive metabolite of acetaminophen, NAPBQI, can be detoxified by sulfation, glucuronidation or GSH conjugation. When these protective pathways are exhausted, NAPBQI reacts with cellular macromolecules resulting in toxicity.

detoxification pathways are overwhelmed, centrilobular hepatic necrosis occurs and is seen in both experimental animals and humans; necrosis is correlated with hepatic protein binding (Hinson and Roberts, 1992). On a molecular level, NAPBQI reacts with cysteine to form the 3-(cystein-S-yl)acetaminophen-protein adduct as determined by mass spectrometry (Hoffmann, et al., 1985). A 44 kDa protein primarily localized in the microsomal fraction has been identified as the earliest protein to undergo modification (Birge, et al., 1991). A 56 kDa selenium binding protein has also been identified as a target of acetaminophen binding (Pumford, et al., 1992). Although these targets have been identified, it is not known if an essential cellular activity has been affected accounting for the observed toxicity because the functions of these proteins are not known.

Idiosyncratic toxicities to xenobiotics are difficult to predict and usually do not develop immediately, but rather have a latent period. Adverse idiosyncratic reactions to xenobiotics are characterized by dose independence, are difficult to reproduce in animals and do not occur in all humans (Pohl, et al., 1988). Some idiosyncratic reactions might have a congenital basis, such as, glucose-6-phosphate dehydrogenase deficiency leading to hemolytic anemia; others are known to have an immunologic basis. Reactive metabolites can combine with protein to form adducts which initially result in the development of antibodies against the altered protein (Hinson, et al., 1994; Pohl, 1993). Subsequent exposure to the chemical produces a toxicity as a result of a hypersensitivity immune response. To date, the most studied drug producing this type of effect has been halothane. Halothane, a halogenated alkane with molecular formula 2,2-dichloro-1,1,1-trifluoroethane, produces "halothane hepatitis" in the liver in a certain fraction of people (1 in 10-20,000) administered this anesthetic (Pohl, Satoh and Christ, 1988). Halothane can be metabolized by P450 2E1 either in a reductive or oxidative process. It is believed that the oxidative pathway, however, is more important to toxicity due to the formation of a reactive trifluoroacetyl halide metabolite (Gandolfi, et al., 1980). After formation, the metabolite is capable of acylating lysine amino acids giving trifluoroacetyl-lysine-protein adducts. Several major protein targets of known function have been identified

using antibodies that recognize trifluoroacetylated proteins which include protein disulfide isomerase (Martin, et al., 1989) and the calcium binding protein, calreticulin (Butler, et al., 1992). The mechanism of cell death is thought due to cytotoxic cell killing by lymphocytes that recognize hepatocytes containing trifluoroacetylated proteins (Vergani, et al., 1980).

Dichloroethane

The 1,2-dihaloalkanes (DHAs) are important industrial chemicals produced on a worldwide basis. The most important of these is DCE which is also known as dichloroethylene and ethylene dichloride; the chemical formula is $C_2H_4Cl_2$. Physical and chemical properties of DCE are presented in Table 1.1. DCE is a clear liquid that evaporates readily at room temperature and has a pleasant smell and a sweet taste (Williams and Diwan, 1994). In the United States DCE is among the largest-volume synthetic organic chemicals manufactured with 1990 production approximately 18.6 billion pounds (Kirschner, 1995). The major use for DCE is as feedstock in the production of vinyl chloride (~ 84%) and to a lesser extent, perchlorethylene, trichloroethylene and 1,1,1-trichloroethane. Additional uses include the manufacture of paint thinners, acrylic adhesives, rubber cement, leather and metal cleaners, and rubber goods (Markowitz, 1994). Occupations with the largest numbers of workers exposed to DCE include automobile mechanics, registered nurses, heavy equipment mechanics, janitors and machinists (Williams and Diwan, 1994). The general population may be exposed to DCE in contaminated air, or by drinking contaminated water. Ingestion of residual DCE on food items is also a possibility. The production of the chemically related DBE has declined in recent years due to its ban as a fungicide and decrease in the use of leaded gasoline to which it is added as a lead scavenger.

Acute toxicological properties of DCE following inhalation exposure involve the central nervous system (CNS) and include symptoms of headache, dizziness, weakness, cyanosis, muscular spasms, hypotonia, vomiting and unconsciousness

(Organization, 1987). Cyanosis is a result of either respiratory depression or obstruction of the airways due to inflammation of the airways. Oral ingestion of DCE produces similar, but more pronounced CNS symptoms. It has been reported that ingestion of 20-50 mL of DCE is lethal in humans (IRPTC, 1984).

DCE is hepatotoxic in humans after acute oral poisoning. A reported dose of ≥ 570 mg/kg/day resulted in severe hepatocellular damage and liver atrophy (Williams and Diwan, 1994). Recently it has been reported that lipoglycoprotein metabolism in rat hepatocytes may be impaired by DCE (Cottalasso et al., 1994). The block in secretion of lipoglycoproteins from the Golgi and endoplasmic reticulum occurs as early as five minutes after administration of DCE and may be responsible for some of the observed hepatotoxic effects such as fatty liver. Renal effects have been observed

Table 1.1 Physical and Chemical Properties of 1,2-Dichloroethane

<u>Property</u>	<u>Information</u>
Molecular weight	98.96 g/mole
Color	colorless
Physical state	heavy liquid
Melting point	-35°C
Boiling point	84°C
Density (20°C)	1.25 g/mL
Odor	Pleasant
Odor threshold:	
Water	20 mg/mL
Air	10-50 ppm
Solubility:	
Water (20°C)	0.87 g/100 ml
organic solvents	Miscible with chlorinated solvents alcohol, chloroform, ether.
Partition Coefficients:	
Log K_{ow}	1.47
Log K_{oc}	1.21
Vapor pressure (20°C)	61 mm Hg
Henry's Law constant (25°C)	1.10×10^{-3} atm-m ³ /mol
Autoignition temperature	413°C
Flashpoint	13°C (closed cup)

in humans following the acute ingestion of DCE. Ingestion of 714mg/kg/day caused renal damage in the form of bleeding and hyperemia of the kidney (Schonborn, et al., 1970). Minimal to moderate renal regeneration was also noted in male mice at a dose of 2,710 mg/kg/day. Bacterial mutagenicity assays have shown that DHAs are mutagenic in vitro (Cmarick, et al., 1992; Inskeep, et al., 1986; Koga, et al., 1986). Recently, Ballering and coworkers used the *vermillion* locus of *Drosophila melanogaster* to investigate the DNA sequence changes due to DCE, DBE and 1-bromo-2-chloroethane (BCE). Their data strongly support the conclusions that DHAs are genotoxic through modification at ring nitrogens—N⁷ of guanine and N¹ of adenine—and that the mutagenic action seems to be the formation of non-coding lesions and/or misrepair (Ballering, et al., 1994). Based upon the available mutagenicity studies, the International Agency for Research on Cancer (WHO) classifies DCE as a class 2B carcinogen (possible human carcinogen) while the EPA classifies it as class B2 (probable human carcinogen). To date, there is no specific evidence relating DCE exposure with the occurrence of cancer in humans.

For some of the toxic and carcinogenic effects of DCE to be expressed, it must be metabolized (Figure 1.8). Both microsomal and cytosolic fractions are capable of metabolic activation resulting in different reactive metabolites so that two pathways of DCE metabolism have been proposed: 1) P450 oxidation and 2) glutathione transferase activity with glutathione conjugation (Guengerich, et al., 1980b; Shi and Hill, 1981). The relative role of these two pathways in the toxicity of DCE has not yet been unequivocally determined. The microsomal pathway is dependent primarily on P450 2E1 which can convert DCE into chloroacetaldehyde (Guengerich, et al., 1980a). This is an alkylating agent that is highly reactive and has been shown capable of binding to protein (Guengerich, et al., 1981). As P450 2E1 is an ethanol inducible cytochrome P450, it is expected that ethanol cotreatment would have a profound effect on metabolism. Low tissue ethanol concentrations were found to stimulate P450 activity towards DCE, while high tissue levels suppress metabolism of DCE (Sato, et al., 1981). Concurrent administration of disulfiram, a compound that inhibits P450

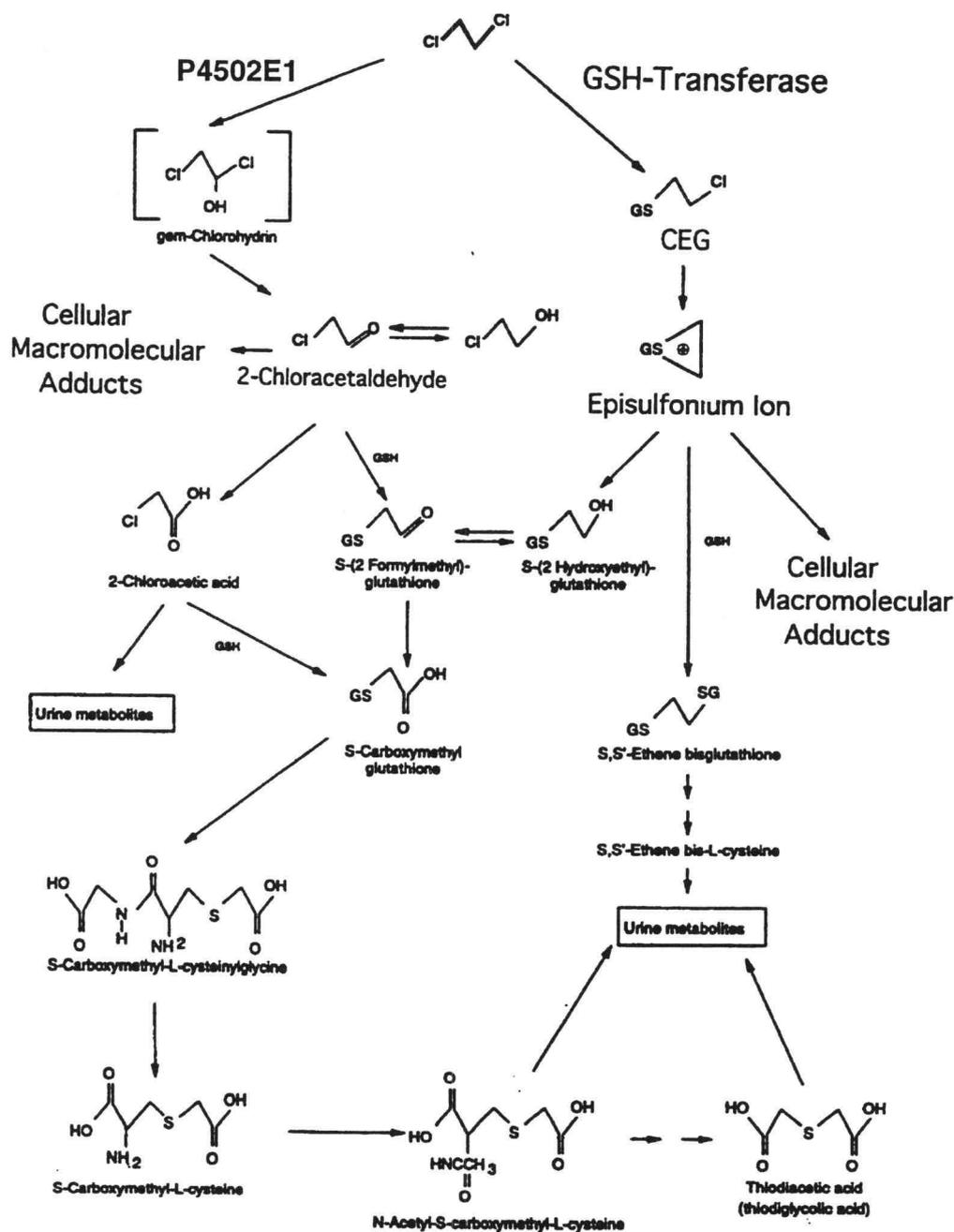


Figure 1.8. Metabolism of 1,2-dichloroethane showing two activation pathways. P450 2E1 leads to 2-chloroacetaldehyde while GST results in CEG. Adapted from Williams, 1994.

metabolism, resulted in an increase in hepatotoxicity that was not observed for DCE treated rats alone (Igwe, et al., 1986) and an increase in DCE blood levels 5-fold over controls (Cheever, et al., 1990). Although in rats the oxidative and conjugation routes occur at a ratio of 4:1 (van Bladeren, et al., 1981b), data strongly implicate the GSH pathway having greater importance regarding carcinogenicity and mutagenicity (Storer and Conolly, 1985; Romert, et al., 1990; Ballering et al., 1993). When DCE is conjugated with GSH the product is S-(2-chloroethyl)glutathione (CEG). CEG has been detected in the bile, by HPLC analysis, of rats treated with BCE (Marchand and Reed, 1989). CEG, and not the cysteine conjugate, is responsible for the nephrotoxicity and proximal tubular lesions observed in rats (Kramer, et al., 1987). CEG was shown by Jean and Reed to be capable of alkylating a variety of functional groups present in amino acids, in particular, the sulfhydryl functionality on cysteine, the hydroxyl group on tyrosine and the imidazole of histidine (Jean and Reed, 1989). Much of my thesis research has focused on identifying the sites of alkylation in the peptide oxytocin, human hemoglobin and *E. coli* thioredoxin following alkylation by CEG (Erve, et al., 1995a; Erve, et al., 1995b).

Biological Mass Spectrometry

In a recent review of mass spectrometry, it was stated that: "This is the era of biological mass spectrometry, the study of macromolecular science in the overall context of human health and disease" (Burlingame, et al., 1994). Biological mass spectrometry is reliant on the capability to ionize large, polar biomolecules, such as, proteins, nucleic acids, carbohydrates and complex lipids. The strength of biological mass spectrometry, relative to other advanced techniques including nuclear magnetic resonance spectroscopy and X-ray crystallography, is the capability to analyze mixtures and obtain accurate molecular weight and structural information on small (picomole and lower) amounts of sample.

Several ionization strategies exist to introduce charged biomolecules into the mass spectrometer for analysis. One of the first developed was fast atom bombardment (FAB), which permitted the mass spectrum of met-lys-bradykinin (M_r 1318) to be recorded in 1981 by Barber and coworkers. FAB employs a beam of atoms (eg. argon or xenon) or ions (eg. cesium) accelerated to kinetic energies on the order of several keV, which collide with sample molecules present in a liquid matrix, typically glycerol. Depending on the chemical nature of the compound, either positive or negative molecular ions are formed in the gas phase and the mass to charge ratio (m/z) of the compound can be measured. One disadvantage of FAB ionization is the creation of a large number of glycerol ions that can obscure the molecular ion of interest. In addition, for peptide mixtures, FAB will often preferentially ionize hydrophobic peptides to a greater extent than hydrophilic peptides (Biemann, 1988).

Two ionization techniques have dominated the field of biological mass spectrometry in the past five to six years. One ionization technique is called electrospray, or when nebulization is utilized to assist droplet formation, ionspray. The combination of electrospray ionization with mass spectrometry has been credited equally to two different groups: Fenn and Yamashita in the US and Aleksandrov in the former USSR (Smith, et al., 1990). The general basis of electrospray ionization is the initial dispersal of multiply charged liquid droplets followed by evaporation of the liquid to produce multiply charged analytes. A high electric potential, typically 3-6 kV, in the region where the analytes elute from a capillary column, leads to a fine mist of highly charged drops containing analyte. Evaporation of the liquid drop is assisted by a counterflow of heated, dry gas, until the drop is so small that a columbic explosion occurs as a result of many like charged molecules present close together (Smith, et al., 1990). This produces molecular ions containing multiple charges which are introduced into the mass spectrometer; the mass spectrum can be described as consisting of a distribution of charged species (Figure 1.9), each representing a different m/z of the compound of interest. Because of this multiplicity in m/z , electrospray ionization enables the molecular weight of proteins to be measured with

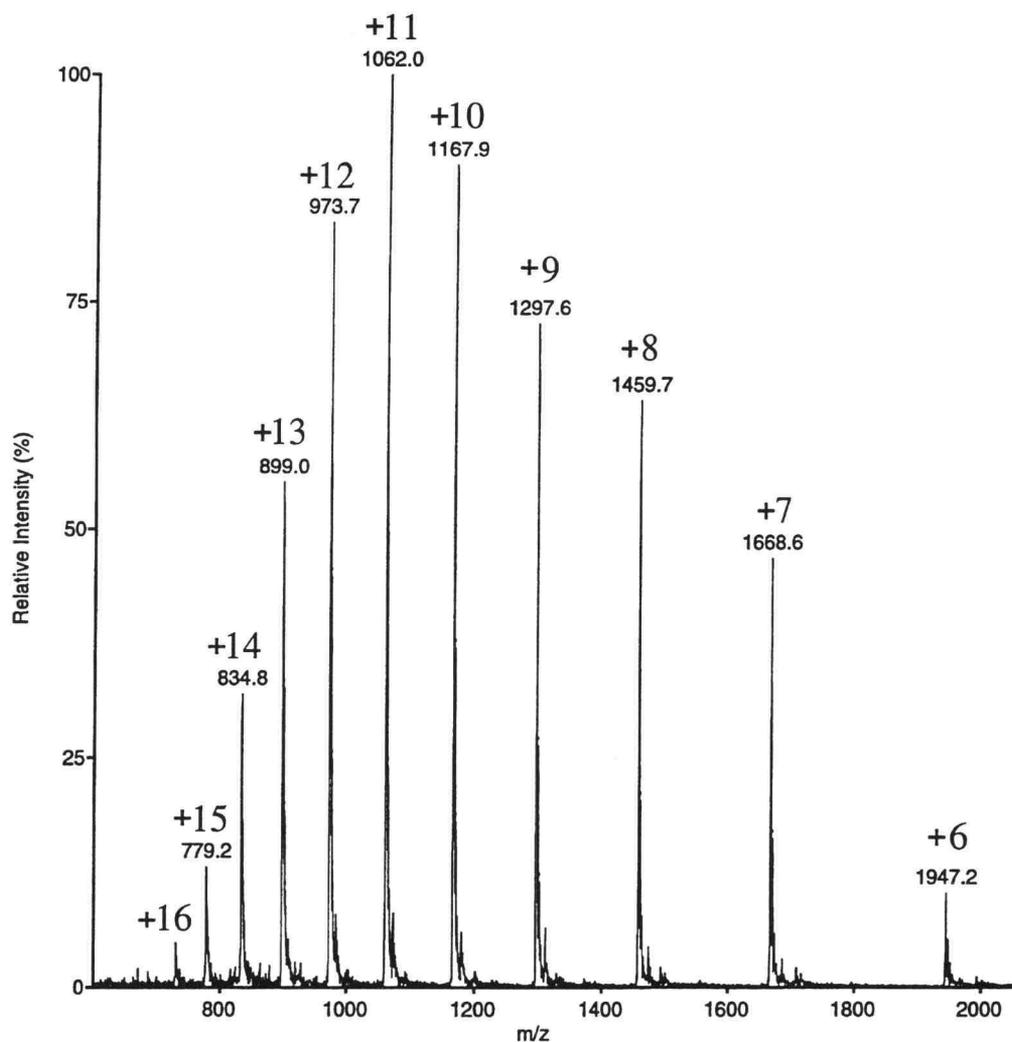


Figure 1.9. Electrospray mass spectrum of *E. coli* thioredoxin obtained on a Sciex III plus triple quadrupole instrument.

high precision: $11,667.4 \pm 1.9$ for *E. coli* thioredoxin (M_r 11,668). Matrix-assisted laser desorption-ionization is the other technique that has had major impact on the mass spectrometry of biomolecules. Tanaka and, independently, Hillenkamp, are attributed with pioneering the idea of dispersing the sample in a radiation absorbing matrix (Burlingame, Boyd and Gaskell, 1994). Laser radiation (266 nm) was used in the experiments of Hillenkamp to irradiate a sample present in nicotinic acid, resulting in desorption of molecular ions up to molecular weight 60 kDa (Biemann, 1988). To overcome the broad peaks characteristic of the ion signals produced with nicotinic

acid, cinnamic acid derivatives were tested and found to have the advantage of less matrix protein-adduct formation. The matrix of choice for many applications is now 3,5-dimethoxy-4-hydroxycinnamic acid (sinnapinic acid); it absorbs at 355 nm in the ultraviolet (Beavis and Chait, 1990). Time of flight mass spectrometers are the ideal mass analyzers when ionization, such as produced by a pulsed Nd:YAG laser, occurs in pulses.

The sequence of basic units in a biopolymer is important information that can give insight into the function of the biological compound in question. For peptides and proteins, the basic units are amino acids linked together by peptide bonds. As all the ionization processes described above are "soft" ionization methods, they generally produce singly or polyprotonated molecular ions that contain too little energy to fragment (Biemann, 1988). Tandem mass spectrometry (MS/MS) is a powerful technique that allows structural information on peptides, and hence proteins, to be obtained by a process known as "collision-induced dissociation" (CID) (Hunt, et al., 1986). By passing a selected molecular ion into a collision cell filled with an inert gas, such as helium or argon, peptide bonds will fragment to produce "product ions" that are mass analyzed in a second mass spectrometer. Nomenclature that identifies a product ion based on whether the C-terminus (y ion) or N-terminus (b ion) carries the charge was developed by Roepstorff and Fohlman (Roepstorff and Fohlmann, 1984), and modified by Biemann (Biemann, 1988) (Figure 1.10). A limited number of sequences for the peptide can be deduced based on the diagnostic b and y ion series observed. The utility of MS/MS in structure elucidation is illustrated by the determination of the complete primary structure of thioredoxin from *Chromatium vinosum* by sequencing the peptides obtained by tryptic digestion (Johnson and Biemann, 1987). Recent work has attempted the direct sequencing of protein with masses as high as 66 kDa by CID (Loo, et al., 1991). However, the complexities caused by the formation of multiply charged product ions makes sequence information difficult to glean from the spectra and further progress, both theoretical and instrumental, is needed before this strategy for protein sequencing becomes common.

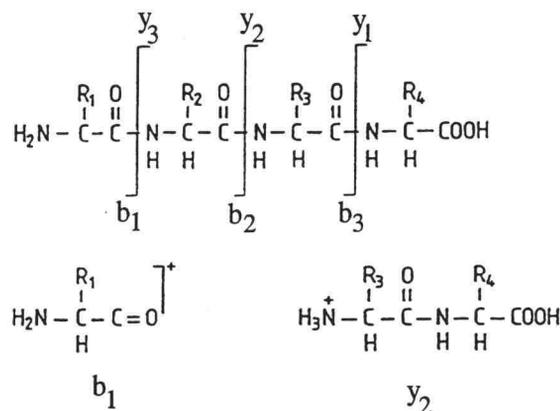


Figure 1.10. Nomenclature for fragment ions obtained during CID.

Some applications of MS/MS in toxicology have been reviewed by Blair (Blair, 1993). These include structural characterization of proteins and peptides covalently modified by xenobiotics, screening of GSH-conjugates of drugs, identification of drug metabolites and reactive intermediates. MS/MS has been applied to identify the nature of carcinogen-DNA adducts derived from polycyclic aromatic hydrocarbons (PAHs). It has been assumed that PAHs are activated by P450s to the diol-epoxide, the ultimate carcinogen, that reacts with DNA. An alternative mechanism is one electron oxidation to form a radical cation; reaction with DNA bases will form an adduct unique to this mechanism (RamaKrishna, et al., 1992). Gross and coworkers first obtained evidence consistent with the radical cation mechanism by *in vitro* activation of benzo[a]pyrene with either electrochemical oxidation, horseradish peroxidase, or P450s, and structural characterization of the modified bases by MS/MS (Wellemans, et al., 1994). *In vivo* evidence for the radical cation mechanism will depend on the ability to measure adducts in the femtomole range and requires new and more sensitive mass spectrometric detection procedures, such as, array detectors with multichannel capability.

