Characterization of the enzymes involved in the glutathione synthesis and the redox enzyme glutathione reductase in *Caenorhabditis elegans* (Maupas, 1900)

Dissertation

Submitted for the attainment of a doctoral degree from the Department of Biology, Faculty of Mathematics, Informatics and Natural Sciences of the University of Hamburg, Germany

by
Caroline Enjuakwei Ajonina Buzie
(Bamenda, Cameroon)

Hamburg 2006

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Professorin Dr. Eva Liebau
Weiterer Gutachter der Dissertation:
Herr Professor Dr. Rolf-D. Walter
Tag der Disputation: 3. November 2006

Hamburg, den 19. Oktober 2006

Professor Dr. Reinhard Lieberei
Leiter des Departments Biologie

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes (DAAD)

Table of contents

Abreviations

AHT	Anhydrotetracycline
Amp	Ampicillin
AP-I	Activator Protein I
APS	Ammonium persulfate
ARE	Antioxidant responsive elements
BLAST	Basic local alignment search tool
bp, kb	base pair, kilobase
Bis-Tris-Propan	1,3-Bis[tris(Hydroxymethyl)-methylamino]propane
BSA	Bovine serum albumin
BSO	L Buthionine-S,R-sulfoximine
bZIP	Basic leucine-zipper proteins,
cDNA	Complementary DNA
Ce	*Caenorhabditis elegans*
Da	Dalton
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide
DTNB	5, 5'-Dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EpRE	Electrophile responsive element
FAD	Flavine adenine dinucleotide
γ-GCS	γ-Glutamylcysteine synthetase
γ-GCT	γ-Glutamyl cyclotransferase
GFP	Green fluorescence protein
GPX	Glutathione peroxidase

His	Histidine
PHGPX	Phospholipid hydroperoxide glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide (oxidized glutathione)
GST	Glutathione transferase
γ-GT	γ-Glutamyl transpeptidase
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
IPTG	Isopropyl-β-thiogalactopyranoside
K_m	Michaelis-Menten constant
LB	Luria-Bertani medium
L1/2/3/4	First, second, third and fourth larval stages
MBP	Maltose binding protein
mRNA	Messenger RNA
MRP	Multidrug resistance associated protein
NF-kB	Nuclear factor –kB
NGM	Nematode growth medium
Ni-NTA	Nickel-nitrilotriacetic acid
NO	Nitric oxide
Nrf	Nuclear factor erythroid-2-related factor
NTB	5-Thio-2-nitrobenzoate
O_2^-	Superoxide anion radical
5-OP	5-Oxoprolinase
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase-chain reaction
PMSF	Phenylmethylsulfonyfluoride
RNA*i*	RNA interference

RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription-coupled polymerase chain reaction
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SSA	5-Sulfo salicyl acid
TAE	Tris-acetate-EDTA-buffer
TBE	Tris-borate-EDTA-buffer
t-butyl	t-butyl hydroperoxide
TEMED	N', N', N', N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
Tween	Polyoxyethylensorbitane-monooleate
5' UTR	5' Untranslated Region
v/v	Volume per volume
v/w	Volume per weight
WHO	World Health Organisation
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER 1 Introduction

1.1 Nematodes

Nematodes are structurally simple round worms belonging to the phylum Nematoda. All nematodes are non-segmented and cylindrical organisms with a smooth cuticle and a body cavity that contains a tubular digestive tract, reproductive system and other organs but lack a discrete circulatory or respiratory systems (Mandell et al., 2005). Most nematodes are so small, between 400 µm to 5 mm long that a microscope is needed to see them. Their small size, resistant cuticle and ability to adapt to severe and changing environments have made nematodes one of the most abundant animals on earth. In spite of their close morphological similarities, nematodes are found in almost every terrestrial and marine biotope including seas, freshwater, soil, plants, animal surfaces and interiors and in decomposing life forms. The parasitic forms have substantial impact on human welfare, through crop damage and diseases of both human and domestic animals (Blaxter et al., 1992, Maizels et al., 1993, Gillespie and Pearson, 2001).

1.1.1 Pathogenesis and host- parasite relationship

Nematodes are the most common human and plant parasites. Intestinal nematodes have been identified as a major source of chronic ill-health, compromising the growth potential and intellectual achievements of children throughout the world (Gillespie and Pearson, 2001). These parasites (*Ascaris lumbricoides*, hook worms and *Trichuris trichiura*) infect approximately 1.4 billion, 1.2 billion and 1.0 billion persons, respectively (Anderson and May, 1991, Mandell et al., 2005). Infections by these nematodes constitute a major health burden in many parts of the world among the tropical and sub-tropical countries (Awasthi et al., 2003). Their eggs or larvae mostly develop on soil before becoming infective to humans. Another complex infectious disease of man and animal is caused by the nematode parasites of the superfamily filariodea. Filariasis is one of the world's leading causes of permanent, severe and long-term disability. This disease has a serious impact on the health and socio-

economic status of people affected (WHO 2000, Guerrant et al., 2006, Gupta and Srivastava, 2006). Millions of inhabitants of tropical and sub-tropical countries are exposed to at least one of the filarial infections. Adult filarial worms live in vessels, tissues or body cavity of the vertebrate host. The larval development takes place in certain blood sucking invertebrate intermediate host. Filarial parasites are classified into three groups by the habitat of the adult worm, cutaneous filaria (*Loa loa*, *Onchocerca volvulus*), lymphatic filaria (*Wuchereria bancrofti*, *Brugia malayi*) and body cavity filaria (*Mansonella perstans*). The clinical picture of filariasis varies from severe lymphoedema or elephantiasis in Bancroftian filariasis through the skin and eye lesions of onchocerciasis and the less pathogenic and more or less benign manifestations loasis (WHO, 1987). Nematodes are also the most common plant parasites. Some plant parasitic nematodes (Paratylenchus, Criconemella, Trichodorus) can cause severe damage that can result in unmarketability, yield decrease or even total crop failure. There are different ways they can harm the plants. Some induce their host to produce nutrients which the nematodes can survive on. Others produce metabolites which kill the host tissues.

1.1.2 *Caenorhabditis elegans*

Caenorrhabditis elegans is a small free living soil nematode, a rapidly growing organism which feeds on bacteria. It is found all over the world and widely used in genetic studies. It has a digestive and a nervous system. It exhibits behavior and is capable of rudimentary learning. The adults are about 1 mm in length and have a reproductive cycle of about three days when grown at room temperature. The body of an adult *C. elegans* hermaphrodite contains exactly 959 somatic cells, whose entire lineage has been traced through its transparent cuticle (Sulston et al., 1983, Kimble and Hirsh, 1979). After reproduction, it gradually ages, loses vigor and finally dies. The average life span is 2-3 weeks. *C. elegans* is a self-fertilizing hermaphrodite, it is possible to readily grow large quantities of organisms in swirling liquid cultures and also on agar plates covered with bacteria.

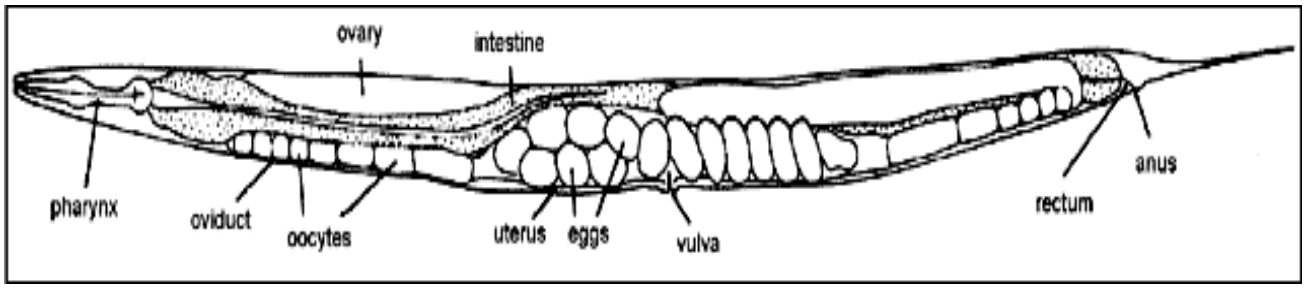


Figure 1. Diagram of a hermaphrodite *C. elegans*.

1.1.2.1 Classification

The classification of *C. elegans* as proposed by Nicholas (1984) is shown below.

Pylum	Nematoda
Class	Secernentea
Order	Rhabditida
Superfamily	Rhabditoidea
Family	Rhabditidae

1.1.2.2 Life cycle of *C. elegans*

C. elegans worms are either males or hermaphrodites. Hermaphrodites, which are the predominant sexual form of *C. elegans,* can reproduce by self-fertilization or can be fertilized by males which arise spontaneously at low frequency. The number of offspring is limited by the number of sperm and can be increased by mating with the male whose sperm preferentially fertilized the oocytes. A single hermaphrodite produces about 280 hermaphrodites' progeny by self-fertilization and more than 1000 hermaphrodite and male worms when mated with males. Fertilized eggs develop internally for several hours with the exact period increasing with the age of the hermaphrodite. After embryonic development and hatching, the worms move through a series of four larval stages (L1-L4) before reaching the adult-hood. Although the size and shape of the animal do not change markedly until the L4 stage, numerous postembryonic cell divisions and cell deaths are taking place internally. If the worms

encounter environmental stress conditions such as starvation and over crowding, they will enter the dauer larval stage, which allows worms to survive adverse conditions. When favorable conditions arise, dauer larvae resume development and proceed to L4 larval stage and subsequently to adulthood (**Figure 2**). In dauer state, worms can survive for up to 6 months, which is much longer than normal life span (2-3 weeks).