Summary of Objectives

1. To examine the alkylation chemistry of CEG towards peptides and proteins in order to determine the extent to which adducts are formed in vitro.
2. To apply tandem mass spectrometry to identify particular amino acids in these peptides and proteins that undergo alkylation by the reactive species CEG.
3. To investigate the influence of protein tertiary structure in influencing which amino acids are targeted by the reactive species CEG.

Chapter III

Alkylation of Oxytocin by S-(2-chloroethyl)glutathione and Characterization of Adducts by Tandem Mass Spectrometry and Edman Degradation

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Abstract

S-(2-chloroethyl)glutathione (CEG), an alkylating agent formed by glutathione conjugation with 1,2-dichloroethane (DCE), is able to alkylate DNA and proteins. As a prelude to identification of specific protein alkylation sites, the peptide oxytocin was alkylated by CEG, and tandem mass spectrometry was used to identify the alkylation sites. It was found that mono-, bis- and tris- adducts can result from alkylation of reduced oxytocin and that tandem mass spectrometry differentiated (S-[2-(cys)¹ethyl]glutathione)oxytocin (mono adduct Cys-1) from (S-[2-(cys)⁶ethyl]glutathione)oxytocin (mono adduct Cys-6). Manual Edman degradation was used to eliminate the possibility that alkylation has occurred at Tyr-2 rather than at Cys-1 in the case of (S-[2-(cys)^{1,6}ethyl]glutathione)oxytocin (bis adduct) and mono adduct Cys-1. A mono-adduct-homo-dimer resulting from alkylation at Cys-6 and disulfide bridge formation through Cys-1 was also identified. Oxidized oxytocin formed two minor adducts, representing less than 5% of the oxytocin present in the reaction mixture. These findings demonstrate that alkylation of oxytocin by the episulfonium ion of CEG did occur, as evidenced by tandem mass spectrometry, and that characterization of these adducts will aid in the identification of alkylated amino acids in proteins exposed to CEG.

Introduction

It is now widely accepted that reduced GSH functions not only to detoxify xenobiotics but can also enhance the toxicity of certain chemicals through the formation of reactive GSH conjugates (Dekant and Vamvakas, 1993; Monks, et al., 1990; Monks and Lau, 1994). The dihaloalkanes are metabolized by both microsomal and GSH transferase (GST) enzyme activity (Guengerich, et al., 1980; van Bladeren, et al., 1981b), but it is thought that the latter pathway is more important with respect to mutagenicity (van Bladeren, et al., 1981a). The conjugation of a dihaloethane (DHE) with GSH by GST, results in the formation of a sulfur half-mustard, S-(2-haloethyl)GSH (Rannug, et al., 1978; Shi and Hill, 1981). The sulfur half-mustard forms a putative episulfonium ion which is a reactive alkylating agent. It has been shown in vivo that the S-(2-chloroethyl)GSH conjugate of dichloroethane (DCE) is found in the bile of rats dosed with bromochloroethane (BCE) (Marchand and Reed, 1989). The GSH conjugate of 1,2-dichloroethane has also been found to cause a high mutation frequency in *Salmonella typhimurium* due to adduct formation at the N⁷ position in guanine (Humphreys, et al., 1990). The GSH conjugate of 1,2-dibromoethane has been identified as an adduct on guanine and is thought to be responsible for the high degree of mutagenicity of this compound (Guengerich and Ozawa, 1983).

Many chemical carcinogens are electrophilic and are capable of reacting with nucleophilic sites in proteins (Skipper and Tannenbaum, 1990). For certain chemicals, a relationship has been found between the degree of DNA alkylation and protein alkylation (Ehrenberg and Osterman-Golkar, 1980). This has found application in biomonitoring exposure to environmental chemicals in the work place (Farmer, et al., 1986). Measuring the differences in degree of alkylation by S-(2-chloroethyl) glutathione (CEG) among a variety of dipeptides can give quantitative data on the relative reactivity among functional groups (Jean and Reed, 1989), but it is expected that reactivity of a particular functional group present in a protein will behave differently due to steric or electrostatic factors. It has been reported, for example, that

the predominant alkylation sites in hemoglobin following alkylation by styrene oxide are at histidine α -20 and β -143 as opposed to the more nucleophilic β -93 cysteine (Kaur, et al., 1989). Consequently, it would be useful to have an analytical technique to answer the question of which amino acid(s) CEG has alkylated on an adducted protein.

Mass spectrometry, including fast atom bombardment (FAB/MS) and, more recently, ionspray MS, is a powerful technique for characterizing modifications on proteins including post translational modifications such as glycosylation (Svoboda, et al., 1991), phosphorylation (Biemann and Scoble, 1987) as well as alkylation (Kaur, et al., 1989). Tandem mass spectrometry allows sequencing of peptides at the femtomole level by collisionally induced dissociation (CID) of the peptide in the mass spectrometer (Arnott, et al., 1993). Therefore, it is possible by mass spectrometry to determine not only the presence of a modification, but the particular amino acid modified as well.

One strategy for determining sequence information in proteins is to enzymatically digest the protein using specific proteases, and to analyze the resulting peptide digest by mass spectrometry in a procedure known as peptide mapping. This technique was applied to the characterization of hemoglobin variants (Jensen, et al., 1991) and to locate disulfide bonds (Morris and Pucci, 1985). The purpose of this investigation was to characterize the structure of a peptide covalently modified with the alkylating agent CEG by tandem mass spectrometry, and to understand better the fragmentation patterns of this alkylated peptide during the CID process. The knowledge gained here should aid in the peptide mapping experiments of proteins modified by CEG. To this end, we selected the peptide, reduced oxytocin, which has been used previously by others to examine the acylation chemistry of S-(2-chloroacetyl)glutathione (Liebler, et al., 1988), and the carbamoylation chemistry of S-(N-methylcarbamoyl)glutathione (Pearson, et al., 1991). The objectives of the present study were to identify which amino acids in oxytocin undergo alkylation at physiologic temperature and pH.

Materials and Methods

Chemicals. Oxytocin (95%) was purchased from Calbiochem-NovaBiochem (LaJolla, CA). Reduced glutathione (GSH), phenylisothiocyanate (PITC), 4,4'-pyridine disulfide (PDS) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO). BCE was purchased from Aldrich (Milwaukee, WI). tris-(2-Carboxyethyl)- phosphine (TCEP) was obtained from Pierce (Rockford, IL). Pyridine was from Mallinckrodt (St. Louis, MO). HPLC solvents were purchased from J. T. Baker (Phillipsburg, NJ), and 18 megohm water was prepared by a Milli-Q system (Waters, Bedford, MA).

Synthetic Procedures. CEG was synthesized according to the method of Reed and Foureman (Reed and Foureman, 1986). CEG, purified by preparative HPLC, contained at most 5% of the hydrolysis product S-2-(hydroxyethyl)glutathione (HEG) as estimated by FAB/MS. The CEG was stored as a lyophilized powder under argon at -80 °C. The alkylating capability of CEG to thiols was assessed by a spectrophotometric technique with 4,4'-dithiopyridine (PDS). PDS permits the determination of sulfhydryl groups in simple compounds (Grassetti and Murray, 1967) and has been used to monitor the disappearance of free sulfhydryl groups in hemoglobin following exposure to alkylating agents (Neis, et al., 1984). Briefly, CEG was reacted with reduced GSH in ammonium bicarbonate buffer at pH 7.4. The thiol concentration was then determined by adding PDS and measuring the absorbance at 324 nm of the resulting 4-thiopyridone. CEG at five fold molar excess over GSH reduced the free thiol concentration by approximately 90%. The hydrolysis product, HEG, did not affect GSH thiol status and was used as a negative control.

Reduction of Oxytocin by TCEP. Oxytocin was reduced with a phosphine derivative, TCEP. In a typical alkylation experiment, two hundred micrograms (0.2 μ mol) of oxytocin was weighed into a 1.5 mL Eppendorf centrifuge tube. It was reduced, by addition of 200 μ L of 4 mM TCEP (8 fold molar excess) and incubation for 10 minutes, to yield a 1 mM solution of reduced oxytocin. Disulphides are reduced by TCEP via a mechanism similar to that of the trialkyl phosphines, which

probe reduction of bovine insulin prior to matrix assisted laser desorption mass spectrometry (Fisher, 1993). The advantages of TCEP include its high solubility in water, activity below neutral pH, selectivity towards disulfide bonds, and small molar excess needed to quantitatively reduce disulfides (Burns, 1991). TCEP is superior to dithiothreitol (DTT) in this experiment because of the need to either extract excess DTT from oxytocin, or to add CEG in molar excess above DTT. In the latter case, the chromatogram of the resulting reaction mixture is very complex, presumably due to CEG reacting with DTT to give a variety of products. In contrast, reaction mixtures of oxytocin reduced with TCEP do not contain other products.

Covalent Modification of Oxytocin by CEG. Aliquots of reduced oxytocin (50 μ L) were placed in separate Eppendorf tubes and 50 μ L 0.4M ammonium bicarbonate buffer (pH 7.7) was added. CEG was weighed and dissolved in 40-70 μ L buffer and quickly added to the oxytocin to give molar ratios ranging from equimolar to 30 fold excess (CEG vs. oxytocin). The pH dropped slightly with the addition of CEG so that the pH of the reaction mixture was between 7.0 - 7.4. The reaction mixture thus consisted of approximately 0.5 mM oxytocin and from 0.5 to 15 mM CEG. The reaction mixtures were incubated for 90 minutes at room temperature to insure that the full alkylating capacity of CEG was utilized. The reaction mixture was then injected onto an HPLC column and adducts were collected as described below.

Instrumentation. Isolation of the oxytocin CEG adducts was accomplished by reverse phase HPLC with an analytical C18 column (250 x 4.6 mm, 5 μ m, 30 Angstroms, Vydac, Hesperia, CA). The elution gradient was linear from 15% B to 30% B in 17 minutes with a flow of 1 mL/min [solvent A = CH₃CN/H₂O/TFA (5:95:1 v/v/v); solvent B = CH₃CN/H₂O/TFA (80:20:0.8 v/v/v)]. However, to increase separation between closely eluting adducts, the gradient was held constant prior to the elution of the more closely spaced adducts. This allowed baseline resolution to be obtained for most of the adducts. Mobile phases were prepared from HPLC grade solvents and Milli-Q water and purged for 15 minutes with helium gas prior to use. The liquid chromatography system included a Spectra Physics Series 8800 pump (Freemont, CA), an Applied Biosystems 759 UV absorbance detector set for 215 nm,

an Hewlett-Packard 3388A integrator/recorder, a dynamic mixer, and a column heater to maintain a temperature of 29⁰ C. Adducts were collected manually and concentrated by roto-evaporation prior to mass spectrometric analysis.

Sequencing of oxytocin-adducts was accomplished with a Perkin-Elmer Sciex API III plus triple-quadrupole mass spectrometer (Sciex, Thornhill, Canada). Samples were introduced into the mass spectrometer by infusion at a rate of 1.2-2.5 $\mu\text{L}/\text{min}$ with a syringe pump (Harvard Apparatus, South Natick, MA) and a solvent mixture of $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{formic acid}$ (50:50:1 v/v/v) via an injector fitted with a 5 μL sample loop. Collision induced dissociation (CID) took place in the second quadrupole (RF only), with argon as a collision gas at a thickness of $2.7\text{-}3.4 \times 10^{14}$ molecules/ cm^2 . The ion spray voltage was 5000 V and the orifice voltage was varied between 60 and 90 volts depending on the adduct. Collision energies were optimized for each adduct and ranged from 16 eV (laboratory frame of reference) for the mono-adducts to 26 eV for the bis-adduct. The mono-adduct-homo-dimer was fragmented at 37 eV.

FAB/MS was performed on a Kratos MS-50 double focussing instrument operated at a resolution of 1000 for raw data collection. A 2 μL sample volume was mixed on the probe tip with 2 μL of matrix consisting of thioglycerol/glycerol (2:1 v/v). Xenon gas was used to generate the primary ionizing beam from an Ion-Tech ion gun operated at 7-8 kV.

Edman Degradation. Samples were reduced to a volume of 5 μL or less in a 0.6 mL Eppendorf tube. Five microliters of a 5% solution of PITC in pyridine was added to each adduct, and the mixture allowed to react for 30 minutes at 45⁰ C after which the products were roto-evaporated. Samples were then hydrolyzed with neat TFA at 37⁰ C for ten minutes and roto-evaporated again. Prior to FAB/MS analysis the samples were reconstituted with 1 μL of acetic acid and 5 μL water.

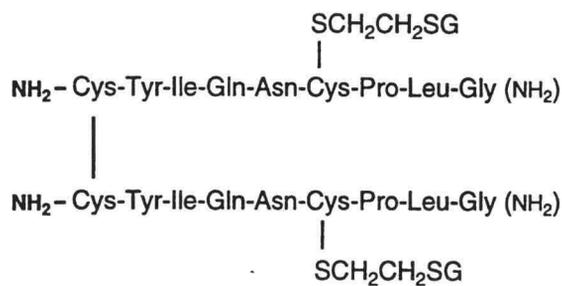
Results

Alkylation of oxytocin by CEG. The reduced form of oxytocin has two cysteinyl sulfhydryl groups, a hydroxyl group on tyrosine, three primary amides and a terminal primary amino group that are potential nucleophiles (Figure 2.1). The alkylated forms of oxytocin were separated by HPLC due mainly to their differences in polarity. The order of elution of the adducts is tris, bis-, mono Cys-6, and mono Cys-1. These all elute prior to reduced oxytocin. The mono-adduct-homo-dimer eluted after reduced oxytocin, presumably because of its higher molecular weight. Differences between the distribution of the alkylated oxytocins can be seen as the amount of CEG is increased. When adding an equimolar amount of CEG to reduced oxytocin, there was approximately 8% bis-adduct, 11% mono adduct at cysteine-6, and 35% mono adduct at Cys-1 compared to 45% unreacted oxytocin. As expected, increasing CEG to give a five fold excess over oxytocin results in a much greater amount of bis adduct (39%). However, the relative abundance of the mono adduct cysteine-6 becomes greater (30%) than that at Cys-1(16%), compared to 8% reduced oxytocin (Figure 2.2). Above 15-fold excess, some tris adduct was detected although most of the adducted oxytocin was the bis adduct. The tris alkylated form was never predominant.

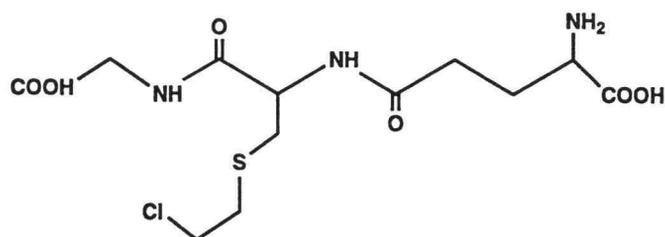
When the reaction mixtures were exposed to air for extended periods of time, some of the reduced oxytocin would reoxidize or polymerize. The mono adducts, which still had a free sulfhydryl group, would, to a certain degree, form intermolecular disulfide bonds. There is tandem-MS evidence that the resulting product is a homo-dimer linked between Cys-1, with the CEG adduct on Cys-6 (Figure 2.3). It is possible that another homo-dimer linked via Cys-6 exists because another HPLC peak, poorly resolved from the Cys-1 homo-dimer, was observed. However, we were unable to acquire mass spectral data to support this possibility.



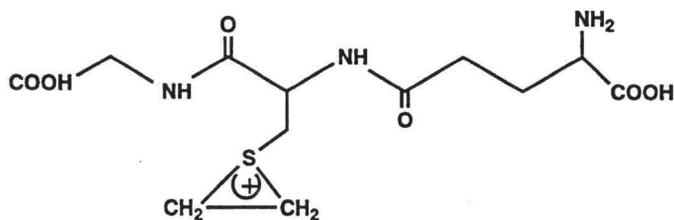
$R_1 = R_2 = \text{H}$	Reduced Oxytocin
$R_1 = R_2 = \text{Disulfide bond}$	Oxidized Oxytocin
$R_1 = -\text{CH}_2\text{CH}_2\text{SG}$ $R_2 = \text{H}$	Mono-Adduct Cys-1
$R_1 = \text{H}$, $R_2 = -\text{CH}_2\text{CH}_2\text{SG}$	Mono-Adduct Cys-6
$R_1 = R_2 = -\text{CH}_2\text{CH}_2\text{SG}$	Bis Adduct



Mono-adduct-homo-dimer



S-(2-Chloroethyl)glutathione (Half-mustard)



Episulfonium ion of S-(2-Chloroethyl)glutathione

Figure 2.1. Structures of compounds referred to in the text.

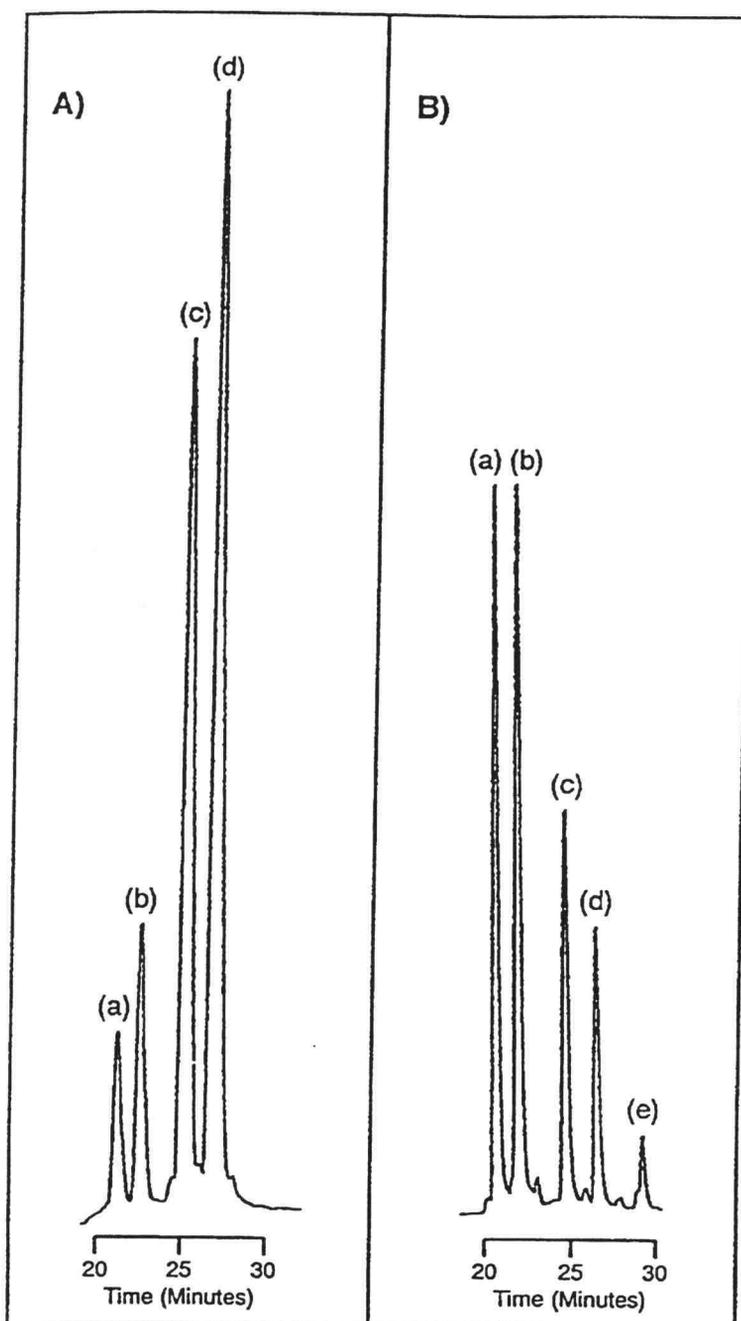
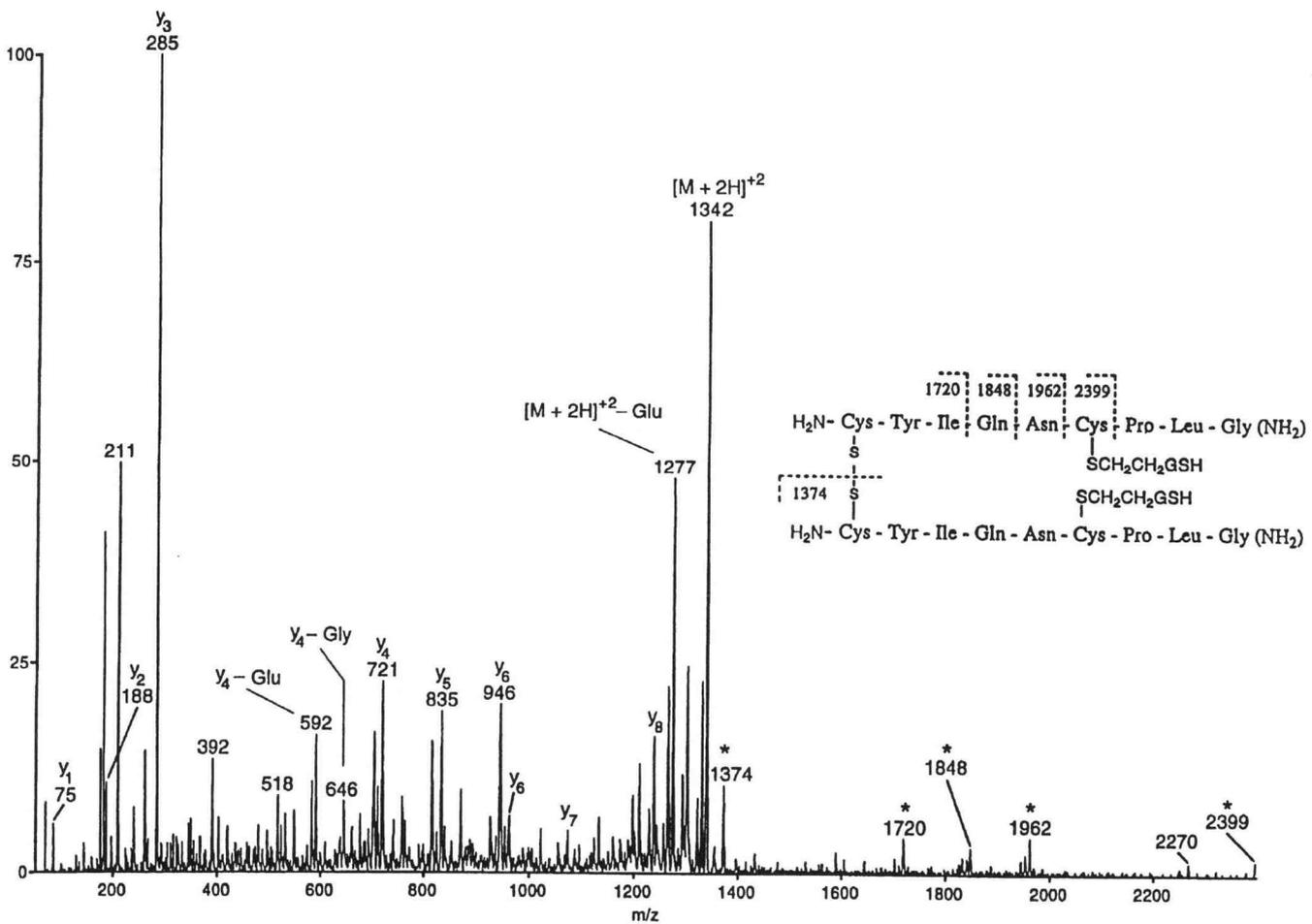


Figure 2.2. HPLC analysis of reduced oxytocin and its adducts for (A) an equimolar mixture of CEG:oxytocin and (B) a 5:1 molar mixture of CEG:oxytocin. Peaks are as follows: (a) bis-adduct; (b) mono-adduct Cys-6; (c) mono adduct Cys-1; (d) reduced oxytocin; (e) mono-adduct-homo-dimer. Chromatograms represent the elution of 215 nm absorbing material from the reverse phase column as described in Materials and Methods.