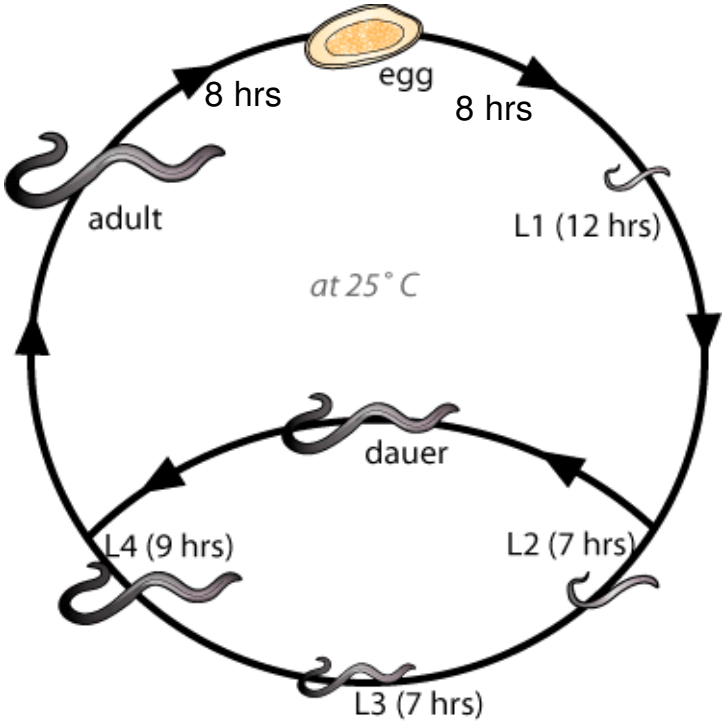


Figure 2. The life cycle of *C. elegans* (Ji Yuan, 2003)

The duration of each developmental stage during growth at 25°C on petri plates seeded with *Escherichia coli* OP50 is given in parentheses. The solid arrows represent molts. Wild-type dauer larvae molt from starved populations from the L_2 stage, and resume growth by molting into L_4 larvae 12 h after being placed in food. Adults start laying eggs 8 h after the final molt, and the eggs hatch 8 h after laying to yield the L_1 larvae. The entire life cycle requires 3 days at 25°C.

1.1.2.3 C*. elegans* as a model system

C. elegans has been the subject of intensive study in recent years because of the advantages this simple metozoa offers for ultrastructural and light microscope analysis (Riddle and Brenner, 1978, Herma and Horvitz, 1994, Shakes and Epstein, 1995). Many features of the worm`s biology make them ideal for genetic analysis.

Firstly, they can be easily raised in the laboratory. Secondly, they are small in size and have a short life generation time which makes it possible to maintain and propagate large number of strains over many generations. *C. elegans* has at least rudiments of the physiological system: a digestive-, excretory-, neuromuscular- and reproductive system found in higher animals like mice and humans. In addition it can be easily transformed with transgenes – DNA injected into animals (Tabara et al., 1999).

1.2 Microinjection in *C. elegans*

DNA transformation assays in *C. elegans* provide experimental links between molecular structure and phenotype. Experiments with transgenic *C. elegans* start in general with the injection of DNA into the adult gonad. Effects on the phenotype or gene expression patterns can be analyzed in F1 progeny derived from the injected animals or in the following generations. Microinjection of *C. elegans* was first carried out by Kimble et al. (1982). Stinchcomb et al. (1985) then showed that injected DNA could be maintained for several generations in transgenic lines. The first selective methods for producing and maintaining transgenic lines were reported by Fire (1986). These methods have been considerably modified by Mello et al. (1991). The *C. elegans* hermaphrodite gonad consists of two arms each of which is reflexed into a U-shape. An adult ovary contains thousands of germ-like nuclei surrounding a common core of cytoplasm. Germ-line transformation is achieved by microinjection of DNA directly into oocyte nuclei (Fire, 1986) or by microinjection of DNA into the cytoplasm of the hermaphrodite syncytial gonad (Stinchcomb et al., 1985, Mello et al., 1991). Three forms of heritable DNA transformation have been observed in *C. elegans*: (1) extrachromosomal transformation, (2) nonhomologous integration, and (3) homologous integration.

1.3 RNA *interference* in *C. elegans*

RNA*i* is the use of double stranded RNA (dSRNA) to silence genes and generate knockdown phenotypes. RNA*i* has become a widely used method for functional

genomic studies in *C. elegans* due to its ease, speed and cost effectiveness (May and Platerk, 2005, Kittler et al., 2005). RNA*i* can be induced using bacteria to deliver dsRNA*i*. In this method, bacteria that are deficient in RNAse II, an enzyme that normally degrades a majority of dsRNAs in the bacterial cells are used to produce high quantities of specific dsRNA segments (Timmons et al., 2001). In the vast majority of species RNA*i* can only be initiated following the microinjection electroporation or transfection. However, Timmons and Fire in 1998 reported that RNA*i* could be initiated in *C. elegans* by feeding with bacteria expressing dsRNA. Following uptake, this dsRNA triggers a systemic effect, initiating RNA*i* against corresponding target gene (May and Plasterk, 2005).

1.3.1 Mechanism of RNA *interference*

Introduction of siRNA into the cell can be achieved by direct incorporation via transfection or by indirect incorporation via a DNA expression vector (Kennerdell and Carthew, 1998, Novina and Sharp, 2004). The double stranded RNA oligonucleotide is then recognized as a potential threat to the cell, possibly being viral genetic material (**Figure. 3**). The cell's response is through the action of Dicer, a member of the RNase III nuclease family, which cuts the dsRNA into 21-23 nucleotide sequences (Zamore et al., 2000). RISC (RNA-induced-silencing-complex), an enzymatic complex of over 12 different molecules, the major one being Argonaut, recognizes the siRNA molecule. RISC uses an ATP molecule to unwind the siRNA molecule, leaving the complement (antisense) strand in the complex. The unwinding of the double strand by a helicase is the activation step for the silencing complex (Zamore et al., 2000). When RISC is activated it is ready to act upon an mRNA with identical sequence to the complementary sequence of the siRNA included in the RISC complex. RISC, as depicted in **Figure 3**, binds through base pairing to the appropriate RNA sequence and cleaves it (Zamore et al., 2000, Novina and Sharp, 2004). The cleaved RNA strands are then digested by nucleases. The RISC applies this process repeatedly.

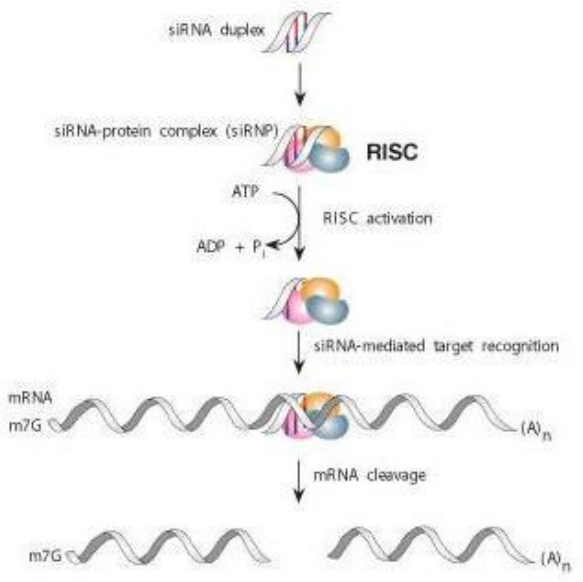


Figure 3. Mechanism of activation of RISC by joining of siRNA, unwinding and removal of sense strand of the siRNA. (www.cellsignal.com)

1.4 Oxidative stress and its effect to the cell

Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or organ caused by reactive oxygen species (ROS) such as superoxides anion radicals (O_2^-), hydrogen peroxides (H_2O_2), hydroxyl free radical ($OH^\cdot$) and peroxynitrite. Oxidative stress can be imposed on cells as a result of an increase in oxidant generation, a decrease in antioxidant protection, or a failure to repair oxidative damage. ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Besides the atmospheric radiation, the respiration chain represents the principal source of oxidative stress. There are four electrons in the cell respiration that require the reduction of molecular oxygen to H_2O. The electron donor cytochrome c transmits only an electron per reaction, in order that O_2 is generated. The superoxide anion has been found to react in aqueous biological systems in the presence of redox metals like Fe (III)/Fe (II), Cu (II)/Cu (I) of the cytochrome c oxidase to prevent free radicals of the molecule (Stryer, 2000). Electron transfer through cytochrome c leads to the reduction of O_2 to O_2^- (Droge, 2002). The effect of this reduction was demonstrated by Melov (2000) using mice,

whose mitochondrial superoxide dismutase (SOD) has been knocked out. Melov found that the mice did not survive the first week of experiment. Differences occur in the cell when SOD converts superoxide to reactive H_2O_2. The complete reduction of molecular oxygen by the univalent pathway therefore results in the formation of the superoxide anion radical, hydrogen peroxide, and hydroxyl radical (**Figure 4**). The anion charge of O_2^- inhibits its effectiveness as an oxidant of electron rich molecules, while reactivity of H_2O_2 is diminished by the stability of its oxygen-oxygen bond. Neither of these features applies to the hydroxyl radical and $HO^{\cdot}$ reacts at virtually diffusion-limited rates with most biomolecules.

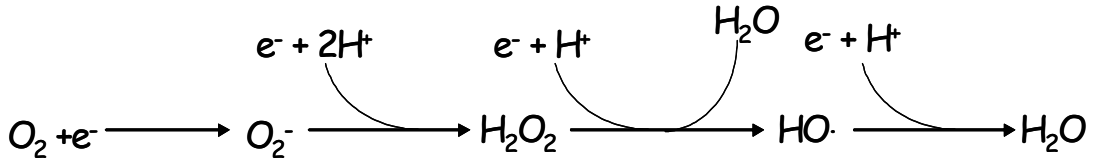


Figure 4. Reduction potentials for oxygen species to water.

The intermediates of this reaction are too reactive to be tolerated in living tissues and their removal involves the intervention of several enzymatic and non-enzymatic mechanisms. Iron and iron compounds may facilitate hydroxyl-radical generation from activated oxygen species. However, in the presence of reduced metals like Fe^{2+} or Cu^{2+} ions, H_2O_2 becomes converted to $HO^{\cdot}$ and OH^-. Hydroxyl radical is a potent oxidant which reacts non-specifically with organic material. By abstracting a hydrogen atom from polyunsaturated fatty acids, it may initiate the process of lipid peroxidation and eventual cell injury or death.