Figure 2.3. Spectrum of daughter ions formed by CID of the $[M+2H]^{+2}$ parent (m/z 1342) of the mono-adduct-homo-dimer. The origin of the diagnostic fragment ions denoted by an asterix (*) are shown in the fragmentation scheme. The site of alkylation was determined to be at Cys-6 and disulfide linkage through Cys-1.



Mass Spectrometry

Spectra of the alkylated forms of reduced oxytocin were obtained by both FAB/MS and ionspray MS. FAB/MS revealed peaks at m/z 2009 (tris), m/z 1676 (bis) and m/z 1343 (mono)(data not shown). Ionspray MS produced both the singly and doubly charged forms of the mono- and bis-adducts, with the doubly charged forms predominating. The tris-adduct formed a triply charged ion in addition to a doubly charged ion. Since neither of these techniques provided sequence information, tandem MS was used to address that question.

The daughter ion spectrum of reduced oxytocin has been recorded by Pearson et al. with FAB/MS/MS (Pearson, et al., 1991). CID spectra of peptides are distinguished by a series of b and y fragment ions that provide amino acid sequence information about the peptide (Biemann, 1988). Noting which peaks in the spectra of the alkylated forms of oxytocin shifted compared to unalkylated oxytocin helped to establish the sites of CEG adducts as described below.

Daughter ions of adducts were produced by fragmenting the doubly charged parent ion. Most spectra revealed a partial set of b_n ions, revealing the sequence from the N-terminus to the C-terminus and an almost complete set of complementary y_n ions, revealing the sequence from the C-terminus to the N-terminus. The y_3 ion was prominent and observed in all the spectra (Figures 2.3, 2.4 and 2.5), due to cleavage of the sequence Pro-Leu-Gly from the C-terminus. Since the regions of the spectra below y_3 (m/z 285) were almost identical, it follows that no alkylation occurred on these three amino acids.

One mono adduct had an HPLC retention time of approximately 21.7 min, and displayed an ion at m/z of 1058, that was shifted by the mass of CEG moiety (334 Da) above the b_6 ion at m/z 725 in unalkylated oxytocin, indicating alkylation somewhere on the first six amino acids. Further analysis of the spectra revealed that y_4 through y_7 and y_9 were also shifted 334 Da, indicating that alkylation occurred at Cys-6 (Figure 2.4B). The other mono adduct eluted with a retention time of approximately 23.5 min, had the identical molecular weight, and also contained a shifted b_6 ion at m/z 1058, but b_3 through b_5 were also shifted 334 Da while y_1

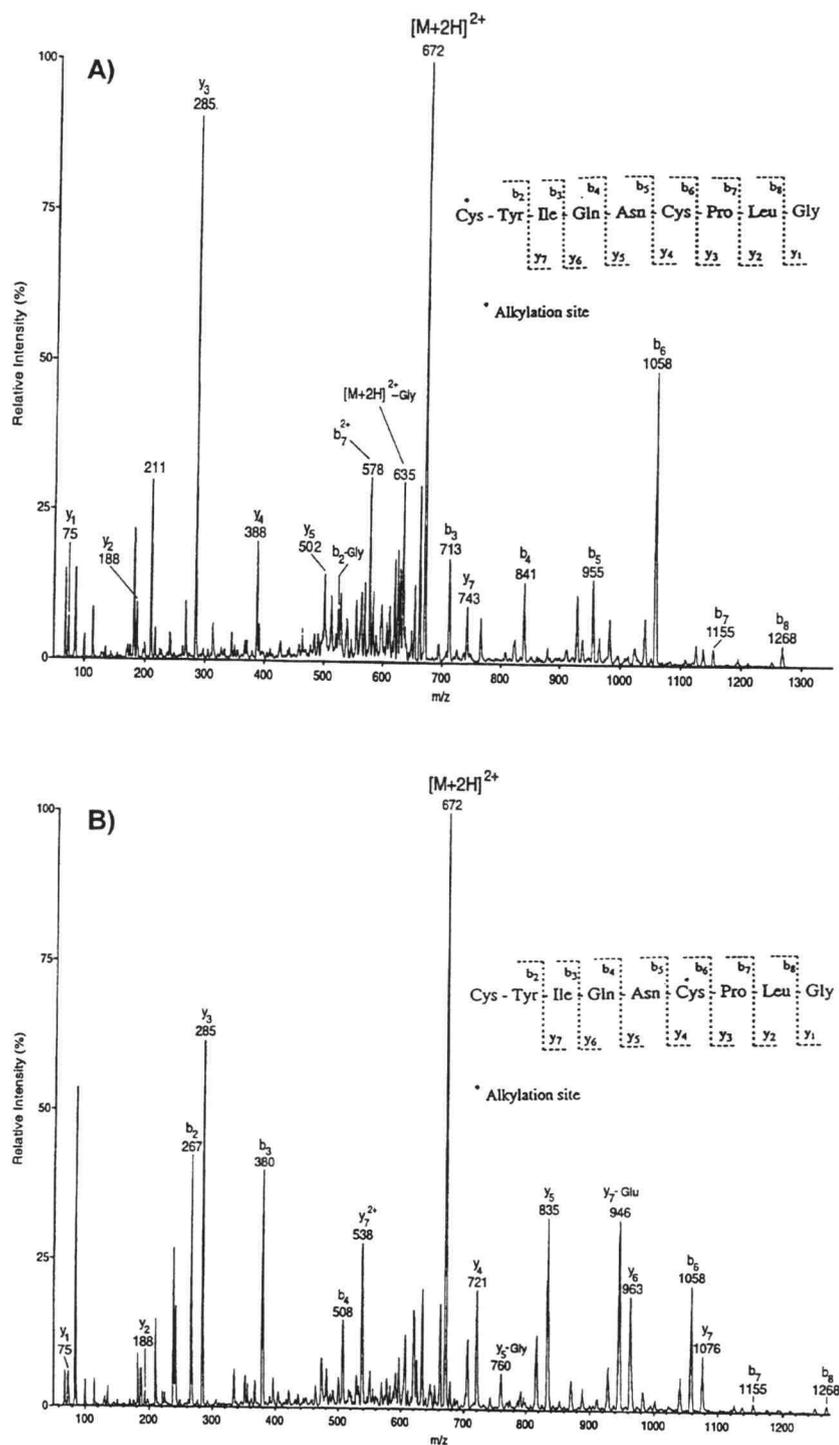


Figure 2.4. Spectrum of daughter ions formed by CID of the $[M+2H]^{2+}$ parent (m/z 672) of mono adduct Cys-1 (A), and mono adduct Cys-6 (B). Both spectra were obtained by ionspray MS/MS on a Sciex API III triple-quadrupole mass spectrometer by direct infusion of the sample into the ion source.

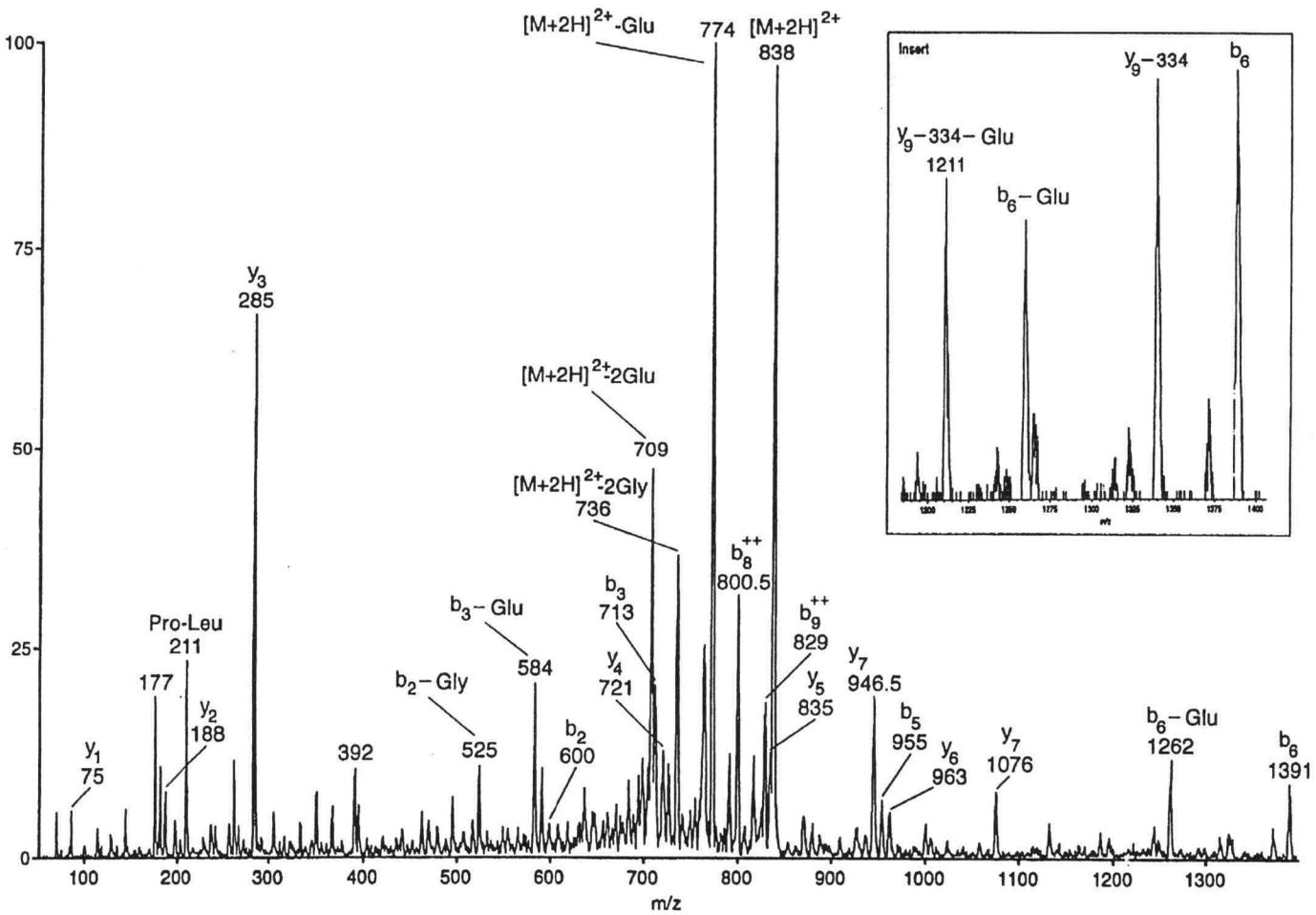


Figure 2.5. Spectrum of daughter ions formed by CID of the $[M+2H]^{2+}$ parent (m/z 838) of the bis-adduct of reduced oxytocin. Insert shows an expanded view of the high m/z region of oxidized oxytocin bis-adduct, revealing loss of the alkylating moiety from the parent ion to yield a peak at m/z 1341, which is not observed in the bis-adduct of reduced oxytocin (not in insert).

through y_7 were not (Figure 2.4A). This condition can only be satisfied if alkylation had occurred at either Cys-1 or Tyr-2. As for native oxytocin, this mono adduct shows little indication of fragmentation between these two residues to produce a b_1 ion (m/z 437) or the corresponding y_8 ion (m/z 906) (Figure 2.4A). This made unambiguous assignment of the alkylation site impossible, and it was necessary to perform a manual Edman degradation to confirm that Cys-1 was alkylated. Comparison of the spectra for both mono adducts reveals clear differences in the peaks that are present, which reflect their different structures (Figure 2.4).

Although the triply charged ion at m/z 895 was more intense, sequencing of the mono-adduct-homo-dimer was accomplished by fragmenting the doubly charged ion at m/z 1342, as this produced a more readily interpretable spectrum containing primarily singly charged ions. The spectrum exhibited a complete set of y_n ions, but no b_n ions were identified (Figure 2.3). The peak at m/z 1374 corresponds to the intact mono adduct plus both sulfur atoms involved in the disulfide bond. The peak at m/z 1720 corresponds to the intact mono adduct plus the amino acids Cys-Tyr-Ile from the other mono adduct. Peaks at m/z 1849 and 1963 reflect additions of the masses of Gln (128) and Asn (114) to m/z 1720, respectively. The highest m/z value at 2399 corresponds to one complete mono adduct plus six amino acids of the other mono adduct, including the alkylating moiety. This ion appears to lose a glutamate residue as indicated by the peak at m/z 2270. Support for the disulfide linkage was obtained by adding the reducing agent TCEP to the dimer, which resulted in its disappearance and simultaneous appearance of the monomer (spectra not shown).

Alkylating oxidized oxytocin with 25 fold molar excess CEG produced an adduct eluting immediately before oxidized oxytocin in an HPLC run, with an area approximately 5% that of oxidized oxytocin (data not shown). Although not evident from the chromatogram, this peak was due to two adducts when analyzed by ionspray MS. The two pairs of singly and doubly charged species corresponded to the mass of a mono alkylated (m/z 1341) and bis-alkylated (m/z 1674) form of oxidized oxytocin, respectively.

The CID spectrum (data not shown) of mono-alkylated oxidized oxytocin revealed a pattern similar to that of mono adduct Cys-1. Differences include a peak at m/z 1007 that corresponds to oxidized oxytocin. This forms by the loss of the intact alkylating, which is seen at m/z 334, from the parent ion. The ions in the spectrum are consistent with alkylation at the N-terminal cysteine. Since the sulfhydryl groups exist as a disulfide bond, alkylation must have occurred at the free amino terminus.

Sequencing the bis-adduct of oxidized oxytocin produced the daughter ion spectrum that is very similar to that of the bis adduct of reduced oxytocin, which is shown in Figure 2.5. The highest value m/z , 1389, observed in the spectrum of the oxidized bis adduct (Figure 2.5, insert) corresponds to the b_6 ion; the b_6 ion in the daughter ion spectrum of bis alkylated reduced oxytocin appears at m/z 1391, i.e. two mass units higher. The peak at m/z 1260 corresponds to b_6 -Glu, also analogous to what is observed in the reduced bis adduct. The prominent peak at m/z 1341 (Figure 2.5, insert), absent in the bis-adduct spectrum of reduced oxytocin, is explained as the loss from y_9 at m/z 1674 (not in insert) of an intact alkylating moiety seen at m/z 334 amino group of cysteine or the hydroxyl of tyrosine and the adduct. This could result from cleavage of the carbon-nitrogen bond between the free amino group of cysteine or the hydroxyl of tyrosine and the adduct. This ion goes on to lose a glutamate residue from the other alkylating moiety to produce the peak at m/z 1211. Another peak also explained by loss of the complete alkylating moiety is m/z 927 (not in insert), which arose from b_6 -Glu.

Removal of N-terminus by manual Edman Degradation.

Several adducts were subjected to manual Edman degradation to provide additional structural information that was absent in the CID spectra. In particular, the absence of the b_1 ion and the complementary y_8 ion for the bis and mono Cys-1 adduct leaves open the possibility that the spectra could be explained by tyrosine alkylation. Edman degradation specifically removes the N-terminal amino acid from a protein, resulting in the formation of a phenylthiohydantoin derivative of the cleaved amino acid and a truncated peptide (Allen, 1989). If it is blocked at the N-terminus, by acetylation or alkylation, for example, the Edman reaction cannot occur. Removing

the alkylated cysteine from the bis adduct gave a peak at m/z 1239, which is consistent with a truncated oxytocin with one alkylating moiety at Cys-6. Similarly, removing the N-terminal amino acid from the mono adduct assigned to Cys-1 gave the same m/z as observed for the Edman degradation product of unalkylated oxytocin, which cannot be explained if alkylation had been at Tyr-2. Furthermore, this supports location of the alkylating moiety on the sulfhydryl group of cysteine and not on the free amino terminus; for reason explained above, alkylation on this latter site would block removal of the N-terminal cysteine. Removal of the N-terminus from the mono adduct assigned to Cys-6 also gave a peak at m/z 1239, which is consistent for this structure (Table 2.1). Thus, cleavage of the N-terminal cysteine from both adducts provided information that was consistent for the structures of the adducts proposed.

In addition, we took both the mono adduct at Cys-1 and bis-adduct and added iodoacetamide (IAA) which is known to react exclusively with free thiols. In agreement with assignments, the mono adduct added one molecule of IAA while the bis-adduct did not react with IAA. Therefore, the mono adduct at Cys-1 has one free thiol while the bis adduct has no free thiols.

Table 2.1 Edman degradation results for oxytocin and adducts.

Compound	Initial (m/z)	Edman ^d (m/z)	+ PITC ^a (m/z)
Oxytocin	1009	906 (-103)	1041 ^b
Mono-Cys1	1343	906 (-437)	1041 ^b
Mono-Cys6	1343	1239 ^b (-103)	1374
Bis adduct	1676	1239 ^b (-437)	1374
GSH	308	n.d. ^c	n.d. ^c
HEG	352	n.d. ^c	487 ^b
S-Ethyl GSH	336	n.d. ^c	471 ^b

^a Addition of PITC (135 amu) to degradation product.

^b Signifies major peak

^c Not detected

^d Measured m/z following Edman degradation. Enclosed in parenthesis is the mass of the residue removed.

Discussion

The results of this *in vitro* study confirm expectations that CEG is a chemically reactive species capable of alkylating nucleophilic sites in peptides at physiological pH and temperature. The focus of the present investigation was to define better the alkylation chemistry by identifying which amino acids became alkylated and to determine the behavior of CEG alkylated peptides during the CID process. Reaction of reduced oxytocin with CEG resulted in the formation of a total of five different adducts which were sequenced by tandem mass spectrometry. Free sulfhydryl groups in oxytocin appear to be the main site of alkylation in reduced oxytocin, since the bis-adduct and both mono-adducts contain the alkylating moiety(ies) on the sulfhydryl of cysteine. It was apparent from the HPLC data that the mono adduct at Cys-6 was formed in preference to the mono adduct at Cys-1 at levels of CEG greater than five times that of oxytocin. This is in agreement with the report of Pearson et al. (22) for N-(S-methyl)carbamoyl GSH, namely, the preferential carbamoylation of the internal cysteine (Cys-6). Baillie and Davis also report that this internal cysteine is the site of disulfide formation with GSH (Baillie and Davis, 1993). However, with an equimolar level of CEG to reduced oxytocin, the data show clear evidence for modification at the N-terminal cysteine (Cys-1), and this species is more abundant than the mono adduct at Cys-6. The data for the mono-adduct-homo-dimer is compatible with two mono adducts alkylated at Cys-6 and joined by a disulfide bridge through Cys-1. Although this product was never a major component, it was often present and could be easily detected during the HPLC runs.

Fragmentation of glutathione conjugates have been reported at both high and low collision energies (Baillie and Davis, 1993; Ballard, et al., 1991; Murphy, et al., 1992). Loss of a glutamate residue, or a glycine residue derivatized to the methyl ester has been used as a screening procedure to detect the presence of glutathione conjugates in the bile of rats (Ballard, et al., 1991; Pearson, et al., 1990). In the CID spectra reported here, loss of both a glycine residue and a glutamate residue from the alkylating moiety could be seen giving rise to a subseries of ions 75 or 129 mass units

below their respective b or y ions, as tabulated for the bis-adduct in Table 2.2. Intensities of these ions were often comparable, and for certain ions, greater than the parent b or y ion. For example, the b_2 ion at m/z 600 in the bis adduct spectrum generates an ion at m/z 525 that is twice as intense. As the bis-adduct contained two alkylating moieties, up to two glutamate residues could be lost from a given ion as seen in Table 2.2; this was observed for y_7 and y_9 . The y_9 ion of the tris adduct lost three glutamate residues. Loss of glutathione was also detected, but the glutathione fragment was not a prominent ion. Rather, loss of a glutamate residue from the glutathione fragment was detected as an intense peak at m/z 177 in the spectra of both bis-adducts, the homo dimer and the oxidized mono adduct.

Oxidized oxytocin also reacted with CEG to give at least two adducts that were sequenced by tandem mass spectrometry. Although these spectra were similar to those of mono adduct Cys-1 and the bis adduct of reduced oxytocin, there were differences. Because oxidized oxytocin contains no free thiols, the number of possible alkylation sites are reduced. The similarity between the spectra of mono Cys-1 of the reduced oxytocin adduct with the mono oxidized adduct suggests that CEG is reacting at the amino terminus of Cys-1. The presence of a bis-adduct of oxidized oxytocin, albeit in low abundance, implies that CEG can react with another nucleophile. The data for the bis-adduct support alkylation at Tyr-2 in addition to Cys-1, based on the observation of y_1 - y_7 ions which are not shifted with respect to unalkylated oxytocin. This is consistent with seven unmodified amino acids from the C-terminus. The y_8 ion was not observed, which may suggest that it has been shifted due to alkylation, and is fragmenting to ions that have not been identified. Tyrosine in the peptide angiotensin has been shown to be a target of alkylation following treatment with styrene oxide (Ferranti, et al., 1992). During mass spectrometry both adducts of oxidized oxytocin showed the loss of a complete adduct, something that was not detected in the spectra of the adducts formed with reduced oxytocin. This may be due to an alternative fragmentation mechanism between the adduct and a nucleophilic nitrogen or tyrosine hydroxyl that is broken in the CID process, which does not occur with the bond between the adduct and sulfur.

Table 2.2. Product ions for the bis-adduct, showing loss of glutamic acid and/or glycine from CEG.

b_n	predicted b_n (m/z)	observed b_n (m/z)	-glutamate ^a	-glycine ^b
1	437.1	n.d.	-	-
2	600.2	n.d.	-	-
3	713.3	712.7	-	+
4	841.3	841.1	-	+
5	955.4	955.1	+	+
6	1391.5	1391.0	+	-
7	1488.5	n.d.	-	-
8	1601.6	1601.0	+	+
9	1658.6	1658.0	+	+

y_n	predicted y_n (m/z)	observed y_n (m/z)	-glutamate ^a	-glycine ^b
1	75.1	74.9	n.a.	n.a.
2	188.1	188.0	n.a.	n.a.
3	285.2	285.2	n.a.	+(Gly9)
4	721.3	721.5	+	+
5	835.3	834.5	-	-
6	963.4	963.2	+	-
7	1076.5	1076.5	++	-
8	1239.6	n.d.	-	-
9	1675.6	1676.0	++	+

^a Loss of glutamic acid is denoted by +; no loss, by -.

^b Loss of glycine is denoted by +; no loss, by -.

n.a. not applicable

n.d. not detected

The Edman degradation results support the assignment of the alkylation sites for the bis-adduct and both mono adducts of reduced oxytocin. Removal of the N-terminus of mono-adduct Cys-1, gives the same mass as observed when Edman degradation is applied to reduced oxytocin. This would not have been observed if tyrosine were alkylated. For each of the adducts and oxytocin, it was observed in the FAB/MS spectra that there was a peak corresponding to an addition of 135 Da, the mass of PITC. Thus, the major peak in the FAB/MS spectrum of the Edman product of oxytocin was not at m/z 906 (oxytocin-Cys) but at m/z 1041, which corresponds to the addition of PITC (+135 amu). Sequencing of this peptide by CID revealed that PITC had added to the free thiol of Cys-6. It is not apparent why PITC can add to both S-ethyl GSH and the hydrolysis product of CEG, namely HEG, and not to the alkylating moiety on the oxytocin adducts.

One requirement of tandem MS as a method for adduct characterization is the necessity of optimizing the fragmentation energy for each adduct to obtain a product ion spectrum. Since the optimal collision energy for the two mono-adducts was not the same as that for the bis-adduct nor that for the tris-adduct, tuning of the instrument was required. An important aspect to the mass spectrometric sequencing of the CEG-alkylated oxytocins was that neither the b_1 nor y_8 ion appeared, making the unambiguous assignment of alkylation at Cys-1 or Tyr-2 by tandem-MS impossible. It was necessary to perform a manual Edman degradation step to answer this question. Nevertheless, using previous knowledge of the alkylating properties of CEG, one can deduce where the most likely site of alkylation is when faced with two possibilities. The strengths of this method are that, although fragmentation of the adduct occurs, the bond between cysteine, the major target of CEG, and the ethylene bridge derived from CEG to the alkylated amino acid does not fragment, allowing identification of alkylation sites on small amounts of sample.

The information provided in this study should help in solving the more difficult task of identifying alkylation sites in proteins. For example, the partial fragmentation of the alkylating moiety derived from CEG could allow for neutral loss screening to identify an alkylated peptide in a mixture of peptides produced in a protein digest.

Acknowledgments

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Chapter IV

Alkylation of *E. Coli* Thioredoxin by S-(2-chloroethyl)glutathione and Identification of the Adduct on the Active Site Cys-32 by Mass Spectrometry

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Abstract

Alkylation of reduced *Escherichia coli* thioredoxin by the episulfonium ion derived from *S*-(2-chloroethyl) glutathione (CEG) at physiologic pH resulted in at least three different alkylation products. These adducts were separated by reverse phase chromatography, digested with trypsin, and peptide-mapped. The peptide containing the active site cysteines was collected and sequenced by tandem mass spectrometry. Results indicate that the site of alkylation was at Cys-32 exclusively with no alkylation at Cys-35. Raising the pH above the pK_a of Cys-35 to ionize the thiol before reacting with the episulfonium ion of CEG did not lead to alkylation at Cys-35, suggesting that a steric factor prevents the episulfonium ion of CEG from accessing this cysteine. A tryptic digest of a minor bis-adduct yielded an alkylated peptide which contained tyrosine, also known to be alkylated by CEG. Sequencing by tandem mass spectrometry, however, was unsuccessful due to fragmentation of the alkylating moiety from the peptide. Results of this study confirm that the episulfonium ion of CEG can adduct thioredoxin at the active site and may have important toxicologic significance regarding the mechanism of 1,2-dichloroethane toxicity.

Introduction

The cellular tripeptide glutathione (GSH) can protect cells from both endogenous and exogenous electrophiles that react with cellular constituents and cause cell damage (Reed, 1985). It is now clear that glutathione conjugation can cause cytotoxicity by enhancing the reactivity of a number of xenobiotic chemicals, such as, some aliphatic halogenated alkenes (Dekant, et al., 1989), quinones (Mertens, et al., 1991), and isothiocyanates (Temmink, et al., 1986). The glutathione conjugate *S*-(2-chloroethyl) glutathione (CEG), formed as a consequence of 1,2-dichloroethane (DCE) conjugation with GSH, is another example of a toxification reaction. Although DCE can be metabolized by an oxidative pathway via P450 2E1 leading to the reactive metabolite 2-chloroacetaldehyde, it is believed that mutagenicity is due to the GSH-conjugate pathway (Storer and Conolly, 1985). CEG can form an electrophilic episulfonium ion that can react with specific nucleophilic sites in DNA (Humphreys, et al., 1990; Ozawa and Guengerich, 1983). DCE metabolites also react with proteins and DCE treated rats show signs of oxidative stress in the form of lipid peroxidation (Sano and Tappel, 1990).

Covalent binding of reactive intermediates to proteins is accepted to be important in the development of many chemical-induced toxicities, although the detailed mechanisms by which these covalent interactions produce cellular toxicity are not completely understood (Nelson and Pearson, 1990). Protein alkylation may cause toxicity by (a) altering the protein such that loss of function occurs or by (b) creating an immunogen that leads to a deleterious immune response (Hinson and Roberts, 1992). It is becoming clear that the amino acids alkylated in a protein are not randomly targeted, but are determined by the nature of the electrophile (hard, soft), available nucleophiles, and steric constraints imposed by the tertiary structure of the protein. Protein adducts are often the focus of molecular dosimetry studies aimed at quantifying exposure to carcinogens in the work place, such as measuring hemoglobin adducts of ethylene oxide (Ehrenberg, et al., 1977; Osterman-Golkar, et al., 1976).

Escherichia coli (*E. coli*) thioredoxin is a small, ubiquitous, redox-active protein that has been studied extensively (Biguet, et al., 1994; Buchanan, et al., 1994; Goto, et al., 1992; Oblong, et al., 1994). One of its many roles is as a hydrogen donor for ribonucleotide reductase in the enzymatic synthesis of deoxyribonucleotides. Due to its protein-disulfide reductase activity, the protective role of thioredoxin against the effects of oxidative stress has been investigated. For example, thioredoxin has been shown to enable lens epithelial cells exposed to hydrogen peroxide to recover from the resulting oxidative damage, based on regeneration of G3PDH and leucine uptake (Spector, et al., 1986). Recently, evidence has been obtained that the thioredoxin/thioredoxin reductase system may regenerate enzymes inactivated by oxidative stress in endothelial cells (Fernando, et al., 1992). Previous work in this laboratory has shown that thioredoxin alkylated by CEG is inactivated, based on the insulin reduction assay (Meyer, et al., 1994). The results of this assay strongly implicate the active site as being targeted by CEG. Both eukaryote and prokaryote thioredoxin contains a conserved active site having the sequence Trp-Cys-Gly-Pro-Cys, which is present on a loop that protrudes from the three dimensional structure of the enzyme (Holmgren, 1985). The purpose of this investigation was to study the alkylation chemistry of CEG towards thioredoxin, and in particular, to identify which amino acids become alkylated following reaction with the episulfonium ion of CEG in vitro. To this end, we used a combination of peptide mapping and mass spectrometry to determine CEG alkylation sites in thioredoxin.

Materials and Methods

Chemicals. Recombinant *E. coli* thioredoxin was purchased from Calbiochem-NovaBiochem (La Jolla, CA). Reduced GSH, 4,4'-dithiopyridine (PDS), and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO). 1-bromo-2-chloroethane (BCE) was purchased from Aldrich (Milwaukee, WI). tris-(2-Carboxyethyl)phosphine (TCEP) was obtained from Pierce (Rockford, IL).

Sequencing grade trypsin was from Boehringer-Mannheim (Indianapolis, IN). Labeled GSH was synthesized from glutamic acid α -t-butyl ester, trityl-cysteine and ^{15}N -glycine using a Wang resin (4-alkoxybenzyl alcohol polystyrene), all purchased from Bachem (Torrance, CA). Bio-Gel P-2 gel (extra fine, $<45\ \mu\text{m}$) with molecular weight cutoff of 2000 Da was from Bio-Rad Laboratories (Richmond, CA). HPLC solvents were purchased from J. T. Baker (Phillipsburg, NJ), and 18 megohm water was prepared by a Milli-Q system (Waters, Bedford, MA).

Synthetic procedures. CEG was synthesized according to the method of Reed and Foureman (Reed and Foureman, 1986). CEG, purified by preparative HPLC, contained at most 5% of the hydrolysis product as estimated by FAB/MS. The alkylating ability of CEG towards thiols was assessed by a spectrophotometric technique with PDS as described previously (Erve, et al., 1995). Some experiments used CEG containing 50% ^{15}N . CEG, consisting of 50 % ^{15}N -labeled CEG, was synthesized from a mixture of GSH containing 50% ^{15}N -Gly. ^{15}N -GSH was synthesized manually, using standard Fmoc chemistry (Snyder, 1992). A Wang resin was used as a solid support, and ^{15}N -glycine was coupled to the resin according to the procedures of Grandas et al. (Grandas, et al., 1988). The final product was purified with preparative HPLC.

Reduction of thioredoxin by TCEP. Thioredoxin was reduced with a phosphine derivative, TCEP. In a typical alkylation experiment, 300 μg (0.03 μmol) of thioredoxin was reduced by addition of 25 μL of 8 mM TCEP (7 fold molar excess) and incubated for 10 minutes to yield a 1.2 mM solution of reduced thioredoxin. Disulfides are reduced by TCEP via a mechanism similar to that of the trialkyl phosphines, which have been used to quantitatively reduce organic disulfides (Fisher, 1993). The advantages of TCEP include its high solubility in water, activity below neutral pH, selectivity towards disulfide bonds, and small molar excess needed to quantitatively reduce disulfides (Burns, 1991).

Modification of Thioredoxin by the episulfonium ion of CEG. Aliquots of reduced thioredoxin (50 μL) were mixed with equal volumes of 0.4M ammonium bicarbonate buffer (pH 7.7). CEG was weighed and dissolved in 40-70 μL buffer and

quickly added to the thioredoxin to give molar ratios ranging from equimolar to 25-fold excess (CEG vs. thioredoxin). The pH dropped slightly with the addition of CEG so that the pH of the reaction mixture was between 7.0 - 7.4. The reaction mixture thus consisted of approximately 0.5 mM thioredoxin and from 0.5 to 12.5 mM CEG. The reaction mixtures were incubated for 90 minutes at room temperature to insure that the full alkylating capacity of CEG was utilized. The reaction mixture was then injected onto an HPLC column and thioredoxin adducts were collected as described in the instrumentation section.

Tryptic Digestion. The lyophilized CEG alkylated thioredoxin products (50 - 100 μ g) were dissolved in 0.4M NH_4HCO_3 buffer (pH 7.8) and reduced with 5 μ L 8 mM TCEP. Free thiols were alkylated by adding 5 μ L of 50 mM iodoacetamide (IAA) and incubating at room temperature for 10 minutes. To denature the protein, 25 μ L 8 M urea was added. Sequencing grade trypsin was dissolved in 10 mM HCl and 5 μ g was added to the sample. The concentration of urea was reduced to about 2M with the addition of 45 μ L H_2O . Digestions were allowed to proceed for 18 hours at 37° C before being stopped with the addition of 2 μ L 50% TFA. Digests were either analyzed immediately or stored at -80° C until analysis.

Bio-Gel separation of IAA from thioredoxin. Reduced thioredoxin was alkylated at Cys-32 with IAA by reacting at pH 7.0. A spin gel column was prepared, using a 1 mL pipette tip as a gel reservoir, that trapped IAA in the gel and allowed thioredoxin to elute in the void volume (Chen, et al., 1992). The column was tested for its ability to remove IAA by measuring the absorbance of IAA at 206 nm. A 50 μ L sample of 50 mM IAA showed no absorbance at 206 nm following filtration through the spin-gel column. The thioredoxin/IAA sample was placed on top of the spin-gel and spun at 2500 g for 6 minutes to remove unreacted IAA. The carboxyamidomethylated-thioredoxin was recovered for latter use.

Instrumentation. Isolation of the CEG alkylated-thioredoxin products was accomplished by reverse phase HPLC on an analytical C8 column (150 x 4.6 mm, 5 μ m, 30 angstroms, Vydac, Hesperia, CA). The elution gradient was linear from 30% B to 64% B in 20 minutes followed by an increase to 85% B over 5 minutes. The

flow was 1 mL/min [solvent A = CH₃CN/H₂O/TFA (5:95:1 v/v/v); solvent B = CH₃CN/TFA (100:0.8 v/v)]. Peptide mapping of the tryptic digests was accomplished by reverse phase HPLC on an analytical C18 column (250 x 4.6 mm, 5 μm, 30 Å, Vydac, Hesperia, CA). The elution gradient was linear increasing from 5% B to 37% B in 37 minutes, then to 70% B over 23 minutes and finally to 98% B in 12 minutes with a flow of 1 mL/min [solvent A = CH₃CN/H₂O/TFA (5:95:1 v/v/v); solvent B = CH₃CN/H₂O/TFA (80:20:0.8 v/v/v)]. Mobile phases were prepared from HPLC grade solvents and Milli-Q water and purged for 15 minutes with helium gas prior to use. The liquid chromatography system included a Spectra Physics Series 8800 pump (Freemont, CA), an Applied Biosystems 759 UV absorbance detector set for 215 nm, an Hewlett-Packard (Palo Alto, CA) 3388A integrator/recorder, a dynamic mixer, and a column heater to maintain a temperature of 29° C. Adducts and peptides were collected manually and concentrated by spin-evaporation prior to further analysis.

Sequencing of the peptide (T3) that contained the alkylating moiety derived from CEG was accomplished with a Perkin-Elmer Sciex API III plus triple-quadrupole mass spectrometer (Sciex, Thornhill, Canada). The sample was introduced via an injector fitted with a 5 μL sample loop and infused into the mass spectrometer at a rate of 1.2-2.5 μL/min with a syringe pump (Harvard Apparatus, South Natick, MA) and a solvent mixture of CH₃OH/H₂O/formic acid (50:50:1 v/v/v). Collision induced dissociation (CID) took place in the second quadrupole (RF only) with argon as a collision gas at a density of 2.5×10^{14} molecules/cm². The ion spray voltage was 5000 V, orifice voltage was 95 V, and collision energy 33eV (laboratory frame of reference). Sequencing of peptide (T4) was at a collision energy of 25 eV, orifice voltage of 45 V, and collision gas density of 3.4×10^{14} molecules/cm².

MALDI mass spectrometry was accomplished with a custom built time-of-flight instrument equipped with a frequency-tripled (355 nm) Nd:YAG laser (Spectra-Physics GCR-11). The ion source was operated at 24 kV. Mass spectra were formed by summing the data generated from 30 individual laser shots. Data analysis was performed with m-over-z software provided by Dr. R. C. Beavis at the Skirball Institute, New York University Medical Center, NY. Lyophilized adducts or tryptic

digests were redissolved in water. Typically, 1 μL of sample was mixed with 3-9 μL of matrix to obtain 1 to 10 μM analyte concentration. For each analyses, 0.5-1 μL was deposited on the sample probe and air-dried at ambient temperature. The dried sample was washed by dipping the probe tip into water and air-dried again. The matrices used were either 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or 4-hydroxy- α -cyanocinnamic acid (HCCA).

FAB/MS was performed on a Kratos MS-50 double focusing instrument operated at medium resolving power for raw data collection. A 2 μL sample volume was mixed on the tip of the sample probe with 2 μL of matrix consisting of thioglycerol/glycerol (2:1 v/v). Xenon gas was used to generate the primary ionizing beam from an Ion-Tech gun operated at 7-8 kV.

Results

Alkylation of thioredoxin by the episulfonium ion derived from CEG. The reduced form of thioredoxin has two cysteines at positions 32 and 35 that were expected to be the major targets of the alkylating moiety derived from CEG (Figure 3.1). The alkylated forms of thioredoxin were separated by reverse-phase HPLC

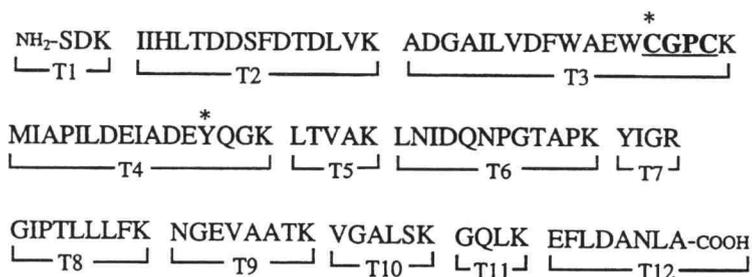


Figure 3.1. *E. coli* thioredoxin protein sequence showing tryptic peptides. The active site containing the two cysteines is underlined. The asterisks denotes the alkylation site on Cys-32 and the proposed alkylation site on Tyr-49.

mainly on the basis of differences in polarity. The order of elution of the adducts is tris-, bis-, and mono-, although there was not much separation between the bis and tris adducts. The alkylated forms of thioredoxin all elute prior to reduced thioredoxin; oxidized thioredoxin had the greatest retention time and eluted last. Differences in the distribution of the alkylated thioredoxins can be seen as the amount of CEG is increased relative to thioredoxin. When adding 5-fold excess CEG over reduced thioredoxin, there was approximately 20% mono adduct and no bis or tris adduct (Figure 3.2). With 10-fold excess there was close to 50% mono adduct formed and a small amount of bis adduct. With 25-fold excess CEG, reduced thioredoxin was converted almost completely to adducted forms consisting primarily of the mono adducted form and about 10% bis and tris adduct.

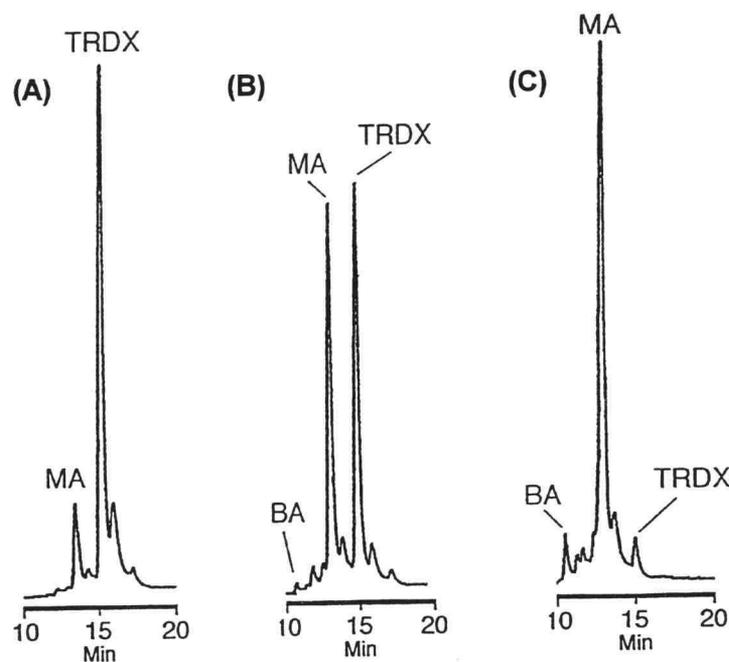


Figure 3.2. HPLC analysis of reduced thioredoxin and its adducts. (A) 5-fold excess CEG:thioredoxin, (B) 10-fold excess CEG:thioredoxin and (C) 25-fold excess CEG:thioredoxin. Chromatograms represent the elution of 220 nm absorbing material from the reverse phase column as described in Materials and Methods. TRX denotes thioredoxin; MA, BA and TA denote mono-, bis- and tris- adducted thioredoxin, respectively.

Peptide mapping of Mono and Bis adducts. The mono adduct collected by HPLC was rechromatographed before enzymatic digestion; as judged by MALDI mass spectrometry the rechromatographed peak was very pure (Figure 3.3A). Following tryptic digestion, a portion of the digest was mass analyzed by MALDI. An intense peak revealed at m/z of 2371 corresponds to the alkylated tryptic peptide T3 containing the adduct derived from CEG and one molecule of IAA from the digestion procedure (Figure 3.4A). A portion of the peptide digest was chromatographed on a C18 column. Comparing the resulting peptide map with that of untreated thioredoxin allowed unknown peaks to be identified and collected (Figure 3.5A and 3.5B). As the alkylated peptide was readily identified, not much effort was expended to determine the identity of the remaining peaks in the peptide digest accounting for some unlabeled peaks. Peaks that were identified are given in Table 3.1. For the mono adduct digest, the peptide of interest had a retention time of approximately 44 minutes; this peptide was collected and lyophilized prior to subsequent analysis.

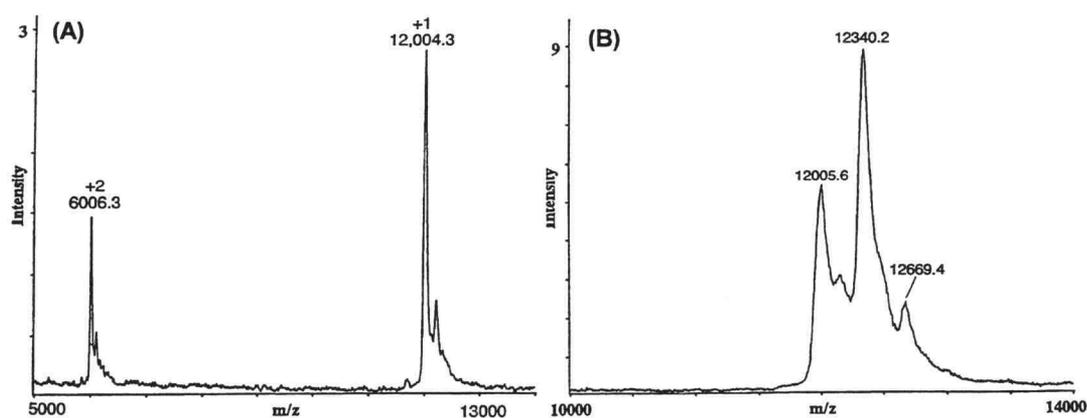


Figure 3.3. MALDI/MS spectrum of (A) mono-alkylated reduced thioredoxin at m/z 12,004 and (B) bis-alkylated thioredoxin at m/z 12,340 following collection off the C8 HPLC column. The bis-alkylated thioredoxin spectrum shows the presence of a mono-adduct as well as a tris-adduct.

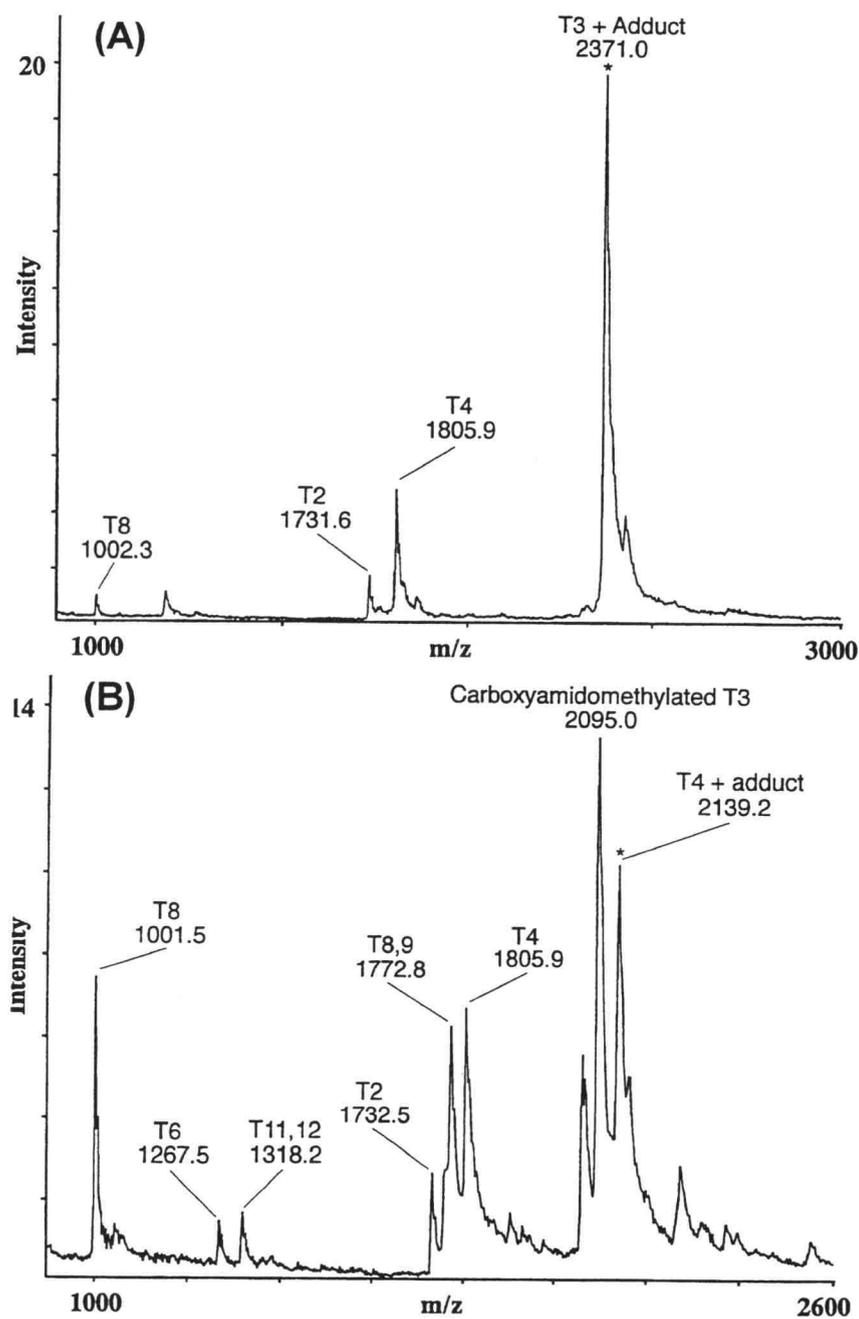


Figure 3.4. MALDI/MS spectrum of the tryptic digests of (A) mono adduct and (B) bis adduct. The digests were allowed to incubate at 37° C for 18 hours before being analyzed.