Free hemoglobin, like other iron-rich substances, might also mediate hydroxyl-radical generation. In the presence of a superoxide anion-generating system (hypoxanthine and xanthine oxidase), hemoglobin promotes hydroxyl-radical formation. Thus, free hemoglobin may be biologically hazardous, in part because it acts as a "Fenton" reagent, having the potential to catalyze hydroxyl-radical generation in areas of inflammation (Sadrzadeh et al., 1984). The reaction between superoxide (O_2^-) and

nitric oxide (NO) results in formation of peroxynitrite which may impede protein function through nitration (Espey et al., 2002).

1.4.1 Sources of reactive oxygen species.

Several endogenous sources of toxic oxygen species have been identified. These include:

i. mitochondrial and microsomal cytochrome P450 metabolism of xenobiotic compounds during normal cell reactions
ii. activated phagocytes and associated oxidative burst due to infection and inflammation
iii. ischemia reperfusion and reoxygenation after hyperoxia
iv. activation of the arachidonic acid cascade by phospholipase A2
v. auto-oxidation of endogenous compounds like catecholamines and monosaccharides (Del Maestro, 1980).

Other important exogenous sources of oxygen radical formation are ionizing radiation, cigarette smoke, redox recycling xenobiotics like paraquat, pumbagin or the cytostatic drug doxorubicin, bacterial, fungi and viral infections (Sies, 1986; Fiers et al., 1999, Droge, 2002)

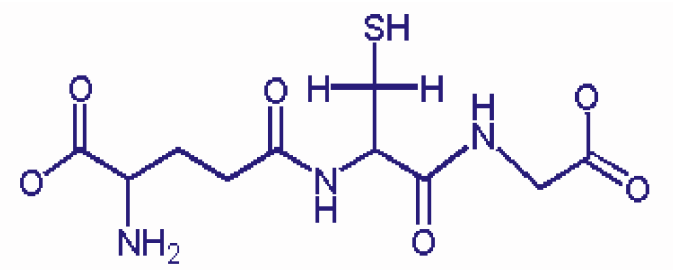
1.4.2 The effect of reactive oxygen species in biological systems

Humans are exposed to many carcinogens, including the reactive species derived from metabolism of oxygen and nitrogen. The noxious consequences of reactive oxygen species in biological systems are manifold. Cell constituents such as nucleic acids, lipids, proteins and carbohydrates are prone to oxidative attack. Peroxidation of membrane phospholipids, a chain radical reaction process, may result in abnormal cell functions like ion exchange and impulse transmission, leading to a score of diseases. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Additionally, oxidative stress and ROS have been implicated in disease states, such as Alzheimer's disease, Parkinson's disease, cancer and aging.

Oxidative scission of DNA may result in mutations, chromosomal aberrations and carcinogenesis. Oxidative destruction of sugars may result in altered functions such as loss of viscosity of joint lubricants. The oxygen rich environments in which proteins exist tend to produce a variety of chemical reactions in proteins. Reactive oxygen species (ROS), which are products of cellular respiration, react with nucleic acids, lipids, proteins and sugars. The oxidation of lipids, reducing sugars and amino acids leads to the formation of carbonyls and carbonyl adducts such as 4-hydroxy-2-nonenal (HNE). In addition to forming carbonyl groups, ROS are responsible for deamidation, racemization and isomerization of protein residues. These oxidatively modified proteins are not repaired and must be removed (Fridovich, 1986, Sies, 1986, Hayes et al., 1999, Arber et al., 1990, Droge, 2002, Espey et al., 2002).

1.5 Glutathione

Glutathione (GSH) is a tripeptide of L-glutamate, L- cysteine, and glycine. Glutathione is widely found in all forms of life and plays an essential role in organisms, particularly aerobic organisms. Being water-soluble, it is found mainly in the cytosol and other aqueous phases of the living system. It is also present in tissues in concentrations as high as one millimolar. In animals and plants, glutathione is the predominant non-protein thiol and functions as a redox buffer, keeping SH-groups of proteins in a reduced condition, among other antioxidant activities (Meister, 1988, Wu et al., 1994, Lomaestro and Malone, 1995). Glutathione exists in two forms: the reduced form (GSH) and the oxidized form (GSSG, two GSH moieties joined by their sulfhydryl groups). The structural formula is as follows:



The cysteine residue in GSH allows it to have redox buffering activity. GSH also plays roles in catalysis, metabolism, signal transduction, gene expression and apoptosis. It is a cofactor for glutathione S-transferases, enzymes which are involved in the detoxification of xenobiotics, including carcinogenic genotoxicants, and for the glutathione peroxidases, crucial selenium-containing antioxidant enzymes. It is also involved in the regeneration of ascorbate from its oxidized form, dehydroascorbate. Some of the metabolic functions include DNA synthesis and repair, protein synthesis, amino acid transports and enhancement of immune system function (Anderson et al., 1985). GSH can be depleted intracellularly by forming a direct complex with electropilic agents such as bromobenzene or diethyl maleate via inhibition of synthesis or by subjecting cells to oxidant stress (Deneke and Fanburg, 1989). Intracellular depletion of GSH appears to be critical for subsequent alteration of protein thiol and calcium homeostasis (Bellomo and Orrenius, 1985). This results in both calcium release from intracellular stores and inhibition of calcium extrusion, producing a marked increase in cytosolic calcium concentration, which triggers cytotoxicity.

1.5.1 Glutathione biosynthesis, metabolism, and utilization

Glutathione synthesis occurs within cells in two closely linked, enzymatically controlled reactions that utilize ATP and draw on nonessential amino acids as substrates. First, cysteine and glutamate are combined by the enzyme γ-glutamyl cysteine synthetase (γ-GCS) (**Figure 5a**), with availability of cysteine usually being the rate-limiting factor. Cysteine is generated from the essential amino acid methionine, through the degradation of dietary protein, or from turnover of endogenous proteins. The build-up of GSH acts to feedback-inhibit this enzyme, thereby helping to ensure homeostatic control over GSH synthesis. In the second step, GSH synthetase (GS) catalyzes the subsequent formation of a peptide bond between the cysteinyl carboxylate of γ-glutamylcysteine and the amino group of glycine (**Figure 5b**). Each of these reactions requires hydrolysis of ATP to drive formation of the peptide bond reaction (Meister and Anderson, 1983, Griffith and

Mulcahy, 1999, Kelly et al., 2002, Copley and Dhillon, 2002). Excessive accumulation of γ-glutamylcysteine can lead to its conversion to 5-oxoproline by the enzyme γ-glutamyl cyclotransferase. Build up of 5-oxoproline can have adverse consequences due to metabolic acidosis. Therefore, de novo GSH synthesis is regulated by the level of γ-GCS present in the cell, feedback inhibition of GSH and the availability of its substrates, particularly L-cysteine (Griffith, 1999).

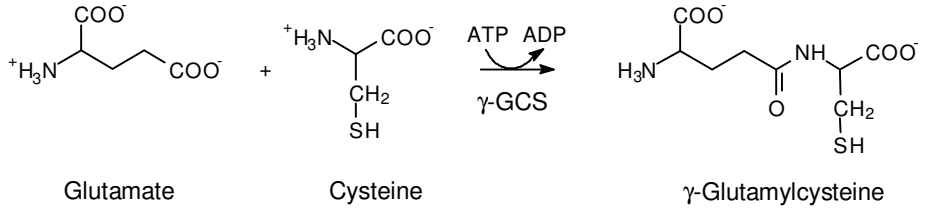


Figure 5a. The formation of γ-glutamylcysteine from L-Glu and Cys catalysed by γ-GCS.

The glutathione synthetase (GS) then adds glycine

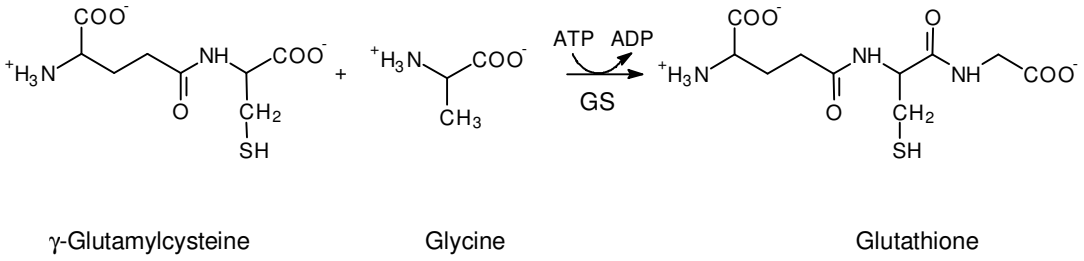


Figure 5b. GSH-synthesis from γ-glutamylcysteine and glycine catalysed by GS.

To maintain adequate levels of GSH, GSSG is reduced at a high rate by glutathione reductase (GR). GSSG is formed in antioxidant reactions and can accumulate with increased oxidative processing. The ratio of GSSG/GSH which lies between 10 and 100 serves as a sensitive index of oxidative stress and its ability to resist toxic challenge (Sies, 1989, Toborek and Hennig, 1994, Jones, 2002, Wu et al., 2004). Because oxidative functions require GSH to be in its reduced form, GSSG is reduced by GR in a reaction that requires reduced nicotinamide adenine dinucleotide

phosphate (NADPH) from the pentose phosphate pathway as a hydrogen donor (**Figure 6**). GSH is used by glutathione peroxidase to detoxify H_2O_2. In this process, the oxidation power of H_2O_2 is transferred to GSSG (Usanga and Luzzatto, 1985; Nagel and Roth, 1989, Toborek and Hennig, 1994, Ruwende and Hill, 1998). The sulfhydryl of GSH can be used to reduce peroxides formed during oxygen transport.

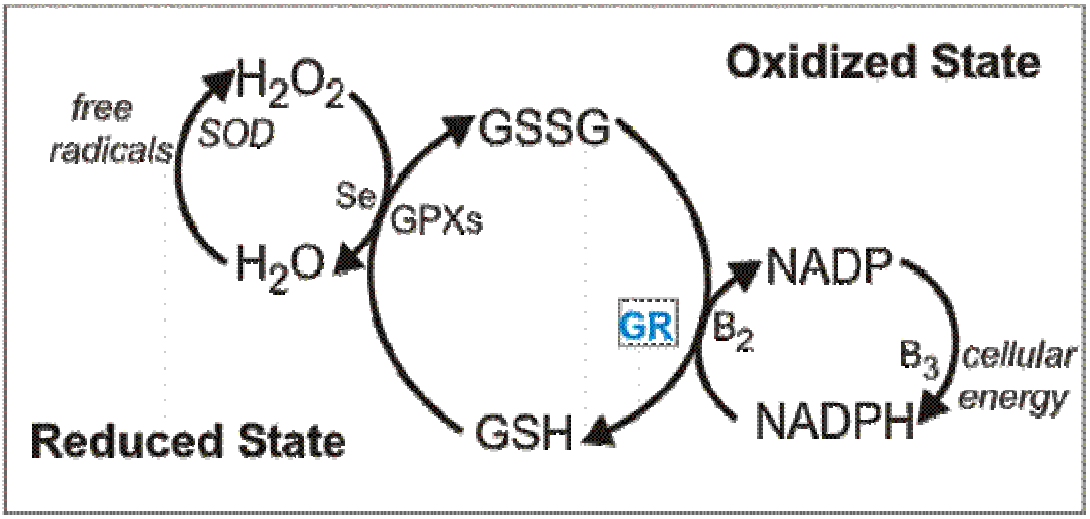


Figure 6. Recycling of GSH

GSH depletion can trigger apoptosis of the cell (Slater et al., 1995, Duke et al., 1996). GSH status is homeostatically controlled, being continually self-adjusting with respect to the balance between GSH synthesis, its recycling from GSSG and its utilization. There is a continuous turnover of GSH in all cells at rates that can vary widely, even in different tissues in the same organism (Kosower and Kosower, 1978, Meister, 1988, Deleve and Kaplowitz, 1991). GSH is regenerated through the γ-glutamyl cycle, which comprises six enzymes that participate in its synthesis and breakdown (**Figure 7**). There are few organisms for example halobacteria that contain millimolar concentrations of γ-Glu-Cys and not GSH (Newton and Javor, 1985). Experiments with viable GS knockout mutants from *Saccharomyces cerevisiae* showed that γ-Glu-Cys can serve as a redox reagent in yeast. The knock out mutants were more sensitive to oxidative stress conditions than the wild type

(Ohtake et al., 1990). The GSSG efflux from cells contributes to a net loss of intracellular GSH. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (Griffith, 1999, Lu, 2000). The GSH and GSSG concentration is usually denoted as total glutathione in cells, a significant amount of which (up to 15%) may be bound to protein. GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, but its value can be affected by other redox couples, including $NADPH/NADP^+$ and $thioredoxin^{red}/thioredoxin^{ox}$ (Jones, 2002). The role of GSH as a reductant is extremely important particularly in highly oxidizing environment. The pentose phosphate pathway is therefore an important pathway for the continuing production of the NADPH needed by GR. Using *C. elegans* as a model system, the metabolic imbalance leading to lactic acidosis and energy depletion, the central mechanisms of pathogenesis in mitochondrial dysfunction was studied, the introduction of an additional pathway for lactate oxidation such as the pentose phosphate pathway is considered as a treatment (Leslie et al., 2005). Generally, GSH deficiency in human has been linked to a number of diseases states (Li et al., 2004). The degradation of extracellular GSH is first catalyzed by γ-glutamyl transpeptidase (γ-GT), which catalyzes transfer of the γ-glutamyl moiety of GSH to an amino acid. γ-GT is largely localized at the outer surface of membranes (Novogrodsky et al., 1976). S-Substituted glutathione derivatives and other γ-glutamyl compounds can also serve as the donor of the γ-glutamyl group. Moreover, dipeptides as well as GSH can also serve as the acceptor. Thus γ-GT acts on exported GSH, GSSG and GSH-S-conjugates (Thompson and Meister, 1977). Following this reaction, GSH being the donor and an amino acid being the acceptor, the γ-glutamylaminoacid and cysteinylglycine are formed. γ-Glutamyl cyclotransferase (γ-GCT) catalyzes the conversion of γ-glutamylaminoacid to 5-oxoproline and free amino acid, whereas cysteinylglycine undergoes hydrolysis by the action of membrane bound cysteinylglycine dipeptidase to yield cysteine and glycine. These can cross the plasma membrane and are re-utilized for intracellular GSH synthesis.

5-Oxoproline is then converted into L-glutamate by the action of 5-oxoprolinase. Now, by the successive action of γ-GCS and GS, GSH is again formed.

GSH can act either directly or indirectly as an antioxidant. Directly it can be converted to the disulfide GSSG form, donating electrons and reducing free radicals. Indirectly it can work with the enzymes glutathione peroxidase (GPXs), phospholipid hydroperoxide glutathione peroxidase (PHGPXs), and glutathione-S-transferase (GSTs) (Zhang et al., 1989, Lomaestro and Malone, 1995). PHGPXs and GPXs are selenium dependent enzymes that are encoded by separate genes with moderate sequence identity and are differentially regulated by dietary selenium (Lei et al., 1995, Cheng et al., 1997). These peroxidases detoxify peroxides by reacting them with GSH to form a water soluble compound. The PHGPXs use GSH to detoxify peroxides generated in the cell membranes and other lipophilic cell phases. Studies have shown that PHGPX2 is the major oxidative stress inducible cellular glutathione peroxidase isoform in the lungs and its basal as well as inducible expression is dependent on the basic leucine zipper transcription factors such as Nrf2 (Rangasamy et al., 2004, Singh et al., 2006).

The GST(s) are widely distributed isoenzymes that perform functions ranging from catalyzing the detoxification of electrophilic compounds to protecting against peroxidative damage (Armstrong, 1991, van Bladeren, 2000). They catalyze the conjugation of the GSH thiolate anion with a large number of exogenously and endogenously derived toxic compounds, including the secondary products of lipid peroxidation (Mannervik, 1985). All eukaryotic species possess multiple cytosolic and membrane-bound GST isoenzymes. The cytosolic enzymes are encoded by at least five distantly related gene families (designated class alpha, mu, pi, sigma, and theta GST), whereas the membrane-bound enzymes, microsomal GST and leukotriene C4 synthetase, are encoded by single genes and both have arisen separately from the soluble GST. The most abundant mammalian GSTs are the class alpha, mu, and pi enzymes. GSTs are regulated by a structurally diverse range of xenobiotics of which a significant number occur naturally as nonnutrient. They are also regulated in vivo by reactive oxygen species (ROS), because not only are some of the most potent

inducers capable of generating free radicals by redox-cycling, but H_2O_2 has been shown to induce GST in plant and mammalian cells. Induction of GST by ROS would appear to represent an adaptive response as these enzymes detoxify some of the toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress. Increased level of alpha, mu, and pi GST isoenzymes contribute to the multidrug-resistant phenotype observed in rat (Xia et al., 1996, Hayes and Pulford, 1995, Hayes and McLellan, 1999).

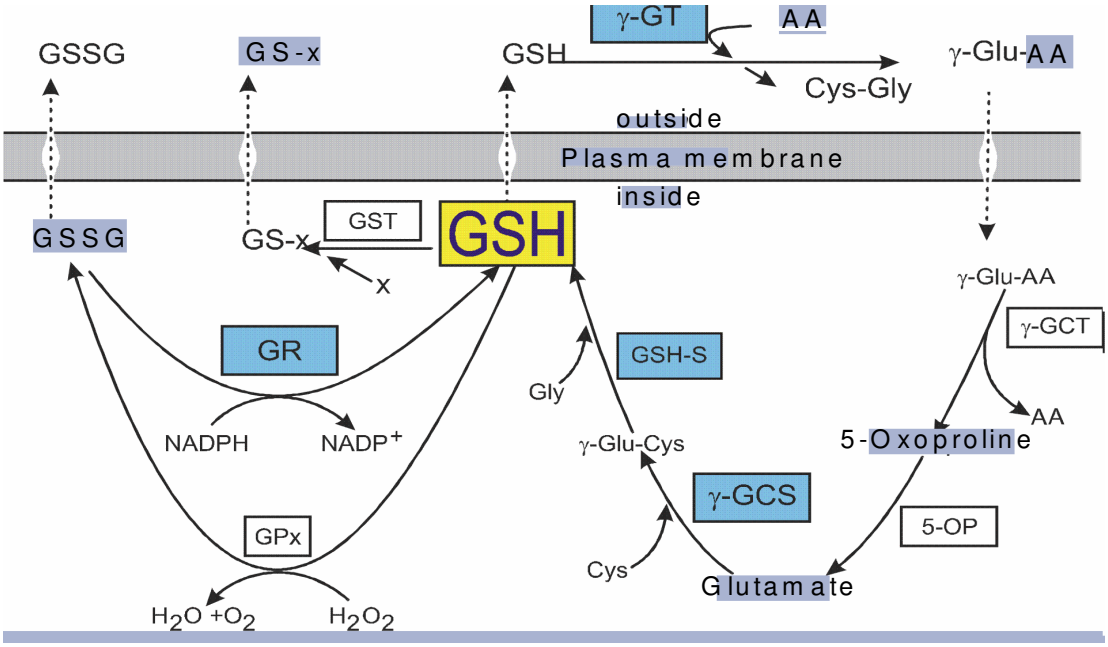


Figure 7. Overview of the glutathione metabolisms in the eukaryotic cell. AA, amino acids; γ-GCS, γ-glutamylcysteine synthetase; γ-GCT, γ-glutamylcyclotransferase; γ-GT, γ-glutamyltransferase; GR, glutathione reductase; GSH-S, glutathione synthetase; GSSG, oxidized glutathione; GST, glutathione-S-transferase; GS-x, conjugate of GSH and xenophile components; 5-OP, 5-oxoprolinase.