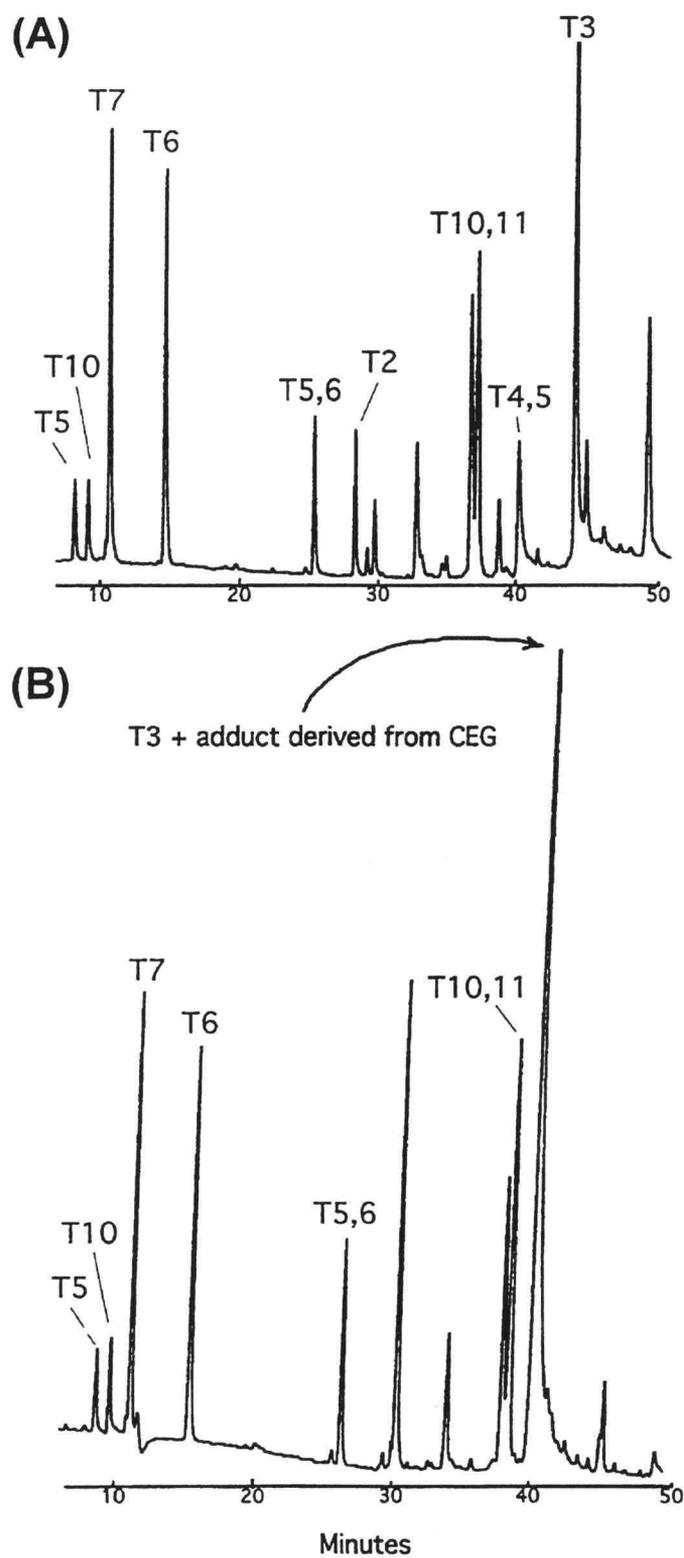


Figure 3.5. Chromatographic separation of peptides produced following the tryptic digestion of (A) native thioredoxin and (B) thioredoxin mono-adduct.

Table 3.1 Predicted and experimental masses of *E. coli* thioredoxin treated with CEG and digested with trypsin.

Peptide	Sequence	Theoretical <i>m/z</i>	Observed <i>m/z</i>
T1	1-3	349.2	n.d.
T11	97-100	445.3	n.d.
T7	70-73	508.3	508.0 ^c
T5	53-57	531.4	531.4 ^a
T10	91-96	574.4	574.4 ^a
T9	83-90	789.4	790.4 ^a
T12	101-018	892.4	892.4 ^a
T8	74-82	1001.6	1001.6 ^a
T6	58-69	1267.7	1267.6 ^a
T11,12	97-108	1318.7	1318.5 ^a
T2	4-18	1731.9	1731.8 ^a
T8,9	74-90	1772.0	1772.8 ^b
T4	37-52	1805.9	1806.0 ^a
T4*	37-52	2140.0	2139.9 ^a , 2139.2 ^b , 2139.4 ^c
T1,2	1-18	2062.0	2062.0 ^a
T3 [^]	19-36	2095.9	2095.0 ^b
T3*	19-36	2370.9	2371.6 ^a , 2371.0 ^b , 2371.2 ^c

* Peptide with adduct derived from CEG

[^] Cysteines are carboxyamidomethylated

^a FAB/MS

^b MALDI/MS

^c Ionspray MS

The bis-adduct of reduced thioredoxin was collected and rechromatographed prior to digestion by trypsin. Although the HPLC peak appeared to represent a single compound, MALDI mass analysis revealed additional compounds that could be assigned to a mono-adduct and a tris-adduct (Figure 3.3B). The tryptic digest was mass analyzed by MALDI prior to being chromatographed; the spectrum revealed an intense peak at m/z 2,139 that could be assigned to the alkylated form of tryptic peptide T4 (Figure 3.4B). This peptide appeared in the chromatographic peptide map at 36 minutes and was well resolved from other known tryptic peptides; hence, it could be collected off the HPLC system with relative ease.

Sequencing modified peptides by CID with Tandem mass spectrometry.

The peptide corresponding to T3 modified by the episulfonium ion of CEG produced doubly (m/z 1,186.2) and triply (m/z 791.2) charged ions when analyzed by ionspray mass spectrometry (Figure 3.6A). The doubly charged form of this peptide was subjected to CID to produce a series of b and y fragment ions. CID spectra of peptides are distinguished by a series of b and y fragment ions that provide sequence information about the peptide (Biemann, 1988). By noting which peaks in the CID spectra shifted compared to the expected fragments for an unalkylated peptide, it was possible to identify the amino acid which had undergone alkylation by CEG. The CID spectrum of alkylated T3 produced y_3 through y_{14} and b_2 through b_9 fragment ions (Figure 3.6B). The $y_5 - y_{14}$ ions were shifted 334 amu above their expected mass values in the unalkylated peptide, whereas y_3 and y_4 were only shifted by 58 amu, the mass increment associated with alkylation by IAA. In addition, y_5 lost a glutamic acid residue from the adduct derived from CEG to produce the observed ion at m/z 768. These observations are consistent with an adduct derived from CEG on Cys-32 and a carboxyamidomethyl moiety on Cys-35. No fragment ions could be identified that would indicate alkylation by CEG's episulfonium ion at Cys-35.

The peptide, which produced a MALDI signal at m/z 2,139 displayed a doubly charged ion at m/z 1,070 and a triply charged ion at m/z 714 in the ionspray mass spectrum. The triply charged ion was subject to CID because the doubly charged ion was too weak for tandem MS analysis. The product ion spectrum produced was not as

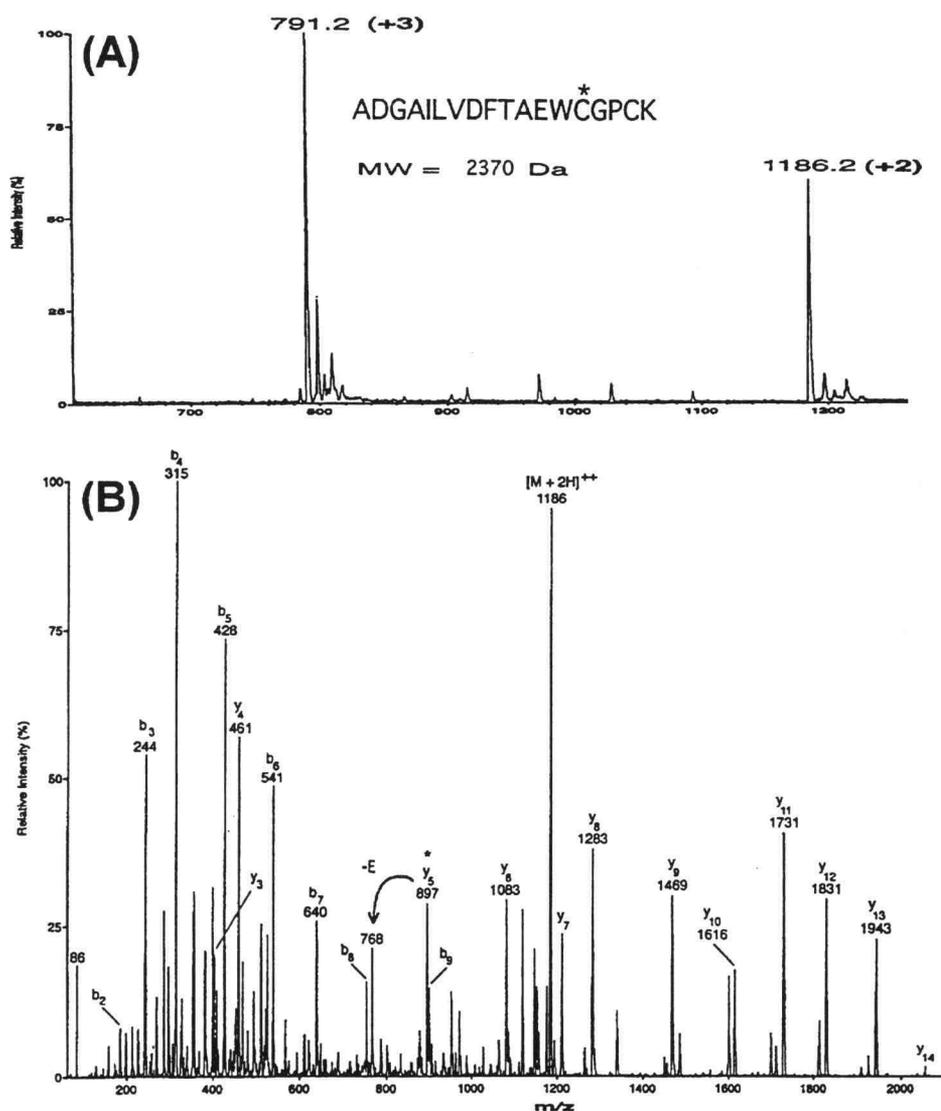


Figure 3.6. (A) Ion-spray mass spectrum of alkylated T3 showing doubly and triply charged species. This peptide was collected from the tryptic digest of the mono-adduct. (B) Spectrum of daughter ions formed by CID of the $[M+2H]^{2+}$ parent (m/z 1,186) of the alkylated peptide T3, obtained by ionspray MS/MS on a Sciex API III triple quadrupole mass spectrometer by direct infusion of the sample into the ion source. The ion denoted by an asterisks corresponds to Cys32-Gly-Pro-Cys35-Lys alkylated by CEG at Cys32 and by IAA at Cys35. This ion loses a glutamic acid residue from the adduct derived from CEG to produce the ion signal at m/z 768.

informative as that obtained for alkylated T3. Fragment ions were formed, but no ions shifted by the mass of the alkylating species could be identified among the ions detected. An ion signal observed at m/z 334 is consistent with extensive loss of the alkylating species ion from the peptide during the CID process; this could account for our inability to ascertain the site of alkylation on this peptide by this method.

Alkylation with ^{15}N -labeled CEG. This work and previous work produced evidence that tris- as well as tetra- adducts of thioredoxin were present (Meyer, et al., 1994), albeit, in low amounts. The tetra-alkylated thioredoxin was formed with a much larger excess of CEG than used in the experiments described in the present work. In an attempt to identify these sites of alkylation, oxidized thioredoxin was alkylated with a mixture of CEG that contained 50% ^{15}N labeled glycine with the expectation that the isotopic pattern of the alkylated peptides would point to possible alkylation sites. Oxidized thioredoxin, which has no free thiols, was used to assure that alkylation could not occur at the cysteines. The reaction products of oxidized thioredoxin alkylated with 35-fold excess CEG were chromatographed by HPLC; the resulting chromatogram showed an adduct peak representing 11% of oxidized thioredoxin and two smaller adduct peaks that account for 2.2% and 1.4% of the area of oxidized thioredoxin, respectively. The largest chromatographic adduct peak consisted primarily of bis-adduct but contained a small contribution from tris-adducted thioredoxin as shown by ionspray mass analysis (data not shown). The mass analysis of the minor chromatographic peaks also showed evidence of coelution of bis- and mono-adducts. Another reaction was carried out with the aim of producing sufficient bis-adduct to allow for peptide mapping of a tryptic digest. Reaction of 300 fold excess CEG over oxidized thioredoxin increased the amount of bis-adduct so that its area now accounted for approximately 45% of that of the unreacted oxidized thioredoxin when chromatographed by HPLC. Mass analysis of the tryptic digest by MALDI showed the presence of alkylated tryptic peptide designated as T4. This peptide was examined at medium resolution by FAB mass spectrometry. The isotopic pattern observed for this peptide matched the theoretically predicted pattern (Figure 3.7), and, thus, gave additional support for this peptide indeed being alkylated by the

episulfonium ion of CEG. No other alkylated peptides were observed, possibly because sensitivity for the CEG-alkylated peptides was not very high in FAB/MS. It may be that CEG adducted peptides at amino acids other than cysteine are not stable and lose the alkylating moiety during the digestion and/or peptide mapping.

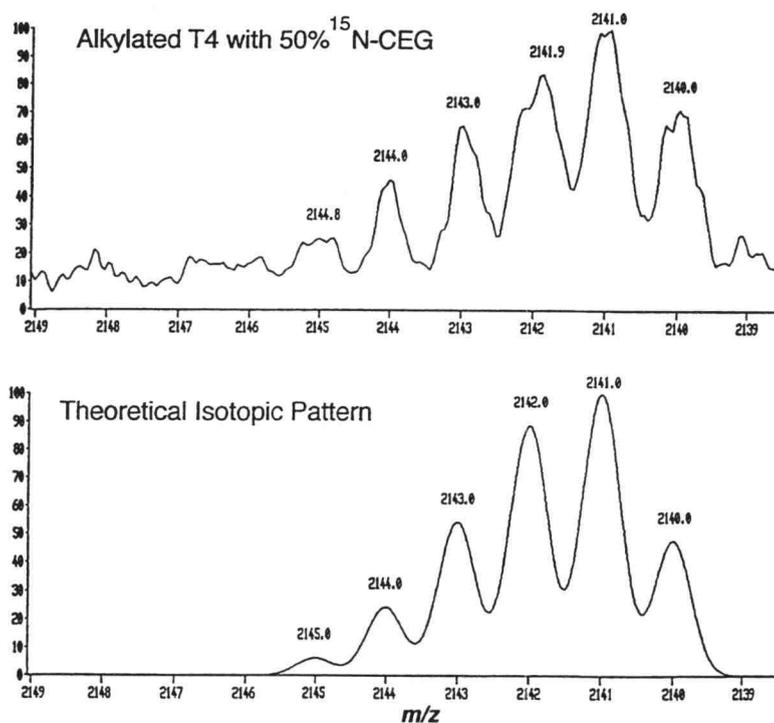


Figure 3.7. Medium resolution FAB mass spectrum of the isotopic pattern of T4 with m/z 2,141 obtained from a tryptic digest of an oxidized thioredoxin adduct alkylated with a 50% mixture of ^{15}N -labeled CEG. Below is the isotopic pattern of the peptide with the expected elemental composition that should be observed, assuming alkylation by the episulfonium ion of CEG.

Reaction of Reduced Thioredoxin at high pH. In order to investigate the reactivity of Cys-35 towards the episulfonium ion of CEG, reduced thioredoxin was first treated with IAA at pH 7.0. Only Cys-32 is present as the thiolate anion and will react with IAA (Kallis and Holmgren, 1980). Unreacted IAA was removed using a P-2 gel. Mass analysis of the resulting product by MALDI revealed a peak at m/z 11,728 that

would correspond to the expected thioredoxin with carboxyamido-methylation at Cys-32 with no alkylation at Cys-35. CEG was then added in excess after the pH had been adjusted to 8.5. The higher pH would result in at least 65% of the Cys-35 thiol ionized, assuming a pK_a of 7.9 (Huimin, et al., 1993). Following this reaction, and addition of more IAA, HPLC showed the formation of one major product (Figure 3.8). A MALDI peak for this thioredoxin product was observed at m/z of 11,786, which corresponds to thioredoxin with two molecules of IAA but no adduct derived from CEG.

Discussion

The results of this study confirm prior findings that the episulfonium ion of CEG is a chemically reactive species capable of alkylating nucleophilic sites in the protein thioredoxin at physiologic pH. The focus of the present investigation was to identify the specific amino acids that undergo alkylation when adducts are formed between the episulfonium ion of CEG and thioredoxin. Reaction of reduced thioredoxin resulted in the formation of at least three adducts the major of which is a mono-adduct that readily forms with only a 10-fold molar excess of CEG over thioredoxin. The mono-adduct was digested with trypsin, and the peptide containing the alkylating moiety was sequenced by tandem mass spectrometry. The sequencing analysis showed the site of alkylation to be exclusively at Cys-32, which is one of the two active site cysteines. The preference for this alkylation site was anticipated because Cys-32 has a lower pK_a than Cys-35. The pK_a values of Cys-32 and Cys-35 have been measured by Raman spectroscopy to be 7.1 ± 0.2 and 7.9 ± 0.2 (Huimin, et al., 1993). The y_5 ion present in the CID spectrum (Figure 3.6B) contains the alkylating moiety and also fragments by loss of a glutamic acid residue from the adduct derived from CEG. A similar observation was observed for the adducts of oxytocin alkylated by CEG at its two cysteines (Erve, Deinzer and Reed, 1995). Cys-35's lack of reactivity at pH 8.5 was unexpected because at this pH it is presumed to be at least 65% ionized. The

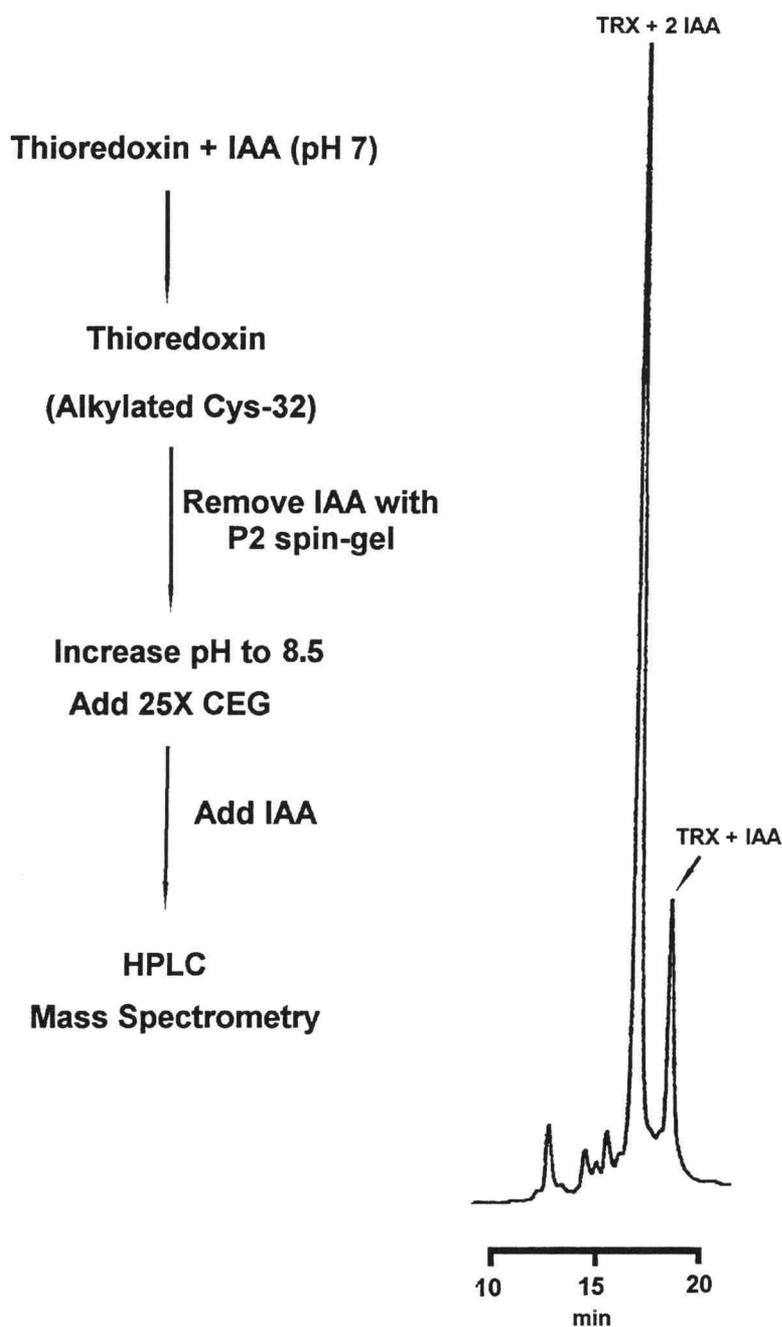


Figure 3.8. HPLC chromatogram of thioredoxin treated with IAA and CEG at pH 8.5. Major peak represents thioredoxin with two adducts of IAA.

alkylating agent IAA, which is smaller relative to CEG, is able to alkylate Cys-35 at this pH ((Kallis and Holmgren, 1980), and this study). The active site of reduced thioredoxin has been determined by NMR (Dyson, et al., 1990) and is known to have the cysteines on a protruding loop with Cys-32 exposed. In comparison, however, Cys-35 is not as exposed (Eklund, et al., 1991). Given that tertiary structure is important in directing alkylation towards specific nucleophiles and away from others, it is possible that the relatively large episulfonium ion of CEG cannot access Cys-35.

A bis-adduct of reduced thioredoxin produced a peptide digest that contained alkylated T3 and T4 tryptic peptides. As for the mono- adduct, this adduct was also alkylated at Cys-32. Attempts to determine the site of alkylation in the peptide T4 by tandem mass spectrometry failed, ostensibly because the adduct derived from CEG fragmented during CID. The most likely amino acid in this peptide to undergo alkylation by the episulfonium ion of CEG on this peptide is Tyr-49, because after thiol groups the hydroxyl functional group on tyrosine is the next most reactive nucleophile towards the alkylating species derived from CEG (Jean and Reed, 1989). Tyrosine in the peptide angiotensin was shown to be alkylated following treatment with styrene oxide (Ferranti, et al., 1992). A molecular model of thioredoxin shows that Tyr-49 is on the surface of the protein, further supporting the plausibility of this target. Tyrosine in the peptide oxytocin was also suspected of being alkylated by the episulfonium ion of CEG; however, this could not be confirmed because the complete adduct fragmented off the peptide (Erve, Deinzer and Reed, 1995). Regardless of where the bond is formed in this second alkylation of thioredoxin, it is definitely more labile under CID conditions (with the collision parameters used) than is the sulfur-adduct bond. The significance of this putative alkylated Tyr-49 on thioredoxin is not known. The tris adduct, being formed in much smaller amounts, could be neither purified nor digested so that the third site of alkylation could not be identified.