There are organisms in which GSH represents only a byproduct, for example in Trypanosomatidae, GSH is not the main molecule in fighting against oxidative stress, but rather trypanothione (bis-glutathionyl-spermidine), in which 2 GSH-molecules over the C-terminus of the glycine are connected with the terminal amino group of spermidine. The trypanothione synthesis has two steps and is catalysed by glutathionylspermidine synthetase and the trypanothione synthetase (in *Crithidia fasciculata*) or a single enzyme, where both tasks are fulfilled (*Trypanosoma cruzi*)

(Tetaud et al., 1998). Trypanosomatidae possess a trypanothione reductase (Steenkamp, 2002) instead of the GR. Trypanothione replaces GSH even as a substrate for the glyoxalase of *T. brucei.* Plants require GSH as a redox-reagent, for it the molecule represents in addition, an intermediate in the phytochelatin metabolism. Phytochelatines are very similar to GSH. They possess the structure formula $(\gamma\text{-Glu-Cys})_{n=2\text{-}11\text{-Gly}}$ and are chelated to heavy metals such as cadmium (Cobbett, 2000). Leguminous plants possess a molecule called homoglutathione $(\gamma\text{-Glu-Cys})_{n\text{-}\beta\text{-Ala}}$ which exists in the cells either together with GSH or alone (Frendo et al., 2001).

GSH content has been analyzed in many nematode species, for example, *Acanthocheilonema viteae*, *Litomosoides carinii*, and *Setaria cervi* (Gupta et al., 2002, Gupta et al., 2005).

The cDNAs encoding two different GSTs from *Ascaris suum* and *Onchocerca volvulus* have been isolated and characterized (Liebau et al., 1994a, Liebau et al., 1994b, Sommer et al., 2001). Besides GST, a functional GR and the gene encoding γ-GCS have been studied (Müller et al., 1997, Luersen et al., 2000, Müller et al 2001). Inhibitor studies have also clarified that the GR and the enzymes involved in the de novo synthesis of GSH play an important role in the survival of parasites (Bhargava et al., 1983, Müller et al., 1995, Hussein and Walter, 1996, Luersen et al., 2000, Liao and Yu, 2005).

1.6 Enzymes of the GSH synthesis

1.6.1 γ-Glutamylcysteine synthetase

The evolutionary history of the glutathione biosynthesis genes showed that the gene for γ-GCS most probably arose in cyanobacteria and was transferred to other bacteria, eukaryotes and at least one archaeon, the eukaryotic gene comes from a mitochondrial progenitor. Based on sequence homology, the γ-GCS sequences fall into three groups: group 1 contains enzymes from gamma-proteobacteria; group 2 contains enzyme from non-plant eukaryotes; and group 3 contains enzyme primarily from alpha-proteobacteria and plants. Although pairwise sequence identities between

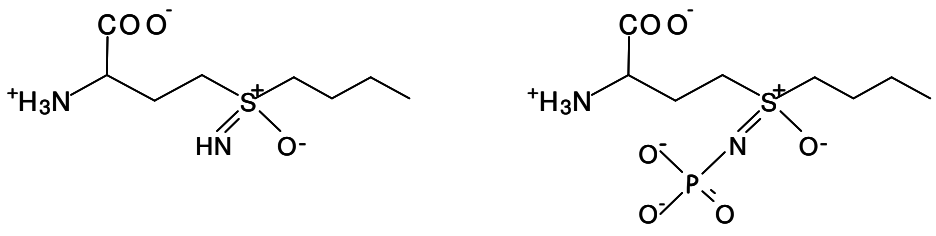
groups are insignificant, conserved sequence motifs are found, suggesting that the proteins are distantly related (Henikoff, 1995, Copley and Dhillon, 2002).

Table 1. Characteristics of γ-GCS among members of different groups.

Organisms	Groups	Molecular mass	Promoter-Elements
E. coli	1	Monomer: 58 kDa	?
H. sapiens	2	Heterodimer: γ-GCS_L: 27 kDa γ-GCS_H: 73 kDa	ARE, EpRE, NF-κB-binding element
S. cerevisiae	2	Monomer: 78 kDa	yAP-responsive element
T. brucei	2	Monomer: 77 kDa	?
C. elegans	2	Monomer: 73 kDa	?
A. thaliana	3	Monomer: 59 kDa	?

The γ-GCS of mammals is a heterodimer which comprises catalytic regulatory lower units of molecular mass 73 kDa (heavy chain: γ-GCS_H) and 27 kDa (light chain: γ-GCS_L). The lower unit genes lay on different chromosomes and are separately regulated. The promoter of γ-GCS_H contains numerous redox sensitive DNA elements such as antioxidant responsive element (ARE) which bind to AP-1 or nuclear factor erythroid-2-related factor 1 and -2 (Nrf-1/2) or electrophilic responsive element (EPRE). Stress conditions such as heat, ionizing radiation or heavy metal lead to the induction of both lower units. In yeast and plants, transcription of γ-GCS is also modulated by stress (Izawa et al., 1995, May et al., 1998, Xiang and Oliver, 1998, Sugiyama et al., 2000, Westwater et al., 2002). In addition to the described mechanisms, mammalian enzymes can be post-translational regulated; for example, in γ-GCS_H there is a binding place for caspase 3 (Franklin et al., 2002). Caspases mediate the cleavage of different target enzymes some of which are involved in the

regulation of apoptosis. Because of the cleavage of the γ-GCS_H the enzyme loses its function, the GSH levels decrease and the process of cellular death of the cell is accelerated. The activity of γ-GCS in all organisms described in the table are controlled by negative feedback inhibition. In mammals, GSH synthesis is modulated by the availability of γ-GCS substrates primarily L-cysteine, by feedback inhibition of γ-GCS, GSH, and by covalent inhibition of γ-GCS by phosphorylation or nitrosation (Huang, 1993, Griffith, 1999). This implies that nitrosation and phosphorylation of enzyme play a role in the regulation of the enzyme (Griffith, 1999). In the formation of peptide bonds the γ-carboxyl group of glutamate is first activated through phosphorylation under ATP consumption. The reaction of γ-glutamyl phosphate with the amino group of cysteine gives rise to γ-glutamylcysteine. The substrate analogue D,L-buthionine-(S,R) sulfoximine (BSO) is an effective specific inhibitor of γ-GCS of man, yeast, trypanosome and nematodes (group 2). The effect is little in *E. coli* and plants (group 1 and 3) (Bailey et al., 1994, Gallo et al., 1995, Bailey et al., 1998). It imitates the structure of γ-glutamylphosphate-cysteine and binds strongly to the catalytic active cysteine residue near the active center (Griffith, 1982).



L-Buthionine-Sulfoximine L-Buthionine-Sulfoximinephosphate

Figure 8. Structure of L-buthionine-sulfoximine and L-buthionine-sulfoximine phosphate

BSO depletes the GSH and has stronger effects on fast proliferating cells like cancer cells. it is used in cancer therapy in combination with other drugs which cause oxidative stress (cisplatin, arsenoxide) in order to increase the sensitivity of malignant

cells against those drugs (Pu et al., 2002a, 2002b). Knock out mutants of γ-GCS in mammals are lethal, while in yeast cells they just cause hypersensitivity against exogenic stress (Inoue and Kimura, 1996; Dalton et al., 2000). In humans, mutants of the γ-GCS have been described whose erythrocytic enzyme activity decreased to 2% of the normal values. Such mutations are very rare and have been observed only in 8 patients yet. They only affect erythrocytes and amino acid changes in the catalytic subdomains thus the reason for the deficiency (Beutler, 1999, Ristoff et al., 2000). The symptoms reach from haemolytic anaemia to neurological disorders, depending on the type of the mutation. Since there is yet no crystal structure of the human γ-GCS available, it is difficult to estimate whether the mutation is concerned with the stability or the catalytic properties of the enzyme and also to what extent that may result to a severity of the symptoms. This enzyme has been characterized in some nematodes, for example, in *O. volvulus*. Inhibition studies carried out showed that the K_i value of *O. volvulus* GCS for BSO and cystamine was lower than that of the mammalian enzyme (Luersen et al., 2000). It has also been found that GCS of *A. suum*, is also more sensitive to known inhibitors such as BSO and cystamine (Hussein and Walter, 1995), thus recommending it as a target for chemotherapy against nematode infections. BSO is the specific transition-state inhibitor of GCS. It shows low toxicity to human tissues and has little intrinsic chemical reactivity (Bailey, 1998). It acts solely through inhibition of GSH synthesis and does not directly affect other cellular thiols. Chemotherapeutic effects of BSO as an inhibitor of GSH synthesis on *Haemonchus contortus* and Trypanosomatidae has also been reported (Arrick et al., 1981, Kerboeuf and Aycardi, 1999).

1.6.2 Glutathione synthetase

In the evolution of the aerobic organisms, the development of an enzyme, that was able to further stabilize the already existing γ-glutamylcysteine, was an additional selection advantage. Glutathione synthetase sequences fall into two distinct groups:

bacterial and eukaryotic GSs proteins in both groups have a common structural fold, but the sequences are so divergent that it is uncertain whether these proteins are homologous or arose by convergent evolution (Copley and Dhillon, 2002).

Table 2. The characteristics of GS of group 1 and 2.

Organisms	Group	Molecular mass	Promoter-element
E. coli	1	Homotetramer: (36 kD)	?
H. sapiens	2	Homodimer: (52 kD)	ARE?
S. cerevisiae	2	Homodimer: (56 kD)	YAP-responsive element
A. thaliana	2	Homodimer: (54 kD)	?
C. elegans	2	Homodimer (52 kD)	?

There is no significant relationship to each other on the basis of pairwise sequence identities. The bacterial GS is known to belong to the ATP-grasp superfamily, that catalyze formation of a bond between a carboxylate group of one substrate and an amino, imino or thiol group of a second substrate. The carboxylate group is then activated at the same time through ATP-mediated phosphorylation. It is not clear whether the eukaryotic GS also belong to the ATP-Grasp superfamily. Table 2 summarizes the characteristics of different GS. The regulation of the genes involved in GSH synthesis through oxidative stress was investigated. Here it was shown that a modulation of the transcription factors Nrf1 in mouse fibroblast has an influence on the transcription of the GS, mediated by the ARE-element in the promoter of this gene (Kwong et al., 1999). In *S. cerevisiae* GS, just like γ-GCS, is regulated by Yap1p under oxidative stress-induced conditions (Sugiyama et al., 2000). Although the primary structures of prokaryotes and eukaryotes in GS hardly resemble each

other, the reaction mechanism and the protein structure are comparable. In the γ-GCS as well as in the GS mediated reaction, an acyl phosphate intermediate is first produced between the phosphate of the ATP and a carboxyl group (in this case the C-terminus of cysteine). This offers the attack point for the α-amino group of glycine through which a tetrahedral bond emerges between the products. The reaction ends with the formation of the peptide bond and a free phosphate. In *E. coli* loss of the GS activity is of no significance. The mutant contains a very low level of the GS and accumulates γ-glutamylcysteine at a concentration approximately equal to the level of GSH found in the wild type. The growth is not impaired in the mutants however the cells are more sensitive to oxidative stress (Fuchs and Warner, 1975). Similarly, Grant and co-workers, 1997 showed that the deletion of the GS and GR genes does not impair the ability of yeast cells to withstand oxidative stress even though the growth of the mutant was poor as compared to the wild type. In plants, the GS plays a larger role, probably due to the fact that the deficiency in phytochelatin biosynthesis can be explained by a deficiency in GSH. Mutants with low GS-activity can not produce phytochelatin, and are therefore sensitive to heavy metal like cadmium (Howden et al., 1995, Clemen, 2006). Studies have shown that the loss of GS activity in humans has been linked to a number of diseases states. (Dahl et al., 1997, Njalsson et al., 2000, Wu et al., 2004). GS deficiency leads to 5-oxoprolinuria (pyroglutamic aciduria) which is an inherited autosomal recessive disorder characterized, in its severe form, by massive urinary excretion of 5-oxoproline, metabolic acidosis, haemolytic anaemia and central nervous system damage. The metabolic defect results in low GSH levels presumably with feedback over-stimulation of gamma-glutamylcysteine synthesis and its subsequent conversion to 5-oxoproline (Shi et al., 1996).

1.6.3 Glutathione reductase

The high intracellular concentration of reduced GSH is maintained by the NADPH-dependent, FAD disulfidoxidoreductase GR. This protein is solely responsible for the reduction of GSSG to GSH and obtains its reduction equivalent from NADPH

(Schirmer et al., 1989, Müller et al., 1997). A high GSH/GSSG ratio is essential for protection against oxidative stress. (Sies, 1989, Dudley et al., 2006) It can be said that GR represents a control point for the thiol redox homeostasis.

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+$$

GR is present in numerous micro-organisms, protozoa, plants and higher animals (Krauth-Siegel et al., 1982, Tutic et al., 1990, Collinson and Dawes, 1995, Stevens et al., 1997). The protein is a homodimer that possesses two identical subunits, each containing 1 FAD and 1 redox-active disulfide / dithiol as components of the catalytic apparatus (Schirmer et al., 1989, Deonarain et al., 1990, Argyrou et al., 2004). The human protein was isolated from erythrocytes by Worthington and Rosemeyer (1974) and its 3D-structure was determined (Schulz et al., 1975, Karplus and Schulz, 1987). The structures and the reaction mechanisms of human enzyme and *E. coli* GR have been intensively examined (Schirmer et al., 1989, Mittl and Schulz, 1994, Perham et al,, 1996). The crystallographic analysis of GR clarifies the construction of the protein from four different domains: an N-terminal lain FAD binding domain, the subsequent NADPH binding domain, the central domain as well as interface domain responsible for dimerization (Pai et al., 1988, Karplus and Schulz, 1989, Perham et al., 1996, Savvides et al., 2002). Glutathione reductase catalyzes the reduction of glutathione disulfide by NADPH and has a redox active disulfide and an FAD cofactor in each monomer. In the reductive half-reaction, FAD is reduced by NADPH and electrons pass from the reduced flavin to the redox active disulfide. The oxidative half-reaction is dithiol-disulfide interchange between the enzyme dithiol and glutathione disulfide. (Schulz and Karplus, 1988, Schirmer et al., 1989, Rietveld et al., 1994, Kanzok et al., 2001, Savvides et al., 2002). The redox active disulfide/dithiol formed from Cys-58 and Cys-63 in human enzyme stands in narrow contact with the protein bound FAD during the catalysis. This is also demonstrated spectrophotometrically (Sahlman and Williams, 1989, Veine et al., 1998). Based on the protein-bound FAD, the oxidized

protein has a maximum absorption of 460 nm. During catalysis, the enzyme is reduced by NADPH to form a stable intermediate with 2 electron reduced state (EH2). This EH2 form of the protein causes a change in adsorption spectrum with an additional peak at 540 nm. This new peak is a characteristic for the interaction of one of the reduced thiolate anion in the active center with the isoalloxazin ring of FAD to form a thiolate-flavin charge-transfer complex (Ghisla and Massey, 1989, Bauer et al., 2003). In the catalytic mechanism of *E. coli* GR, the thiolate form of Cys-42 acts as a nucleophile to initiate disulphide exchange with enzyme-bound GSH and the thiolate form of Cys-47 generates an essential charge-transfer complex with enzyme-bound FAD (Deonarain et al., 1990, Kunert et al., 1990). In human GR, Cys-63 is responsible for the formation of the thiolate-flavin charge-transfer complex, during the process, the Cys-63 is called exchange thiol and it transfers electron to the GSSG (Schirmer et al., 1989). The GR from *Onchocerca volvulus* has been cloned and characterised by Müller et al. (1997). The GR from two cattle filariae, *Setaria digitata* and *O. gutturosa*, have also been isolated and their properties have been compared to those of human erythrocyte GR. Inhibition studies carried out showed that GR is much more susceptible to inhibition with melarsen oxide than the enzyme isolated from human erythrocytes (Müller et al., 1995).

1.7 Research objectives

Parasitic nematodes are responsible for many of the major debilitating chronic diseases of man including onchocerciasis caused by the filarial parasite *O. volvulus*. Current chemotherapy programs are not ideal, with several of the drugs of choice showing a limiting efficacy and/or adverse reactions. Therefore a strategy based on rational drug development should be favoured, including the identification and validation of potential biochemical targets. It is not possible to elucidate the essential functions of potential targets in *O. volvulus* since this human parasite cannot be maintained in the laboratory and genetic manipulations cannot be performed. Therefore, *C. elegans* was chosen in this study, since the wealth of techniques available predestinates this model organism to evaluate drug targets.

Glutathione (GSH) has been identified as an important part of the antioxidant system of many, if not all living cells, and together with glutathione reductase it maintains the correct intracellular redox balance. To investigate whether the GSH metabolism of nematodes represents a potential target for the design and synthesis of novel anthelminthic drugs, the essential function of the GR, GS and GCS will be investigated in *C. elegans*. Here the genes will be identified, cloned and recombinantly expressed in *E. coli*. Following purification, the recombinant proteins will be characterized. In addition, their expression pattern will be analysed in transgenic worms via reporter gene analysis and their minimal promoter region will be investigated by deletion analysis.

GCS, the rate limiting enzyme of GSH biosynthesis, has been characterized in *O. vovulus* and studies using the specific inhibitor BSO showed that the *O. volvulus* GCS is essential for the survival of the parasite. In this study, the up regulation of *Ce*GCS under various stress conditions will be analysed. Finally, by knock down using RNA*i*, the essential role of *Ce*GCS, *Ce*GS and *Ce*GR for the survival of *C. elegans* will be investigated.

Chapter 2 Materials and Methods

2.1 Chemicals and Bioreagents

Aldrich (Steinheim)

Calcium chloride, methyl viologen (gramoxone paraquat dichloride), sodium hypochlorite.

Amersham-Biosciences (Freiburg)

[α-^{35}S]dATP (1000 Ci mmol^{-1}), ECL plus Western blotting detection reagents, Sequenase version 2.0 DNA sequencing Kit.

Bachem (Heidelberg)

γ-Glutamyl-aminobutyrate.

Becton Dickinson (Cockeysville)

BBL Agar granulated, BBL Bacto Yeast Extract, BBL Bacto-Tryptone.

Biomol (Hamburg)

Bovine serum albumin (BSA), fraction V, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), glycine, glycerol, imidazole, isopropyl-ß-D-thiogalactopyranoside (IPTG), lysozyme, phenol.

Bio-Rad Laboratories (München)

Electrophoresis standards for SDS-PAGE.

Boehringer (Mannheim)

Deoxyribonucleotides (dNTP), restriction enzymes, RNAse, T_4 DNA ligase.

Eurogentec

Agarose.

Fluka AG (Neu-Ulm)

L-Glutamic acid-mono sodium salt, juglone, Nonidet P 40 (NP40), phosphoenol pyruvate**,** sodium dodecyl sulfate.

Gerbu (Gaiberg)

N', N', N', N'-Tetramethylethylendiamine (TEMED).

Institute of Bioanalytical (IBA) (Göttingen)

Anhydrous tetracycline.

Invitrogen (Karlsruhe)

Competent cells, PCR supermix, Taq DNA polymerase, TOPO TA cloning kit, Trizol reagent.

Jackson Immuno Research Laboratories, Inc. (USA)

Alkaline phosphatase-conjugated anti rabbit IgG (H+L).

MBI (St. Leon-Rot)

High Fidelity PCR enzyme mix, dNTP.

Merck (Darmstadt)

Acetic acid (glacial), ammonium persulfate (APS), boric acid, calcium chloride, cellulose thin layer chromatography plates, chloroform, coomassie-Blue (R250 G250), ethanol, formaldehyde, D(+)-glucose-monohydrate, hydrochloric acid, Iron (II) chloride, magnesium chloride, magnesium sulfate, maltose, ß-mercaptoethanol, methanol, peptone, *ortho*-phosphoric acid, potassium chloride, potassium dehydrogen phosphate, silver nitrate, sodium carbonate, sodium chloride, sodium

dehydrogen phosphate, sodium hydrogen phosphate, sodium hydroxide, silica gel 60 TLC plates, 5- Sulfosalicylic acid dehydrate (SSA) , trichloroacetic acid, Triton X-100, Tween 20.

New England Biolab (Frankfurt am Main)

Amylose resin, ligation buffer, pMalp2x vector, proteinase K, restriction enzymes, T4-DNA-ligase, M-MulV-reverse transcriptase.

Pharmacia (Freiburg)

HiLoad 16/60 Superdex 75 Column.

Pierce

Inclusion body solubilisation reagent.

Qiagen (Hilden)

Ni-NTA His bind resin, QIAquick PCR purification kit, QIAGEN plasmid Mini, Midi and Maxi kit.