When oxidized thioredoxin was alkylated by the episulfonium ion of CEG and then chromatographed, several smaller adduct peaks eluted before oxidized thioredoxin. These adducts were formed in much smaller amount than the major mono-adduct formed with reduced thioredoxin, indicating that nucleophilic sites other

than the cysteine thiol are much less reactive towards CEG's episulfonium ion. MALDI mass analysis shows that the largest chromatographic peak is actually the result of two coeluting peaks. The tryptic digest was mass analyzed by MALDI and an alkylated T4 was identified. There is not much difference between the tertiary structure of reduced and oxidized thioredoxin (Dyson, et al., 1990), and it is probable, therefore, that this T4 peptide adduct of oxidized thioredoxin is alkylated at the same site (putatively at Tyr-49) as that on reduced thioredoxin. We were not able to identify other alkylated peptides in the tryptic digests of these adducts by either FAB or MALDI mass spectrometry. It is possible that the adduct formed is unstable and is cleaved off the amino acid during proteolytic digestion. This possibility is supported by our attempt to further digest the T4 peptide adduct with endoproteinase Glu-C. A 12 hour incubation of the peptide with enzyme at 50° C produced no observable digestion of the peptide but it did result in loss of the adduct derived from CEG. That is, we no longer observed alkylated T4 but instead only unalkylated T4, suggesting that the adduct was unstable at this incubation temperature.

Protein alkylation is known to play a role in the mechanism of toxicity of a number of drugs, such as, acetaminophen (Mitchell, et al., 1973) and xenobiotics, such as, bromobenzene (Jollow, et al., 1974). It is possible, however, that a given protein adduct has no toxicologic consequence and therefore, it is not sufficient to simply show that a protein has been adducted to infer a toxicologic mechanism. For example, alkylation of alcohol dehydrogenase by the active metabolite of N, N-dimethyl-4-aminoazobenzene causes only minor alterations in enzyme activity and has been regarded as a detoxification pathway for this compound (Coles, et al., 1987). Therefore, the capability to identify the particular amino acid that has undergone alkylation by CEG's episulfonium ion in a protein might point to a toxicological role for protein alkylation as a mechanism of toxicity, especially if the amino acid is vital to the functioning of the protein. In this work, we have used a combination of MALDI and ionspray tandem mass spectrometry to show that one of the active site cysteines of the enzyme thioredoxin, Cys-32, is alkylated by CEG's episulfonium ion at physiologic pH. This finding supports previous work in this laboratory that showed

the partial loss of thioredoxin insulin reduction activity following *in vitro* treatment with CEG (Meyer, et al., 1994). Such loss of activity suggests that if levels of CEG in target cells were high enough an important enzyme required for a number of functions *in vivo* might be inactivated.

To ascertain the consequences of our findings, an antibody against CEG-alkylated thioredoxin would aid in determining if thioredoxin is alkylated in isolated hepatocytes or *in vivo* following the administration of a toxic dose of DCE. Demonstration of alkylated thioredoxin as a critical target *in vivo* would strengthen the implication that CEG plays a role in the toxicity of DCE. Microsomal metabolites of DCE are known to be reactive towards protein, eg. lung microsomal proteins (Banerjee, et al., 1980). Hence, it would be useful to compare the reactivity of 2-chloroacetaldehyde, a P450 metabolite of DCE, towards thioredoxin in order to better understand the relative importance of the GSH-conjugation pathway and the microsomal oxidation pathway in the toxicity of DCE. It would also be of interest to investigate the fate of thioredoxin alkylated by CEG in cells. Is it possible, for instance, that alkylated thioredoxin will be degraded more quickly than unalkylated thioredoxin? Eventually, it will be important to know whether human thioredoxin is alkylated by the episulfonium ion of CEG, because exposure to DCE occurs for certain workers (Williams and Diwan, 1994).

Acknowledgements

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Chapter V

Determination of Hemoglobin Sulfhydryl Status Following Alkylation by S-(2-chloroethyl)glutathione with Mass Spectrometry and Spectrophotometry

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Abstract

Human hemoglobin was alkylated at physiologic pH by the episulfonium ion of S-(2-chloroethyl)glutathione (CEG) in order to investigate if hemoglobin might serve as a biomarker of exposure for 1,2-dichloroethane encountered in the workplace. *In vitro* alkylation resulted in three alkylation products on the α chain and at least two alkylation products on the β chain as determined by MALDI mass spectrometry. To determine if the site of alkylation was the reactive sulfhydryl present at Cys-93 on the β chain of hemoglobin, a spectrophotometric assay using 4,4'-dithiopyridine was used to measure the free sulfhydryl groups before and after treatment with various amounts of CEG. Results indicate that the episulfonium ion of CEG did not react at the sulfhydryl group, as there was no decrease in the sulfhydryl to hemoglobin ratio, even at very high ratios of CEG to hemoglobin. In contrast, iodoacetamide did react with the sulfhydryl groups and gave a dose dependent decrease in the sulfhydryl to hemoglobin ratio as measured by this assay. CEG treated hemoglobin was digested with *Staphylococcus aureus* endoproteinase Glu-C and the digest analyzed by FAB mass spectrometry. Efforts to identify specific alkylated peptides on hemoglobin were unsuccessful due to the difficulty in isolating a pure adducted protein prior to digestion. These results indicate that although the episulfonium ion of CEG does alkylate human hemoglobin, the levels required are much higher than what would be expected *in vivo* and do not encourage further study with respect to biomonitoring based on measuring hemoglobin adducts.

Introduction

Hemoglobin, an oxygen transport protein, is found in erythrocytes and carries oxygen from the lungs to tissues throughout the body. It is a tetramer with a molecular weight of 64,450 Da, and is composed of two dimers each consisting of two distinct polypeptide chains (Dickerson and Geis, 1983). In the fields of toxicology and molecular epidemiology, hemoglobin has been used as a biomarker for chemical exposure in the work place to measure the amounts of carcinogens in the body such as ethylene oxide (Farmer, et al., 1986), propylene oxide (Osterman-Golkar, et al., 1984), acrylamide (Bergmark, et al., 1993), and acrylonitrile (Osterman-Golkar, et al., 1994). Biomonitoring has also been used to quantify the adducts of the chemotherapeutic agent 1-(2-chloroethyl)-1-nitrosourea in cancer patients receiving this drug in order to monitor therapy (Bailey, et al., 1991). The purpose of hemoglobin biomonitoring is ultimately to determine whether an individual has suffered genetic damage and to estimate the possible risk of cancer from this carcinogen; the amount of hemoglobin adduction in the exposed individual is important in arriving at these conclusions (Calleman, et al., 1978; Ehrenberg, et al., 1974). Biomonitoring relies on a variety of nucleophilic sites in hemoglobin which can combine with reactive electrophilic metabolites. The amino acids—cysteine, histidine, lysine, aspartic acid and N-terminal valine—react specifically with a number of chemicals. Hemoglobin is easily sampled and, because the erythrocyte has a life-time of 120 days, it integrates a person's cumulative exposure to the carcinogen over that time (van Welie, et al., 1992). Many analytical techniques have been developed to quantify hemoglobin adducts of certain chemicals. Examples include a modification of Edman degradation which has been applied to N-terminal valine adducts of ethylene oxide (Tornquist, et al., 1986), mild basic hydrolysis followed by derivatization with pentafluoropropionamide and GC/MS analysis for 4-aminobiphenyl adducts on cysteine (Bryant, et al., 1987), and Raney nickel to cleave the bond between cysteine and styrene oxide (Ting, et al., 1990).

The compound DCE is subject to both microsomal metabolism mediated by cytochrome P450 enzymes and glutathione conjugation mediated by cytosolic GST activity (Storer and Conolly, 1985). The product of the latter pathway is CEG, which can form an episulfonium ion by the intramolecular displacement of chlorine by sulfur. The episulfonium ion of CEG can alkylate nucleophilic sites on DNA which have been identified primarily at the N⁷ of guanine (Humphreys, et al., 1990). This is thought responsible for the mutagenicity and carcinogenicity of DCE (Storer and Conolly, 1985). It has been shown that the episulfonium ion of CEG reacts readily with the sulfhydryl group of cysteine, and to a lesser extent, the hydroxyl group of tyrosine residues in the peptide oxytocin (Erve, et al., 1995b) and the protein *E. coli* thioredoxin (Erve, et al., 1995a; Meyer, et al., 1994). CEG formed *in vivo* can be detected in the bile of rats dosed with BCE (Marchand and Reed, 1989) and can cause renal proximal tubular damage in rats (Kramer, et al., 1987).

In erythrocytes, hemoglobin is the predominant protein representing ~ 90% of the total cytosolic protein. Erythrocytes also contain reduced GSH in millimolar concentrations, which has been shown to be important for maintaining sulfhydryls in the reduced state and detoxifying trace amounts of hydrogen peroxide (Srivastava and Beutler, 1970). GST enzymes have been identified in human erythrocytes that are capable of metabolizing a number of small molecular weight halogenated hydrocarbons such as methylbromide, methylchloride and methyliodide (Hallier, et al., 1990). It appears that a polymorphism exists with respect to this activity with 75% of the population thought to be conjugators and the remaining 25% non-conjugators (Hallier, et al., 1993). We decided to investigate the ability of the episulfonium ion of CEG to alkylate human hemoglobin *in vitro* since this is requisite if hemoglobin were to serve as a biomarker of human exposure to DCE. GSH protects hemoglobin from alkylation by IAA, signifying that GSH lowers the sensitivity of hemoglobin biomonitoring towards electrophilic agents that can be detoxified by GSH (Evelo and Henderson, 1988). In the case of DCE, GSH is used in the process of forming the reactive conjugate, possibly diminishing its protective role. The purpose of the present investigation was to determine if the episulfonium ion of CEG forms adducts on

hemoglobin at physiologic pH, and if the site of adduction is the highly nucleophilic β -93 cysteine sulfhydryl. To this end, we used reverse-phase HPLC to chromatograph reaction mixtures resulting from the treatment of a hemolysate with CEG, and MALDI mass spectrometry to measure the masses of the individual α and β chains of hemoglobin. The β -93 cysteine was monitored with a spectrophotometric assay utilizing PDS.

Materials and Methods

Chemicals. Lyophilized human methemoglobin standard, Drabkin's solution (20 parts $K_3Fe(CN)_6$, 100 parts sodium bicarbonate, 5 parts KCN), TFA, PDS and IAA were obtained from Sigma (St. Louis, MO). Endoproteinase Glu-C from *Staphylococcus aureus*, also known as V8, was purchased from Boehringer Mannheim (Mannheim, Germany). Human blood was obtained from the first author by venepuncture and collected into a 5 mL EDTA containing Vacutainer tubes from Beckton Dickinson. HPLC solvents were purchased from J. T. Baker (Phillipsburg, NJ) and 18 megohm water was prepared by a Milli-Q system (Waters, Bedford, MA). Dialysis tubing (1.4 cm width) with a molecular weight cut-off of 6 to 8 kDa was from Spectra/Por (Spectrum, Houston, TX)

Synthetic procedures. CEG was synthesized, according to the method of Reed and Foureman (Reed and Foureman, 1986), in a three-necked round-bottomed flask starting with 0.5 g GSH. 50 ml of liquid ammonia was obtained by condensing gaseous anhydrous ammonia with an ice-bath of ethanol and dry ice. At a temperature of approximately $-40^\circ C$, pellets of metallic sodium were added until there was a permanent blue color; this usually required 4-6 pellets. Approximately 0.80 mL of BCE was added dropwise via a 1 mL glass syringe. With the addition of BCE the reaction mixture would turn white. The reaction was maintained at -40° for 45 minutes after which the ammonia was evaporated. The crude CEG was lyophilized at least 12 hours before further purification by preparative HPLC (Figure 4.1A).

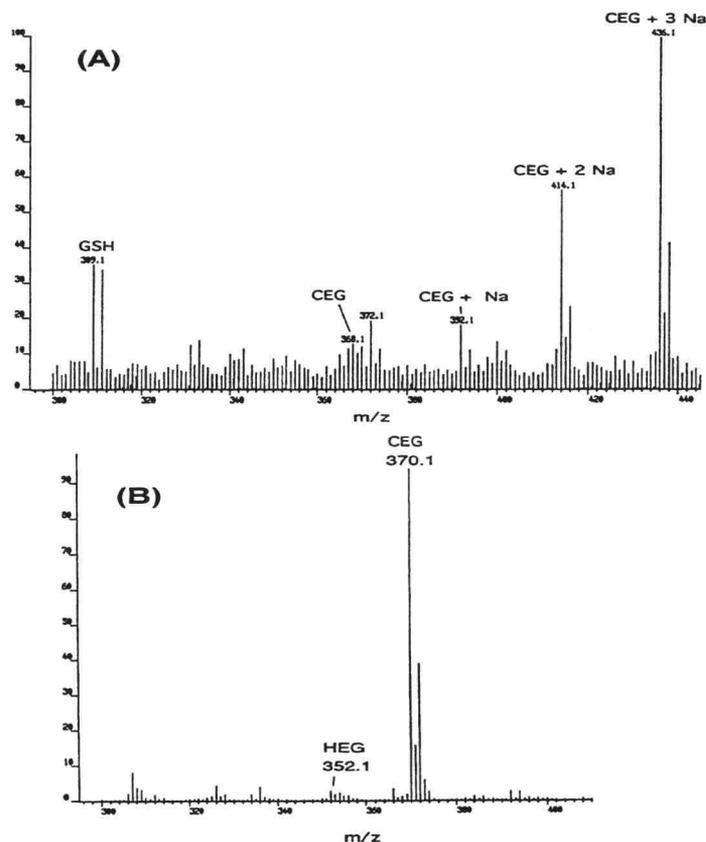


Figure 4.1. FAB mass spectrum of (A) crude CEG preparation showing CEG present as sodium adducts and (B) purified CEG. The purified CEG is visible at m/z 370; the small peak at m/z 352 represents the hydrolysis product, HEG.

Hemolysate Preparation. Hemolysate was prepared according to the procedure of Attoni and Brunori (Antonini and Brunori, 1971). Briefly, approximately 4 mL of whole blood was centrifuged at 200 x G to separate red blood cells (RBCs) from plasma. The RBCs were washed three times with cold isotonic saline. To lyse the RBCs, a volume of cold water three times the volume of the RBCs was added and allowed to stand 30 minutes at 4°C. Membrane proteins were precipitated by adding one volume saturated ammonium sulfate for every four volumes of hemolysate and allowing to stand for 45 minutes. The lysate was then centrifuged at 10,000 x G for 30 minutes on a Sorvall centrifuge (4°C) after which the clear supernatant

(hemolysate) was transferred to dialysis tubing. The hemolysate was dialyzed against three changes of 0.05 M phosphate buffer, pH 7.4 for 36 hours. The hemolysate was stored at 4°C and used within two weeks.

Instrumentation. Preparative HPLC purification of crude CEG was achieved with a C18 reverse phase column (22mm x 28 cm, Whatman). Crude CEG elution was accomplished with an isocratic solvent containing methanol/H₂O/glacial acetic acid (9:91:4 v/v/v) at a flow of 10 mL/min. The liquid chromatography system included twin model 110 Altex pumps and a fixed wavelength UV detector (LKB-Produkter AB, Bromma, Sweden) set at 220 nm and a 750 µL sample injection loop. Chromatograms were recorded with an Spectra-Physics (SP4200) computing integrator. Between 40 and 50 mg crude CEG was dissolved in 650 µL 0.2% acetic acid and immediately injected onto the column. The CEG, which eluted in 35-40 minutes, was collected in a lyophilization jar partially submersed in liquid nitrogen. At the end of the day, the frozen mixture of CEG was lyophilized to remove the solvent. The final purified CEG contained at most 5% impurity of the hydrolysis product, HEG, as estimated by FAB mass spectrometry (Figure 4.1B). Up to 400 mg of crude preparation could be purified per day (Figure 4.2).

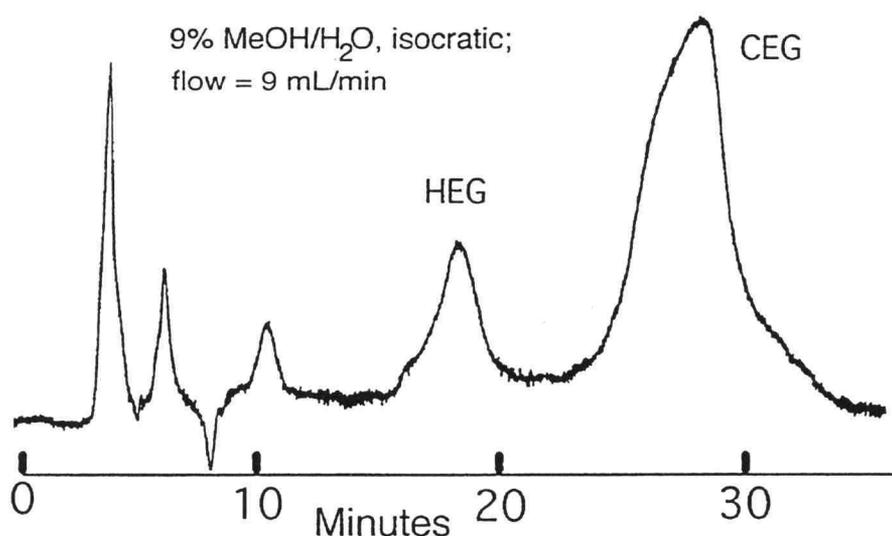


Figure 4.2. Preparative HPLC chromatogram recorded during purification of CEG.

Hemoglobin was chromatographed on a C4 analytical HPLC column (250 x 4.6 mm, 30 angstroms, Custom LC, Houston, TX). The elution gradient was linear from 42% B to 85% B over 45 minutes with a flow of 1 mL/min [Solvent A= CH₃CN/H₂O/TFA (20/80/1 v:v:v); solvent B = CH₃CN/H₂O/TFA (80:20:0.8 v/v/v)]. The liquid chromatography system consisted of a Spectraseries P200 HPLC pump (Spectra Physics, Fremont, CA), a variable wavelength UV absorbance detector set for 220 nm (Spectraflow 757) and an integrator (Hewlett Packard, Palo Alto, CA). Hemoglobin α and β chains and their adducts were collected manually and concentrated by roto-evaporation prior to mass spectrometric analysis.

Matrix-assisted laser desorption-ionization (MALDI) mass spectrometry was accomplished with a custom built time-of-flight instrument equipped with a frequency-tripled (355 nm) Nd:YAG laser (Spectra-Physics GCR-11). Sample preparation and data analysis were described elsewhere (Erve, et al., 1995a). Ionspray mass spectrometry was accomplished with a Sciex III plus triple-quadrupole mass spectrometer (Sciex, Thornhill, Canada); conditions were as described elsewhere (Erve, Deinzer and Reed, 1995b). Fast atom bombardment (FAB) mass spectrometry was performed on a Kratos MS-50 double focusing instrument operated at a resolution of 1000 for raw data collection. A 2 μ L sample volume was mixed on the probe tip with 2 μ L of matrix consisting of thioglycerol/glycerol (2:1 v/v). Xenon gas was used to generate the primary ionizing beam from an Ion-Tech ion gun operated at 7-8 kV.

Spectrophotometric measurements was performed on either a SLM AMINCO DW2000 UV-vis spectrophotometer (Milton Roy, Rochester, New York) or a Uvikon 810 UV-vis spectrophotometer. Quartz cuvettes (1 mL for hemoglobin standards; 3 mL for sulfhydryl determinations) were used in all measurements. The instrument was zeroed with a phosphate buffer blank.

Hemoglobin Concentration Measurements. Hemolysate hemoglobin concentrations were determined by Drabkin's method as modified by van Kampen and Zijlstra (van Kampen and Zijlstra, 1983). Hemoglobin standards were prepared by dissolving lyophilized cyanomethemoglobin in Drabkin's reagent to give the following concentrations: 0, 20, 40, 60, 120 and 180 mg/mL. Each standard was measured in

triplicate at 540 nm to create a calibration line (Figure 4.3). A 10 μL sample of hemolysate was diluted with 1 mL of Drabkin's reagent and measured in triplicate. The resulting absorbance was used to calculate the hemolysate hemoglobin concentration from the calibration line or with the molar extinction coefficient of

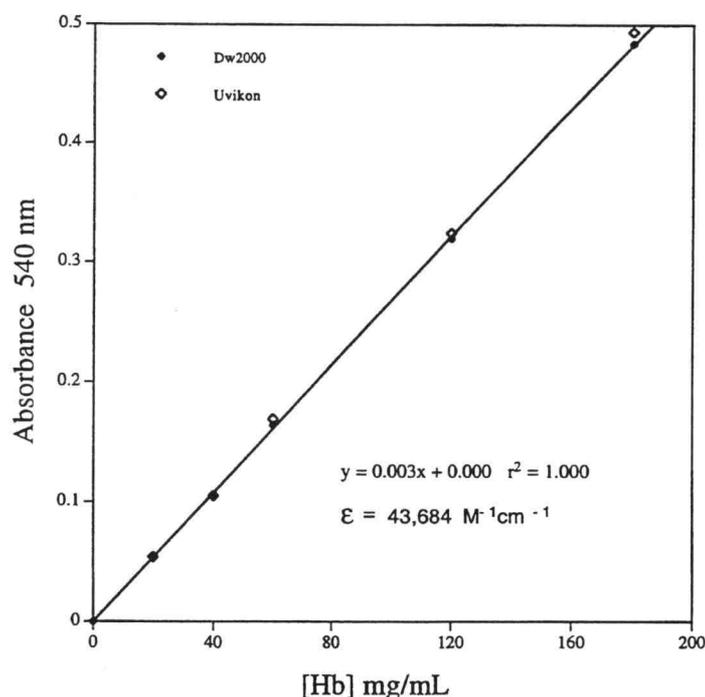


Figure 4.3. Calibration lines for cyanomethemoglobin standards at 540 nm.

cyanomethemoglobin, $44,000 \text{ M}^{-1} \text{ cm}^{-1}$ (van Kampen and Zijlstra, 1983). After determining the concentration of the hemolysate, it was diluted with phosphate buffer, pH 7.4 to approximately $2-4 \times 10^{-4} \text{ M}$ for use in experiments. Buffer concentration was approximately 200 mM phosphate. In order that hemoglobin concentrations could be determined by absorbance at 324 nm, some hemolysate was carefully diluted to make a set of standards of known concentration. These were used to construct a calibration curve based on absorbance at 324 nm, which gave a molar extinction

coefficient of $71,562 \text{ M}^{-1} \text{ cm}^{-1}$ (Figure 4.4A); Neis and coworkers reported a value of $80,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Neis, et al., 1984).

Hemoglobin alkylation by IAA or the episulfonium ion of CEG.

Hemolysate was reacted with equimolar, 2, 5, 10 and 20 fold excess IAA over hemoglobin. CEG was added in 100, 250 and 500 fold excess over hemoglobin. Both mixtures were allowed to react at room temperature; IAA was allowed to react for 30 minutes while CEG was allowed to react for 90 minutes. For some experiments, the hemoglobin CEG reaction mixtures were injected onto a C4 column so that α and β chains and their adducts could be collected for MALDI mass spectrometry analysis. For other experiments, the reaction mixtures were analyzed for protein sulfhydryl content as described below.

Hemoglobin sulfhydryl determination. To determine sulfhydryl concentrations following reaction with either IAA or CEG, 100 μL of the corresponding reaction mixture was diluted to 10 mL with 0.05 M phosphate buffer (pH 7.4). This was divided into three 2.90 mL aliquots. To one aliquot, 100 μL of water were added and hemoglobin concentration calculated based on its absorbance at 324 nm. To the other two aliquots, 100 μL of 1 mM PDS were added. A PDS blank was also prepared. The vials were stoppered and incubated in the dark for 40 minutes at 37°C . After incubation, the vials were allowed to reach room temperature before measuring the absorbance at 324 nm. Sulfhydryl concentrations were determined by dividing the absorbance by the molar extinction coefficient for PDS, determined by cysteine titration to be $20,117 \text{ M}^{-1} \text{ cm}^{-1}$ (Figure 4.4B). Reported values of the molar extinction of PDS were $19,800 \text{ M}^{-1} \text{ cm}^{-1}$ (Grassetti and Murray, 1967) and $20,500 \text{ M}^{-1} \text{ cm}^{-1}$ (Neis, et al., 1984). Ratios of hemoglobin sulfhydryl concentration to hemoglobin concentration were calculated by dividing the appropriate values.