Roche (Mannheim)

Alkaline phosphatase, DNA-Polymerase I (Klenow-fragment), DNase, nylon membranes positively charged, random primed DNA labeling kit, RNase, T7 RNA polymerase.

Roth (Karlsruhe)

Ampicillin, IPTG, roti-marker protein standard, Rotiphorese® NF-Acrylamide/Bis-LSG 40% (29:1), sodium iodide, tris-(hydroxymethyl)-aminomethan, oligonucleotides,.

Serva (Heidelberg)

Acrylamide, bromophenol blue-sodium salts, dextran blue, dodecyl sulfate sodium salt.

Sigma (München)

Adenosin-5-triphophate (ATP), α-L-aminobutyrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP), buthionine-S-,R-sulfoximine (BSO), chloramphenicol, creatin-phosphokinase, L-cysteine, enhanced avian RT-PCR kit, ethidium bromide, gel filtration molecular weight marker kit, glucose, glucose oxidase, glutathione (reduced), glutathione (oxidized), glutathione reductase (GR), N,N-bis[2-hydroxylethyl]glycine (bicine), kanamycin, Kodak BioMax MR film**,** lactate dehydrogenase, mercury chloride, methylene blue, mineral oil, 2-[N-morpholino] ethane sulfonic acid (MES), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), nitrobluetetrazoliumchloride (NBT), phenylmethylsulfonylfluoride (PMSF), pyruvate kinase, sodium bicarbonate, tris-(hydroxymethyl)-aminomethane, bis-tris-propane, 2-vinylpyridin, zinc chloride.

Schleicher & Schuell (Dassel)

Nitrocellulose transfer membrane Protran R.

Whatman (Göttingen)

Chromatography Paper 1.

2.2 Standard Solutions and Buffers

Agarose Gel Electrophoresis:

5 X TBE buffer	450 mM Tris, 450 mM boric acid, 10 mM EDTA, pH 8.0.
5 X TAE buffer	200 mM Tris, 1.5 M sodium acetate, 5 mM EDTA, pH 8.0.
6 X Loading buffer	0.25% bromophenol blue, 0.25% xylene-cyanol, 40% glycerol.

Coomassie-Staining

Fixer solution20% trichloroacetic acid.

Staining solution Stain solution A and B in ratio 1:1.

Stain solution A: 0.2% (w/v) Coomassie brilliant blue G-250 in 96% ethanol.

Stain solution B: 20% (w/v) glacial acetic acid in dH_2O.

Destaining solution 20% (v/v) ethanol, 10% (v/v) glacial acetic acid.

Solution for DNA Preparations

STET-buffer 50 mM Tris/HCl, pH 8.0, 50 mM Na^+-EDTA, 5% (v/v) Triton X-100, 8% (v/v) sucrose.

New Wash 50% ethanol, 10 mM Tris, pH 7.5, 0.05 mM Na^+-EDTA, pH 8.0, 50 mM NaCl.

Glass milk 12% (w/v) silicon dioxide, 0.12% HCl (v/v) in H_2O.

TE-Buffer for DNA

10 mM Tris/HCl, 1 mM EDTA, pH 7.5.

Protein determination

Bradford reagent 0.01% (w/v) Coomassie brilliant blue G-250, 4.7% (w/v) ethanol, 8% (w/v) H_3PO_4.

SDS-gel electrophoresis

2 x SDS sample buffer 70 mM sodium dodecylsulfate (SDS), 5% mercaptoethanol, 125 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue (w/v).

M9 buffer.

6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4$ +7H_2O add 1 l dH_20.

Freezing solution

5.85 g NaCl, 6.8 g KH_2PO_4, 300 g glycerol, 5.6 mM NaOH, add 1 l H_2O, after autoclaved: 0.3 mM $MgSO_4$.

PBS Buffer

10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, 2.6 mM KCl, 136.9 mM NaCl, pH 7.4.

Solutions for competent cells

80 mM $CaCl_2$.

100 mM $CaCl_2$, 20% glycerol,

2.2.1 Media

Luria-Bertani-Medium(LB-Medium)

1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl. **(**for LB-Agar:

LB-medium + 1.5% Agar)

Basal medium

3% (w/v) Yeast extract,

3% (w/v) Soy peptone,

5 µg/ml Cholesterol and autoclaved.

Nematode growth medium (NGM-medium)

3 g NaCl, 2.5 g peptone, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4 pH 6.0 autoclaved and 5 µg/ml cholesterol. (For NGM- agar 1.5% agar added).

2.3 Vectors and bacterial cells

2.3.1 Plasmid vectors

pASK-IBA3	(Institut für Bioanalytik, Göttingen).
pJC40	(Clos and Brandau, 1994)
pMalp2x	(NEB Biolabs)
pPD95.77	(Fire, 1986)

pD49.26	(Fire, 1986)
pBX	(Schnabel and Schnabel, 1990)
PCR-blunt II TOPO	(Invitrogen)
L4440	(Timmons, 2000)
DUOx	(Timmons, 2000)

2.3.2 *Escherichia coli* strains

BL21-(DE3)	(Studier and Moffat, 1986)
BL21 star (DE3)	(Invitrogen)
DH5α	(Hanahan, 1985)
OP50	(Strategene)
HT115	(Timmons 2000)
XL Gold 10	(Strategene)

2.3.3 *C. elegans* strains

Bristol N2 (wild type), ok436 (gcs-1 allele obtained from the *C. elegans* knock out consortium), Vc337 Knock out mutants, pha-*1*(*e2123ts*) (Schnabel and Schnabel, 1990).

2.4 Culture of *C. elegans*

2.4.1 Growth conditions

Worms were reared on 9 cm NGM nutrient petric dishes seeded with OP50 *E. coli* bacteria according to standard protocols (Brenner, 1974). The wild type strain (N2 var Bristol) and mutant strains were kept at 20°C except for the thermosensitive pha-*1*(*e2123ts*) strain that was maintained at 16°C. The culture was subcultured every 10 days.

2.4.2 Separation of *C. elegans* from bacteria

Worms from plates were separated from bacteria by sucrose floatation as described by Sulston and Hodgkin (1988). Worms were resuspended from plates with NGM medium, put in Falkon tube and incubated on ice for 1 h. The worms were then centrifuged at 1500 xg for 10 min at 4°C. The pellet was resuspended in 14 ml 0.1 M NaCl and centrifuged further for 10 min at 1500 xg, after which 5 ml 0.1 M NaCl and 5 ml 60% sucrose were added to the pellet and mixed carefully. The suspension was centrifuged at 1200 xg for 10 min. The floating worm ring was carefully taken out and washed twice with 50 ml 0.1 M NaCl and centrifuged at 1500 xg for 10 min. The pellet was then resuspended in M9 buffer or NGM medium and maintained in plastic dishes at 20°C.

2.4.3 Axenic *C. elegans* culture

Synchronised worm cultures were initialised from eggs obtained from gravid adults in a mixed population by alkaline hypochlorite treatment as described by Fabian and Johnson (1994) and Lewis and Fleming (1995). Adult worms from NGM plates or medium were suspended in M9 buffer and put in a Falkon tube. Worms were then centrifuged at 2000 xg for 3 min and the supernatant discarded. The worm pellet was resuspended with 5 ml of freshly prepared chlorox solution (5.5 ml H_2O, 4 ml chlorox, 0.5 ml 10 N NaOH), incubated at RT for 7 min and then centrifuged at 2000 xg for 3 min. The pellet containing eggs was washed 3 times with sterile M9 buffer under sterile conditions at 2000 xg for 3 min. The pellet was then resuspended in M9 buffer and maintained at 20°C overnight. Worm growth was arrested 2 days following hatching of the eggs in medium, resulting in an essentially L1 larval stage population. The following day experiments can be carried out with L1 worms, and worm can be cultured in axenic culture using basal medium containing 50 mg/ml haemoglobin.

2.4.4 Freezing of worms

C. elegans worms are stored frozen in liquid nitrogen according to Sulston and Brenner (1974). Worms were washed off the surface of a petric dish culture using 1.5

ml of M9 buffer. 1 ml of freezing solution is added to 1 ml of worm suspension and mixed. Aliquots are then dispensed into Eppendorf tubes. These tubes were placed in styropore boxes and put at –70°C for 1 day in order that initial freezing takes place slowly. For further storage these tubes were transferred to boxes at –70°C.

2.5 Photometric determination of GSH concentration in *C. elegans*

The total GSH concentration in *C. elegans* was determined spectrophotometrically according to the method of Anderson (1985). This method is based on a DTNB-GSSG reductase recycling assay which couples the oxidation of GSH by DTNB and the formation of TNB as shown in **Figure 9**

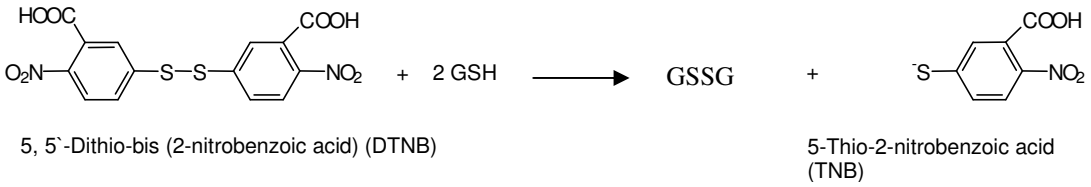


Figure 9. Reaction of GSH with DTNB.

Worm preparation

About 40 ml axenic culture of *C. elegans* was placed on ice for 1 1/2 h and centrifuged at 2000 xg for 10 min at 4°C. The pellet was washed two times with 20 ml PBS. The worms were then suspended with 500 µl PBS and transferred to an Eppendorf tube. The suspension was placed on dry ice for 1 h and the worms were homogenized intensively using a glass/glass homogenizer. The homogenate was then transferred to a fresh Eppendorf tube and centrifuged at 1400 xg for 30 min. The supernatant was used for both protein and GSH determination, according to the methods of Bradford (1976) and Griffith (1980) modified by Anderson (1985), respectively.