Enzymatic digestion of hemoglobin. Hemoglobin, both native and CEG-treated, were digested with Endoproteinase Glu-C (also known as V8). Approximately 1500 pmole hemoglobin was dissolved in 50 μL 50 mM ammonium bicarbonate (pH 7.8). 20 μL 8M urea was diluted to about 2M with water to denature the protein.

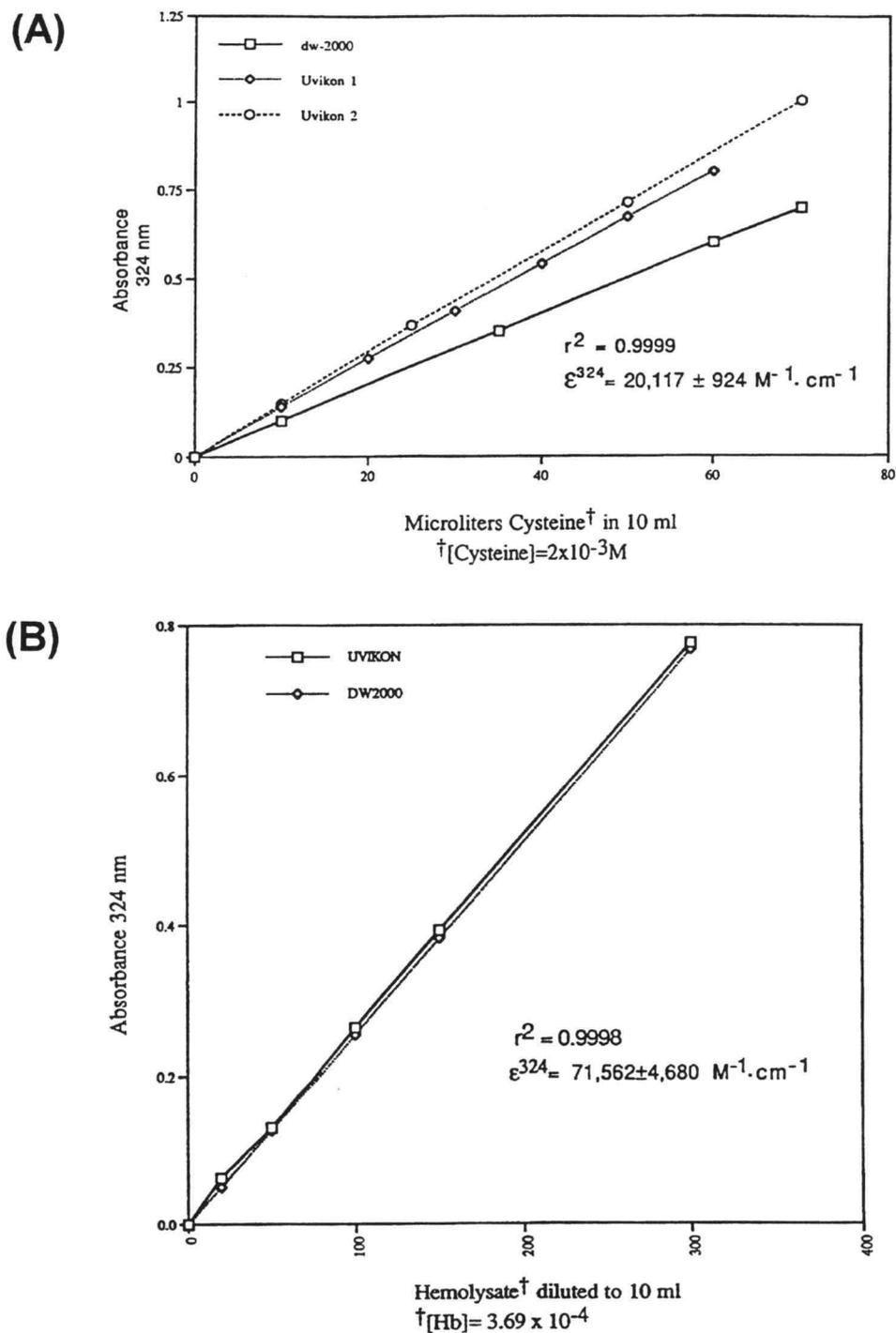


Figure 4.4. Measurement of the molar extinction coefficient (ϵ) at 324 nm for (A) PDS and (B) hemoglobin. The 3 calibration lines in (A) were obtained by titration with 2 mM cysteine and averaged to give the calculated value. The line in (B) was obtained by diluting a known concentration of hemolysate and measuring absorbance of hemoglobin at 324 nm.

Enzyme was added in a ratio of 1:10 (enzyme:protein) and the digest was allowed to incubate 4-6 hours at 37°C. The digestion was terminated by adding 5 μ L of 50% TFA before analyzing by FAB mass spectrometry.

Results

Human hemoglobin reacted with CEG *in vitro* at pH 7.4 resulted in the formation of a number of adducts as determined by both ionspray and MALDI mass spectrometry. With acetonitrile as a solvent, hemoglobin denatures when chromatographed, resulting in the separation of heme from the two polypeptide chains. Under these conditions, heme elutes first as a sharp peak followed by the β chain and α chain. However, when hemoglobin treated with CEG was chromatographed, no distinct adduct peaks appeared as was observed for the adducts of oxytocin (Erve, Deinzer and Reed, 1995b) or the adducts of thioredoxin (Erve, et al., 1995a). Rather, the peaks representing the α and β chains became less sharply defined. The β chain appeared to become broader at a lower level of CEG compared to the α chain (Figure 4.5). At the highest level of CEG, both chains chromatographed as very broad peaks. Other efforts to achieve separation between native and alkylated hemoglobin included using RP-HPLC chromatography with a C8 column, and a combination of cation-exchange chromatography followed by RP-HPLC chromatography with a C4 column; both attempts were without success.

When these peaks were analyzed by MALDI mass spectrometry it was apparent that both chains of hemoglobin had undergone alkylation by the episulfonium ion of CEG. In particular, mass spectral evidence supported the assignment of three adducts on the α chain and two on the β chain (Figure 4.6A and 4.6B). Ionspray mass spectrometry also produced evidence of adducts derived from CEG on both chains (data not shown).

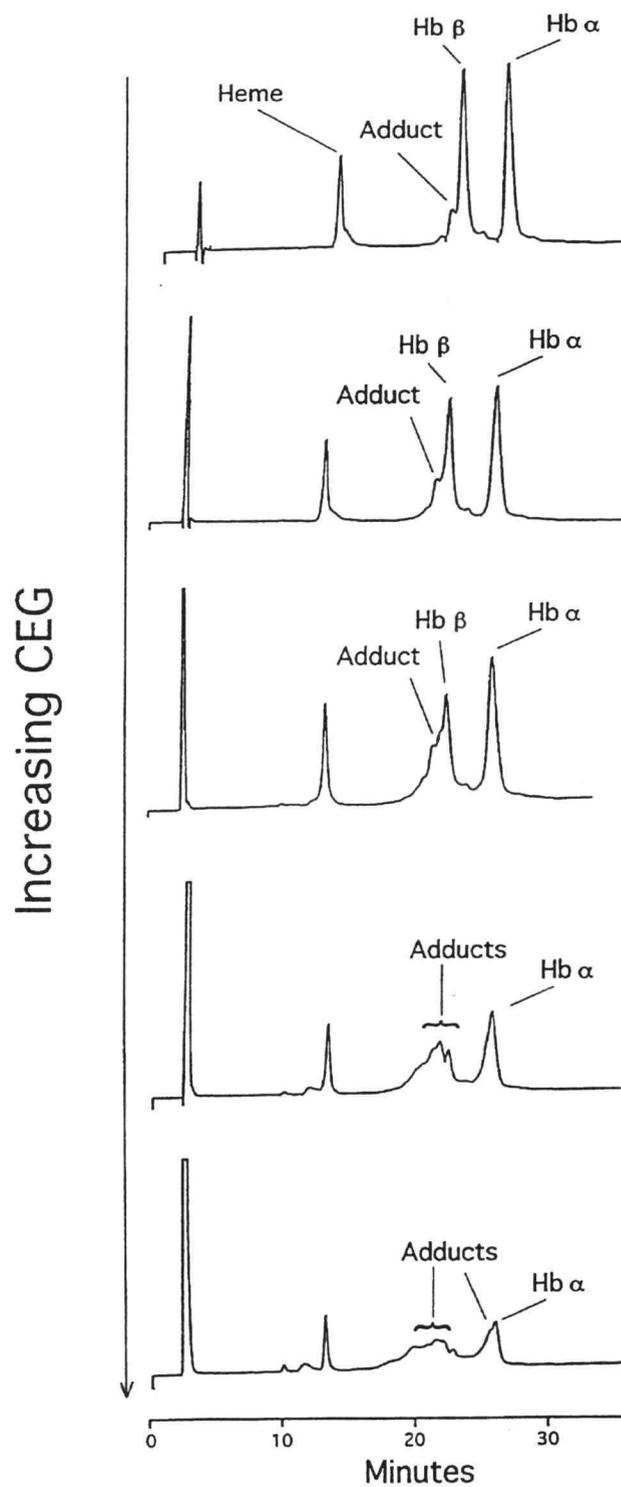


Figure 4.5. HPLC chromatograms of hemoglobin treated with increasing amounts of CEG. The heme elutes first followed by the β chain and α chain. The chromatogram represents the elution of UV absorbing material at 220 nm.

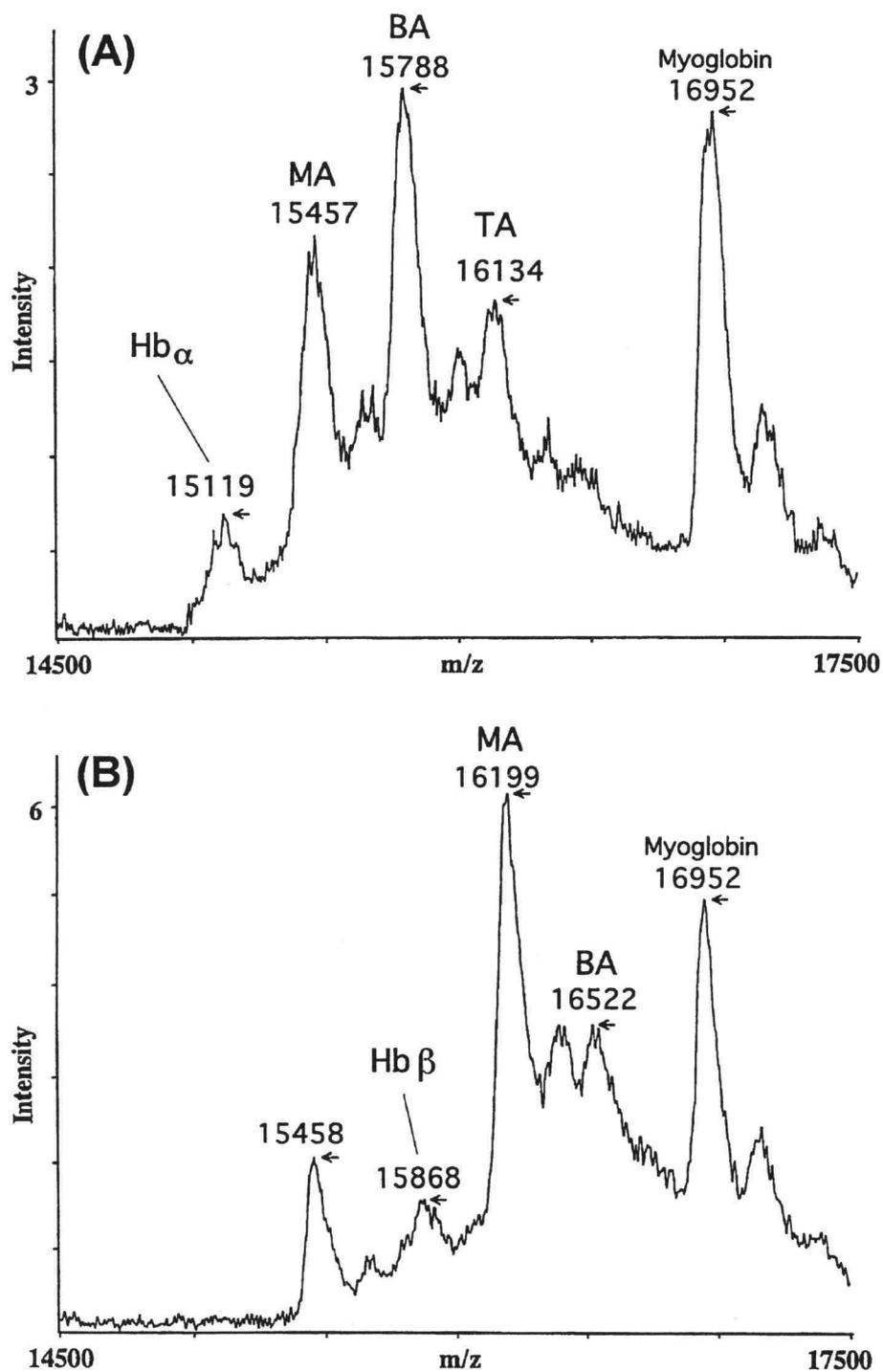


Figure 4.6. MALDI mass spectrum of (A) α -chain and three adducts and (B) β -chain and two adducts. The adducts were collected manually from the HPLC and roto-evaporated to dryness prior to analysis. The adducts were dissolved in water and mixed with sinnapinic acid as a matrix.

The hemoglobin reaction mixtures were also analyzed to determine the extent of reaction of either IAA or CEG with the two sulfhydryl groups in hemoglobin. A similar experimental design has been used to monitor the disappearance of free sulfhydryl groups after exposure to a number of different alkylating agents *in vitro* (Neis, et al., 1984). Following the reaction between IAA and hemoglobin, there was a clear dose dependent decrease in sulfhydryl concentration with increasing amounts of IAA (Figure 4.7, insert). With 2-fold excess IAA to hemoglobin, the sulfhydryl concentration had decreased to 75% of the control. At the highest dose, 20-fold excess, the sulfhydryl concentration was only 7% of the control indicating extensive modification of the two reactive sulfhydryl groups. After reaction with CEG, however, no such decrease was observed even at 500-fold excess CEG to hemoglobin

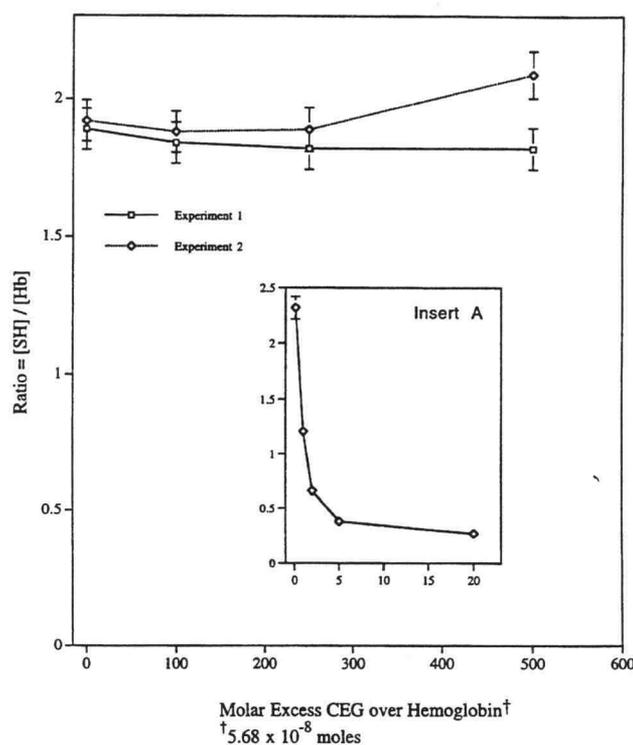


Figure 4.7. The ratio of sulfhydryl to hemoglobin concentration following treatment with 100X, 250X and 500X fold excess CEG. Measurements were made spectrophotometrically with PDS. Insert A show the ratio of sulfhydryl to hemoglobin concentration following treatment with up to 20 fold excess IAA.

(Figure 4.7). That CEG did react with sulfhydryl groups was demonstrated by reaction with GSH. Reacting 5-fold excess CEG over GSH reduced the free sulfhydryl concentration by over 90% as determined by the decrease in PDS absorbance. On examination of an absorption spectrum between 200 and 550 nm of hemoglobin present in the reaction mixtures, however, it was clear there were changes for CEG treated hemoglobin when compared to native hemoglobin. In particular, there was both a hypsochromic shift (shift to lower λ) and decrease in intensity of the Soret band observed for the hemoglobin samples treated with 250- and 500-fold excess CEG (Figure 4.8). In the case of the 500-fold CEG treated sample, there was also a change around 280 nm corresponding to the region of tyrosine absorption. For hemoglobin treated with the same excess IAA (100, 250, 500) there was neither a hypsochromic shift nor decrease in the intensity of the Soret band. There were changes in the tyrosine absorption region (data not shown).

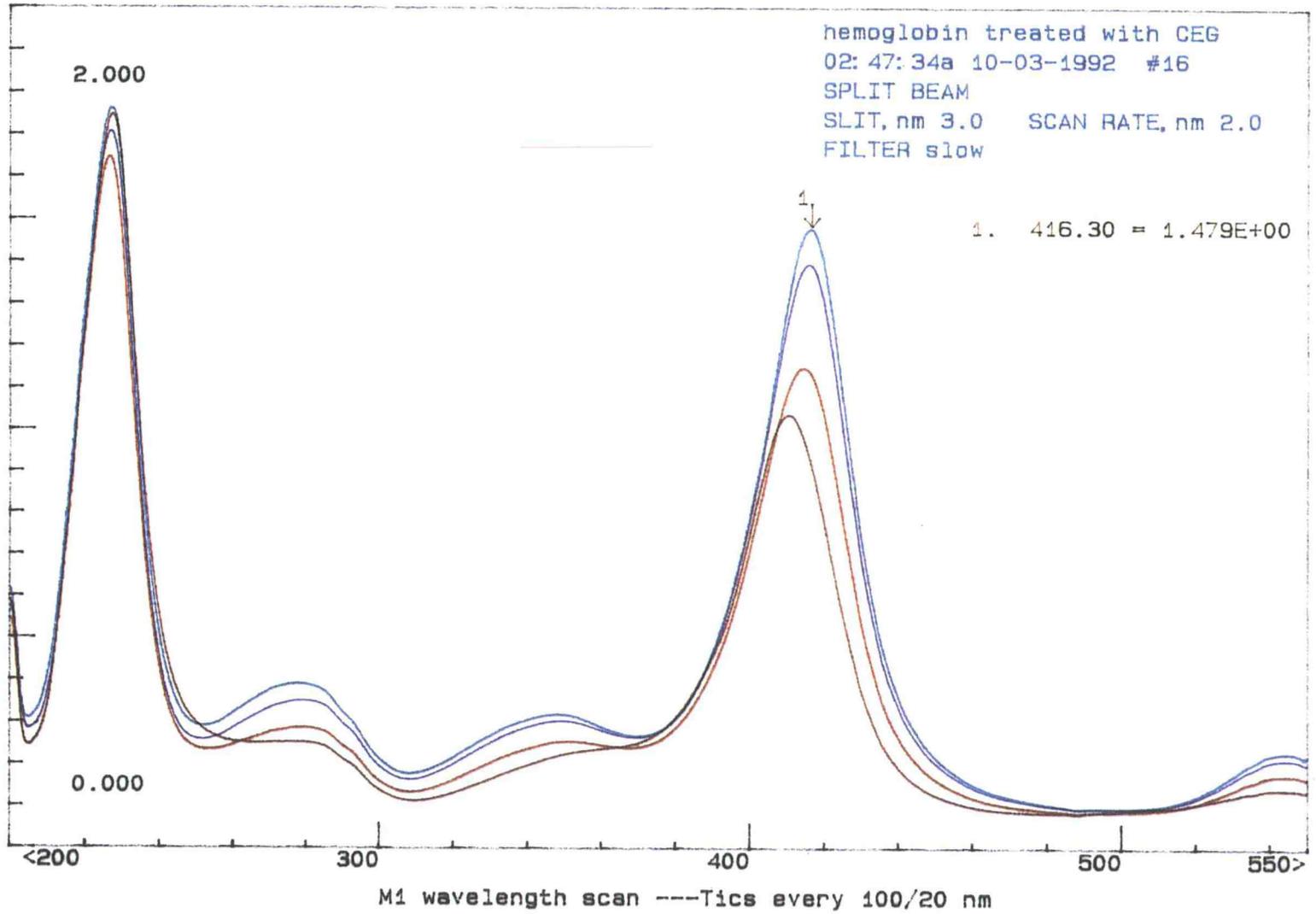
Enzymatic digestion by V8 of both native β chain, and a peak containing predominantly CEG-alkylated hemoglobin β chain produced a number of expected peptide fragments (Table 4.1). The treated sample contained many of the same

Table 4.1 Calculated and observed V8 digest fragments of CEG alkylated hemoglobin by FAB mass spectrometry.

Fragment	Sequence	Theoretical m/z	Observed m/z
V1	1-6	695.4	695.5
V1,2	1-7	824.4	824.5
V7	91-101	1305.6	1305.7
V3	8-22	1616.9	1616.9
V5	27-43	2095.2	2096.1
V8	102-121	2266.2	2267.1
V1,2,3	1-22	2427.2	n.d.
V9	122-146	2680.4	2681.6
V6	44-90	4843.5	4843.2
V7*	91-101	1639.7	1638.7

* The mass of this peptide could correspond to the addition of the alkylating moiety derived from CEG to V7, ie. V7 + 334 Da.

Figure 4.8 Absorption spectra of hemoglobin and CEG-treated hemoglobin. Native hemoglobin is colored turquoise; 100X, 250X, and 500X are colored purple, orange and red, respectively.



peptides fragments as observed for the control. Some of the unassigned peptides correspond to auto-digestion of the enzyme as deduced from a blank digest (data not shown). One peak at m/z 1639 could be assigned to the peptide V7 with an adduct derived from the episulfonium ion of CEG (Figure 4.9). However, no other peptides could be interpreted as originating from hemoglobin with the addition of one or more alkylating moieties derived from CEG.

Discussion

The results of this study demonstrate that the episulfonium ion of CEG is a chemically reactive species towards hemoglobin at physiologic pH, but that very high amounts of CEG are necessary before alkylation is observed. Attempts to alkylate hemoglobin with molar excesses of CEG on the order of 10- to 20-fold did not result in any apparent adduct formation. It was not until CEG was in the range of 100-fold excess that alkylation could be detected by MALDI mass spectrometry. In comparison, *E. coli* thioredoxin showed evidence for adducts with a 5-fold molar excess of CEG to thioredoxin (Erve, et al., 1995a). This implies that at least 20 times more CEG is required to show signs of alkylation on hemoglobin as compared with thioredoxin. When adducts were observed, they were found on both chains of hemoglobin. This observation implies that CEG is capable of reacting with a nucleophilic amino acid on hemoglobin other than cysteine since there are no accessible cysteines on the α -chain. The spectrophotometric experiment was to specifically determine whether Cys-93 on the β -chain of hemoglobin was a target of alkylation by the episulfonium ion of CEG. Reaction with IAA, which combines readily with sulfhydryl groups in the thiolate form, produced the expected results: a decrease in the number of free sulfhydryl groups per mole of hemoglobin with increasing amounts of IAA. This observation confirmed both the validity of the assay and that the sulfhydryl groups were indeed free and available to react. Performing an identical

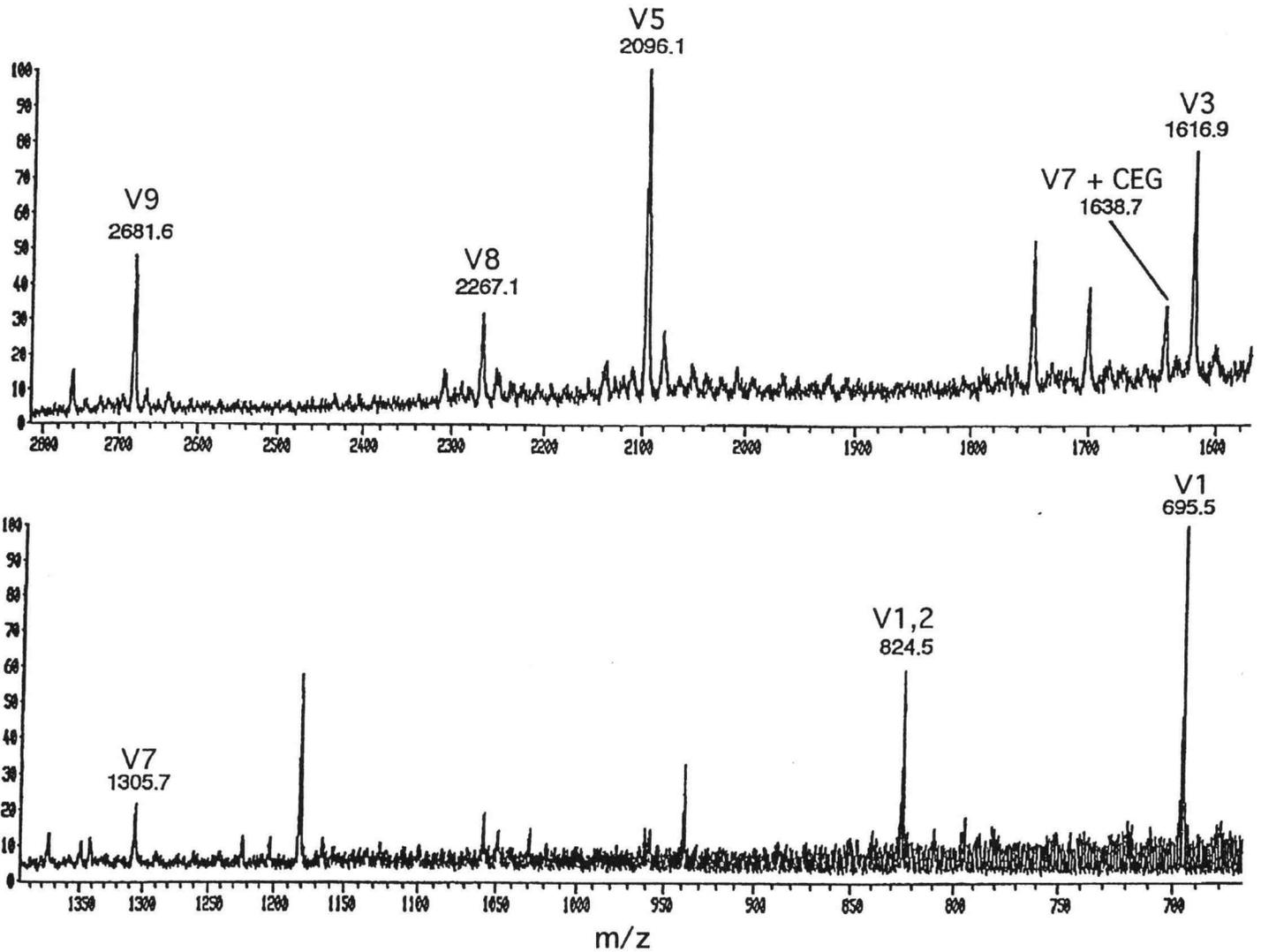


Figure 4.9. FAB/MS of a digest from a CEG-treated hemoglobin β chain. The peak at m/z 1639 may correspond to a peptide fragment containing the alkylating moiety derived from CEG.

alkylation experiment with CEG instead of IAA did not show an analogous decrease in the number of free sulfhydryl groups per mole of hemoglobin with increasing amounts of CEG. These results strongly suggest that the Cys-93 on the β -chain of hemoglobin is not a target for alkylation by the episulfonium ion of CEG. A possible explanation for this lack of reactivity might be that a combination of electrostatic and steric factors prevent the positively charged episulfonium ion from accessing this cysteine. The lack of reactivity of iodoacetic acid towards Cys-93 on the β -chain of hemoglobin is thought to be due to charge interactions with certain amino acid side chains that prevents access to Cys-93 (Garel, et al., 1982). Moreover, the β -93 cysteine sits in the heme pocket which has substantial hydrophobic character. This allows a number of aromatic alkylating species such as 4-aminobiphenyl to react at this cysteine (Ringe, et al., 1988), but may result in the exclusion of a charged hydrophilic species such as the episulfonium ion of CEG. The absorption spectra for CEG treated hemoglobin showed changes not seen in the control. In particular, a shift in the Soret band was seen for two of the three treated samples from 416 nm to 413 nm and 411 nm for the 250- and 500-fold excess CEG treated samples, respectively. The Soret band is an intense peak that represents absorption of the heme group. In oxyhemoglobin it is at 415 nm, whereas oxidation to methemoglobin shifts this to 406 nm (van Kampen and Zijlstra, 1983). It is thus possible that a trace metal impurity present in CEG caused oxidation, by Fenton chemistry, of ferrous iron to ferric iron with the resulting shift in wavelength. An alternative possibility is that CEG was bound near the heme pocket and caused a perturbation of the conjugated heme. In addition, a flattening of the aromatic absorption region at 280 nm for the highest level CEG treated sample was observed. These changes could reflect modification of tyrosine by the episulfonium ion of CEG. Strong evidence exists for alkylation by the episulfonium ion of CEG of tyrosine in oxytocin (Erve, Deinzer and Reed, 1995b) and *E. coli* thioredoxin (Erve, et al., 1995a). There are a total of 38 histidine residues in hemoglobin, some of which are on the surface of the protein and, therefore, exposed to solvent (Dickerson and Geis, 1983). Two of these histidines, β -His-143 and α -His-20, were shown to be alkylated by styrene oxide (Kaur, et al., 1989). There is also

evidence that the imidazole ring of histidine may undergo alkylation by the episulfonium ion of CEG (Jean and Reed, 1989). Therefore, it seems reasonable to propose that at least some of the adducts observed in these experiments are due to alkylation at histidine. Interestingly, the single peptide in the FAB mass spectrum of the CEG-treated hemoglobin digest that might be alkylated by the episulfonium ion of CEG was V7. This peptide contains the β -93 cysteine and two histidines at position 92 and 97, all of which are theoretical targets. However, the histidines correspond to the proximal and distal histidines and are involved in heme and oxygen binding, respectively (Dickerson and Geis, 1983). Consequently, they would not seem likely targets for alkylation. In light of the fact that Cys-93 did not show evidence for reaction, it remains questionable whether this peptide at m/z 1639 is indeed alkylated by the episulfonium ion of CEG. In mouse hemoglobin treated with radiolabelled DCE, cysteine was found to be alkylated by CEG three times greater than histidine, as determined by ion-exchange chromatography (Svensson and Osterman-Golkar, 1986). These workers also determined that the microsomal metabolic product, 2-chloroacetaldehyde, bound to cysteine about 20 times greater than CEG. It should be noted, however, that it is quite possible that the mouse hemoglobin tertiary structure may differ significantly from human hemoglobin which could explain the observed differences in work reported here with the findings of Svensson (Svensson and Osterman-Golkar, 1986).

Further work is needed in order to make any definitive statements regarding the actual alkylation sites of the episulfonium ion of CEG on hemoglobin. The strategy might consist of further enzymatic digestion and attempts to identifying alkylated peptides by MALDI mass spectrometry, which seems to have better sensitivity towards CEG-alkylated peptides than FAB mass spectrometry as suggested by work with thioredoxin (Erve, et al., 1995a). Specific enzymatic digestion coupled with mass spectrometry has been used to identify single amino acid substitutions present in an abnormal hemoglobin (Jensen, et al., 1991). However, in these applications the experiments were conducted on a pure α or β chain variant. A problem that remains to be solved here is the adequate separation of an individual adduct from unreacted

hemoglobin or other adducts. Until this is accomplished, it will be difficult to obtain conclusive evidence for alkylation at a specific amino acid. As adducts are only formed at very high levels of CEG over hemoglobin, there seems little chance that hemoglobin adducts could serve as biomarkers of exposure to DCE in the work environment. Nevertheless, this work illustrates the importance of considering the protein tertiary structure surrounding a functional group, in addition to its nucleophilicity, when deciding whether an alkylating agent will alkylate a particular amino acid.

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Chapter VI

Conclusion

Summary

Based on the work of Paul Jean, it was shown that certain functional groups of dipeptides are extensively alkylated to a larger degree than DNA (Jean, 1991). The investigations described in my thesis have extended his work to show that CEG can indeed react with peptides and proteins and that the sulfhydryl group is the major target when it is available, as is the case for reduced oxytocin and Cys-32 of *E. coli* thioredoxin. It is possible, however, that protein sulfhydryl groups will not react, ostensibly due to steric hindrance as was observed for Cys-35 of *E. coli* thioredoxin. Electrostatic repulsion between the positively charged episulfonium ion of CEG and positively charged residues on hemoglobin may explain the lack of reactivity of β -93 Cys for human hemoglobin. Thus, CEG is selective for protein thiols over other functional groups in proteins and is even selective amongst proteins containing thiols. Tandem mass spectrometry was demonstrated capable of identifying some alkylation sites in oxytocin and *E. coli* thioredoxin. Extensive fragmentation of the alkylating moiety was noted, specifically, the loss of a glycyl or a glutamyl residue. Fortunately, the sulfur adduct bond was not broken during CID allowing cysteine to be unequivocally identified as an alkylation site. In contrast, other nucleophilic sites in peptides and proteins, such as, tyrosine and N-terminal amino groups appear to be more labile. A possible explanation is that a mechanism exists by which these bonds can be more easily broken than the sulfur adduct bond; this possibility has not been further explored.

The significance of the finding that proteins can be alkylated by CEG in vitro at physiologic pH indicates that protein alkylation by CEG may be an important facet

of the hepatotoxic and nephrotoxic effects observed for DCE in vivo. Modification and loss of critical thiols is one possibility that has been used to explain the mechanism of action of some chemicals (Snyder, 1990). Regarding CEG, the finding that an active site cysteine in the important redox enzyme thioredoxin is the major amino acid target in vitro, at molar levels of CEG only slightly higher than that of the enzyme, suggest that irreversible alkylation could inactivate a critical cytosolic enzyme. It remains to be seen whether a major portion of toxicity of DCE in vivo is associated with the loss of this specific protein function due to this alkylation event. It is known, however, that the capability of thioredoxin to reduce the disulfide bonds in insulin is lost following CEG alkylation in vitro (Meyer, et al., 1994). Nevertheless, understanding the toxicologic significance of protein covalent binding requires not only identification of the target protein, but also the amino acid residue that has been modified as well. My thesis work has demonstrated, with *E. coli* thioredoxin, the feasibility of using mass spectrometry to identify alkylation sites that may be applied to other proteins as well.

Future Work

Based on the results of *E. coli* thioredoxin alkylation, and what is known about this multi-faceted enzyme, I would recommend that future research investigate whether thioredoxin is selectively alkylated in vivo following treatment with a toxic dose of DCE or BCE. A strategy used to accomplish this could entail the use of an antibody directed towards mammalian thioredoxin which would allow its isolation from a mixture of cytosolic hepatic proteins. Following isolation, MALDI mass spectrometry could confirm whether or not it was alkylated by CEG. Immunologic approaches for adduct detection and quantitation by enzyme-linked immunosorbent assay were first applied to DNA adducts (Leng, 1985; Strickland and Boyle, 1984). Today, immunologic approaches are frequently applied towards detection of protein adducts as well (Poirier, 1993). For example, antibodies have been produced and used to detect

acetaldehyde adducts present on hemoglobin resulting from ethanol metabolism as a means of monitoring alcohol consumption (Lin, et al., 1993; Niemela, et al., 1990) and to detect adducts arising from benzene exposure (Grassman and Haas, 1993). A difficulty encountered isolating a particular protein adduct, however, is that the adduct might not be easily accessible to the antibody due to steric reasons, resulting in a decrease in sensitivity. To overcome this problem, Day and coworkers have developed a procedure that first enzymatically digests the protein to expose the adduct before application of immunoaffinity chromatography to isolate the adduct of interest (Day, et al., 1990). Therefore, an alternative strategy could involve digestion of cytosolic proteins with trypsin to generate a mixture of peptides. The peptides would then be applied to an immunoaffinity column that recognizes CEG adducted cysteine which would hopefully allow isolation of a CEG adducted tryptic peptide. If the peptide were derived from thioredoxin, it would be identified from the sequence of the peptide. Other protein targets might be identified as well by attempting to match the tryptic peptide sequence with a protein data base. In order for this approach to succeed it will be necessary to generate an antibody that recognizes the adduct derived from the episulfonium ion of CEG on protein.

In light of the fact that chloroacetaldehyde has been postulated to be involved in the toxicity of DCE, it would be of interest to use *E. coli* thioredoxin as a model to compare the alkylating ability of CEG to chloroacetaldehyde. Questions that could be answered include: 1) does chloroacetaldehyde alkylate thioredoxin?; if it does, 2) how do the alkylation levels compare to those of CEG, and, 3) does chloroacetaldehyde alkylate the active site cysteines? With the techniques already developed to study thioredoxin/CEG alkylation, it would be a simple matter to perform the necessary experiments to answer these questions. Information gathered would help to clarify the possible contribution to protein alkylation from the P450 pathway as compared to the GSH pathway.

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APPENDICES

APPENDIX A

CEG alkylation Assay

Introduction:

The purpose of this assay is to determine the reactivity of purified CEG towards thiols by measuring the disappearance of the glutathione thiol as a function of added CEG.

Materials & Method:

Purified CEG, approximately 3 mg.

10 mM GSH in 0.2M NH_4HCO_3 pH 7.4. I also add 1 mM Bathophenanthroline-disulfonic acid (BPDS) (sodium salt) to help stabilize the GSH towards oxidation.

1 mM 4,4'-pyridine disulfide (PDS)

Make up a 10 mM GSH solution by adding 30.7 mg GSH and 4.9 mg BPDS to 10 milliliters of buffer.

Assay:

Part A: CEG alkylation of GSH

Use at least 3 levels of CEG (Table A1). The molar ratios of CEG to GSH were: 0.5, 1.0 and 5.0. Weigh out the CEG into an Eppendorf tube and then add 100 μL of the GSH solution. Let react for 90 minutes to ensure that the full alkylating capacity of the CEG is utilized. Also take some CEG and hydrolyze it before adding the GSH. This is a negative control.

Table A1. Samples used in the CEG assay.

Sample	Excess	GSH (μL)	CEG (μG)
A	1/2	200	370
B	1	100	370
C	5	100	1850
D	-	100	370*
GSH	0	100	-

* Hydrolyzed to HEG.

Part B: Spectrometric Determination of GSH

Take 20 μL of each reaction mixture and add it to 10 μL of 0.05M phosphate buffer, pH 7.4 in a volumetric flask. Pour solution into a ten mL beaker and then aliquot 2.9 mL into 3 glass vials. Then add 50 μL PDS solution and let incubate for 10 minutes. Record absorbance at 324 nm. A PDS blank is also prepared and its absorbance *subtracted*. Calculate [SH] using $\epsilon^{324} = 20,117 \text{ cm}^{-1}\text{M}^{-1}$.

Plot [SH] versus CEG/GSH ratio.

APPENDIX B

Derivatization of Wang Resin with Fmoc-glycine

Introduction:

This method will describe the procedure for derivatizing the Wang resin, the first step in the procedure for synthesizing labeled glutathione. Reference: Grandas et al.

Anchoring of Fmoc-amino acids to hydroxymethyl resins (1989) International Journal of Protein and Peptide Research 33, pp 386-390.

Materials:

from Bachem, Torrance, CA.-

Fmoc glycine 297.3 g/mole or Fmoc ¹⁵N-glycine from Isotec,
4-alkoxybenzyl alcohol polystyrene 'Wang Resin' 0.95 mequiv/g
diisopropylcarbodiimide (DIC) 126.2 g/mole density= 0.81 g/mL

N-methylmorpholine (NMM) 101.1 g/mole density= 0.92 g/mL

1-hydroxybenzotriazole (HOBt) 135.1 g/mole

Solvents: dimethylacetamide (DMA), dichloromethane (DCM), methanol

1-acetylamidazole, from Aldrich.

3:3:2.25:0.3:1 (aa:DIC:HOBt:NMM:Resin)

Method:

Step One: Weigh out 508 mg Fmoc glycine and dissolve in 3 mL of DMA. Weigh out 173.0 mg HOBt and dissolve in 0.6 mL DMA and then mix with the Fmoc glycine with stirring and chill on an ice bath. Add 0.266 mL DIC in 0.4 mL DMA, also chilled, to the above mixture and stir for 5 to 10 minutes. To 0.6 g (0.57 mequiv) of Wang resin in a ten milliliter conical flask, add the prepared mixture and place in a sonicator and let react at room temp. for ~ 17 hours. (temp will actually be about 45°C) After about five hours, test the level of substitution.

Step Two: To wash the resin prior to performing the spectrophotometric assay, withdraw ~ 0.5 mL of the resin mixture with a glass pipette and place on a filter flask

connected to a vacuum. Wash with 15 mL DMA, 15 mL DCM, 15 mL MeOH followed by another 15 mL of DCM. If the resin is clumped, it can be ground gently with a glass stir rod. After completely washing the resin, it should appear white and the faint yellow color seen initially should have disappeared. Next, cover with a Kimwipe (using a rubber-band to fasten to the funnel) and place in a vacuum desiccator for a minimum of 30 minutes. When measuring the final resin, maintain under vacuum for 3 hours or until it has a constant dry weight.

Step Three: To perform the spectrophotometric assay to measure the substitution level, weigh into a 5 mL glass test tube approximately 5-6 mg of the dry resin. Then dissolve it in 0.4 mL of DCM and add 0.4 mL of piperidine. Seal the test tube with parafilm and let react for 30 minutes. Stop the reaction by adding 1.6 mL of methanol. Filter out the resin by passing mixture through a small piece of glass wool placed in the end of a glass pipette. Take 1 mL of the filtrate and dilute volumetrically to 25 mL with DCM. Measure absorbance at $\lambda=301$ nm.

Calculate: substitution level (mmole/g) = $A_{301} / \epsilon \times (25 \text{ mL} / \text{mg resin})$
 $\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$ for fulvene-piperidine adduct.

Step Four: Wash the resin by placing it on the frit in the reaction vessel. The reaction vessel is connected to an aspirator and a nitrogen tank. Adjust the nitrogen so that the flow causes semi-vigorous mixing. Wash the resin with 1) 10X 10 mL DMA, 2) 10X 10 mL wash A (1:1 DMA/DCM) 3) 5X 10 mL DMA.

Step Five: Prepare a 0.3M solution of 1-acetylamidazole in DMA. (0.51g in 15 mL). Add ten milliliters to the reaction vessel, adjust the nitrogen to give gentle mixing, and attach a drying tube to the top of the reaction vessel. Let the acetylation reaction proceed 30 minutes so that any free hydroxyl groups are end-capped. After the reaction is completed, wash the acetylated resin again: 1)10X 10 mL DMA, 2)10X 10 mL Wash A, 3) 5X 10 mL DCM, 4) 5X 10 mL MeOH. Transfer the resin to a

pre-weighed vial, rubberband a kimwipe over the mouth of the vial and place in the vacuum desiccator overnight. The next day, re measure the substitution level of the end-capped resin prior to using it for peptide synthesis.

Glutathione Synthesis using the Wang Resin

Introduction:

This method will describe the procedure for manual synthesis of ^{15}N labeled glutathione using the Wang resin derivatized with Fmoc ^{15}N -glycine.

Materials:

from Bachem, Torrance, CA.-
N-Fmoc glutamic acid α -t-butyl ester 585.7 g/mole
N-Fmoc trityl-cysteine 425.5 g/mole
N-Fmoc ^{15}N -glycine derivatized Wang resin

Method:

Part A-Coupling-

Add the derivatized dry resin to the reaction vessel using some DMA as necessary. The reaction vessel should be connected to the vacuum and have the nitrogen gas adjusted to provide gentle mixing during the steps that follow. Perform the following steps in order: (1) Wash the resin with 10 X 10 mL wash A. Let resin swell in DMA. (2) Add 5 mL of piperidine/toluene/DMA (30:35:35 v/v/v). Mix for three minutes and filter. Repeat but allow 10 minutes of mixing. This step removes the Fmoc group ie. is a deprotection step (3) Wash resin again with 10 X 10 mL wash A. (4) Add the amino acid to be coupled with HOBt and DIC in DCM. Prepare as follows: Mix 4 eq. amino acid + 4 eq. HOBt in enough DMA to give a 0.4 M solution of the amino acid. (the volume of DMA should be ~ 1.5 to 2.5 mL) Separately, prepare a 0.4 M solution of DIC in DCM, using the same volume as that used to dissolve the amino acid. (623 mL DIC in ten mL DCM) Mix these two solutions and allow to stand for two minutes before adding to the resin. (5) Let the coupling reaction proceed for two

hours and filter. (6) Wash resin with 10 X 10ml wash A. (7) Using a spatula, remove some of the resin by dipping it into the reaction vessel and transferring it to a 5 mL glass test-tube. Add the 5 drops each of the three reagents comprising the Ninhydrin solution. Incubate for five minutes at 100°C. A yellow color indicates a successful coupling. Proceed with next coupling by repeating steps (1) through (5). A dark blue color means that the coupling has not occurred. Filter the resin and go back to step (4). A light blue color means that the reaction is not quite complete. Let reaction go for another hour. (9) When the last coupling is finished after step (7) repeat steps (2) and (3). (10) Wash resin with 5 X 10 mL DCM. (11) Wash resin 5 X 5 mL MeOH. (12) Transfer resin to a pre-weighed glass vial and dry in a vacuum desiccator overnight.

Resin Cleavage and Work-up

Introduction:

This method will describe how the peptide GSH is cleaved from the resin and recovered.

Materials:

Mix the following chemicals to make reagent K-

TFA	4.125 mL
H ₂ O	0.25 mL
ethanedithiol	0.125 mL (Warning -Stench Hazard!!)
thioanisole	0.25 mL
phenol	0.25 g

Method:

Step one: Place the resin in a 10 mL round bottom glass vial. Prepare reagent K as a cocktail. Mix the smaller volumes first before combining with TFA. Add to the resin with stirring using a magnetic stir bar. To a 12 gauge needle, attach a balloon and put it through the septum. Fill the vessel with N₂ gas until the balloon

fills before evacuating the vessel by opening the vacuum. Close the vacuum and refill with N_2 gas. Repeat once again. Make sure that the balloon is filled with gas as the cleavage reaction proceeds over the next 2 hours.

Step Two: Filter resin using a small filter funnel. To the vessel into which the filtrate will go, add an attachment that will allow the vacuum to be used to aid in getting every drop off the filtrate (it contains the precious peptide!) Wash the filtrate with ~ 2 mL of TFA. Rinse the reaction vessel with TFA and pass the wash through the filter to combine with the filtrate.

Step Three: Prepare ~ 40 mL of 10% acetic acid in water. Add ~10 mL to the filtrate which will immediately turn the filtrate milky white. Transfer to a separatory funnel. Use the rest of the acetic acid to wash the vessel and transfer to the sep funnel.

Step Four: Extract with 50 mL of anhydrous ether. Shake gently to avoid forming an emulsion. Drain the bottom aqueous layer into another separatory funnel and repeat the ether extraction twice, draining the aqueous layer into the other sep funnel. After the third extraction, put the aqueous layer into a lyophilization jar. Freeze sample and lyophilize overnight.