Calibration

In order to generate a calibration curve, a 100 mM GSH stock solution was diluted with 3% SSA to a final concentration of 100 µM. The GSH equivalent of 1, 2, 3, and 4 nmoles was pipetted into cuvettes and the control was without GSH. 700 µl of daily buffer (143 mM NaH_3PO_4, 6.3 mM EDTA pH 7.5, 0.248 mg/ml NADPH), 100 µl DTNB solution and water was added to a final volume of 1 ml and mixed. The cuvettes were pre-incubated for 12-15 min at 25°C and GSSG reductase (266 U/ml) was added with mixing to initiate the assay. The assay was followed for 10 min in a spectrophotometer at 412 nm. The calibration curve was obtained by plotting the GSH equivalents against the rate of change of absorbance at 412 nm.

GSH determination

The supernatant of the worm homogenate was diluted in 1/3 volume of 5% SSA in order to precipitate the protein. Subsequently, the mixture was centrifuged at 10,000 xg for 20 min at 4°C. Aliquots (25 µl, 50 µl and 100 µl) of the deproteinised supernatant were used in the spectrophotometrical assay described above. The amount of GSH was determined from the standard curve. The concentration of GSH in *C. elegans* was calculated in nmoles/mg protein of the supernatant.

2.6 Preparation of nucleic acids

2.6.1 Preparation of genomic DNA

Genomic DNA was prepared from *C. elegans* worms as described by Jowett et al. (1986). Briefly, 1 g *C. elegans* worms were crushed in a glass/glass motar under liquid nitrogen and reususpended with 10 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine). Following digestion with proteinase K (100 µg/ml) at 48 °C for 2 h, the preparation was extracted twice with phenol-chloroform-isoamyl alcohol and washed once in ether. The DNA was then ethanol precipitated, treated with RNAse I (100 µg/ml) and re-extracted with phenol-chloroform-isoamyl alcohol. The purified DNA was ethanol precipitated and resuspended with TE buffer. An aliquot of the DNA was

quantitatively and qualitatively analysed by spectrophotometric and electrophoretic methods, respectively.

2.6.2 Preparation of RNA from *C. elegans*

C. elegans worms were lysed in TRIzol reagent. The homogenized sample was first incubated at 37°C for 5 min in order to completely dissociate the nucleoprotein complexes. 1 ml of chloroform per 5 ml of TRIzol was then added to the tube and the sample mixed vigorously for 15 sec and incubated at 30°C for 2 min. The sample was centrifuged at 12.000 xg for 30 min at 4°C. Following centrifugation, the supernatant was separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA is only found in the aqueous phase and this was transferred to a fresh tube. The RNA was then precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent was used. The samples were incubated at room temperature for 10 min and then centrifuged at 12,000 xg for 10 min at 4°C. The RNA precipitate formed a gel-like pellet on the side and at the bottom of the tube. The supernatant was then removed carefully and the pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol reagent. The sample was mixed by vortexing and centrifuged at 7,500 xg for 5 min at 4°C. The supernatant was removed and the RNA pellet was allowed to air dry for 5-10 min. The RNA was then redissolved in 20 µl of 100% formamide and stored at –70°C until used.

2.6.3 Ethanol precipitation

Ethanol precipitation of nucleic acids was performed as described by Sambrook et al (1989) using 3 M ammonium acetate, pH 5.5.

2.6.5 Phenol-chloroform extraction

DNA and RNA were extracted with phenol-chloroform using a modified method of Salzman and Weissbach (1967), using Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v).

2.7 Polymerase chain reaction (PCR)

Routine PCRs were performed in 50 µl reaction volumes containing 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 200 µM dNTPs, 10 pmol of each oligonucleotide, 100 ng DNA-template and 0.5 U of high fidelity enzyme. 35 PCR cycles were performed, consisting of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of: denaturation at 94°C for 45 sec to 1 min, annealing of the oligonucleotide at 48°C to 55°C for 45 sec to 1 min, followed by an extension at 68-72°C for 1.5 min. After a final 10 min extension step at 72°C and a 4°C hold, the samples were analyzed by agarose gel electrophoresis.

In order to purify PCR products, the QIAquick-PCR purification kit (Qiagen) was used according to the manufacturer's instructions. With this method, enzymes and salts, as well as free nucleotides and DNA fragments up to a size of 100 bp were separated.

2.7.1 Single worm PCR

Single worms were selected and allowed to reproduce on fresh NGM plates at 20°C, before they were picked from plate, 2.5 µl lysis buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP 40, 0.45% Tween, 0.01% gelatin) with 100 µg/ ml proteinase K was pipetted into sterile 0.5 ml thin wall tubes, a single worm was then put in each tube and transferred for 20 min on dry ice. The worms were subsequently lysed through incubation at 60°C for 1 h and the proteinase was inactivated for 15 min at 95°C. A PCR was then followed by adding 100 pmol of the appropriate sense and antisense oligonucleotides together with 45 µl supermix to the worm lysate. The PCR-conditions were: 95°C, 3 min; 95°C, 1 min; 48°C, 1 min; 72°C, 1-3 min (x 35) depending on the expected DNA-fragment and a final extension of 72°C for 8 min.

2.8 Reverse transcription

In order to synthesize the cDNA from mRNA, the M-MulV-reverse transcriptase (New England Biolab) was used. Briefly annealing was done by adding 1 µg of total RNA with 1 µl primer dT in a sterile microfuge tube with nuclease-free H_2O to final volume

of 17 µl and incubated for 7 min at 70°C, the mixture was spin briefly and placed promptly on ice. After that, 1 µl of 10 mM dNTPs, 2 µl of 10X reverse transcriptase buffer, 1 µl of RNAse inhibitor and 1 µl of M-MuLV Reverse Transcriptase were added to a final volume of 20 µl. The mixture was incubated at 42°C for one hour, and the inactivation step of enzyme was done at 10 min at 70°C. The product was stored at -20°C. 1 µl of the cDNA was later used as a template for PCR reaction.

2.9 Preparations of plasmid DNA

2.9.1 Mini preparation of plasmid DNA

An aliquot of 2 ml overnight *E. coli* culture was centrifuged at 13,000 xg for 2 min. The bacterial pellet was resuspended in 250 µl STET-buffer. 100 µg of lysozyme was added to this suspension and mixed by shaking. The sample was placed on ice for 10 min after which it was boiled in water bath at 100°C for 1 min. The tube was placed on ice for 30 sec, and then centrifuged for 15 min at 13,000 xg. The supernatant was transferred in a new tube and mixed with 500 µl of 6 M sodium iodide solution and 10 µl of glass milk (silicon dioxide). To bind the DNA, the suspension was shaken at 37°C for 5 min. The pellet was washed once with 1 ml NEW WASH solution and then dried at 56°C for 5-10 min. The glass milk pellet was resuspended in 80 µl water, incubated for 5-10 min at 56°C. The DNA was separated then from the glass milk by centrifugation.

2.9.2 Midi and Maxi preparation of plasmid DNA

Large scale Midi and Maxi DNA preparations, were done as described by the manufacturer (Qiagen plasmid Maxi/Midi kit, Qiagen or Nucleobond PC 100 kit, Macherey and Nagel). For the Midi preparation, 100 ml of overnight culture of *E. coli* was used and approximately 150 µg plasmid DNA was obtained. For the maxi DNA preparation method, 200-500 ml of overnight culture of *E. coli* was used and a maximum of 500 µg of plasmid DNA was obtained.

2.10 Restriction enzyme digestion of DNA

DNA plasmid, PCR products and vectors constructs were digested with the appropriate restriction endonucleases in the suitable buffer system and at the correct temperature, according to the manufacturer's instructions (MBI, NEB). As desired, 1-10 µg of DNA were digested in a 15-100 µl reaction volume for 1-3 h (for analytical purposes) and 24 h (for preparatory purposes).

2.11 Agarose gel electrophoresis of DNA

For analytical purposes, DNA samples were mixed with 1/6 volume loading buffer (0.25% bromophenol blue, 0.25% xylene-cyanol, 40% glycerol) and depending on the size of the DNA fragments to be resolved, separated on 0.8–1.5% agarose gels containing 0.5 µg/ml ethidium bromide in TBE buffer at 7.5 volts/cm. A 1 kb ladder was used as the molecular weight marker and the resolved DNA was visualized over UV light. Low melting point agarose (Gibco BRL) in TAE buffer at 5 volts/cm was used to resolve DNA fragments for preparative purposes. The DNA bands of interest were then excised from the gel using a new scalpel and extracted from the agarose by the Gene-Clean technique.

2.12 Recovery of DNA from agarose gels

The gel slices excised from TAE agarose gels were mixed with sodium iodide (1:3 w/v) and incubated at 56°C until the agarose completely dissolved. Glass milk (10 µl) was then added and the mixture was shaken at room temperature for 10 min. The matrix was then centrifuged at 10,000 xg for 30 sec and the pellet washed twice with NEW WASH buffer. The glass milk was dried at 56°C and the bound DNA was eluted from the matrix by resuspending the pellet in distilled water at 56°C for 5 min.

2.13 Ligation of DNA fragments sub-cloning in plasmid vectors

DNA fragments were cloned into plasmid vectors as described by Sambrook et al. (1989). The vectors were digested by double digestion with the appropriate restriction

enzymes. The vector DNA and the DNA fragment to be cloned were then mixed in a molar ratio of 1:3 and ligated in a 10 µl ligation volume overnight at 14°C, using 1 unit of T4 DNA-ligase and 1µl 10x ligation buffer.

2.14 Transformation

2.14.1 Competent bacteria production

Bacteria were made competent for transformation by the method of Hanahan (1985). A bacterial colony was inoculated in 10 ml LB-medium and left to grow overnight at 37°C. The overnight *E. coli* culture was diluted 1:100 in 100 ml LB-medium and allowed to grow until OD_{600} = 0.5. The cells were chilled on ice for 15 min and then centrifuged for 10 min at 4000 xg at 4°C. The resulting bacterial pellet was resuspended in ice-cold 80 mM $CaCl_2$ and incubated on ice for 30 min. The cells were then pelleted and resuspended in 10 ml ice-cold 100 mM $CaCl_2$ 20% glycerol solution, aliquoted at 4°C and frozen at –70°C.

2.14.2 Transformation of competent cells

The transformation of the bacterial cells was performed according to the method of Cohen *et al.* (1972). The competent cells were allowed to thaw on ice slowly. Ligation mixtures or plasmids were then added and the sample was incubated for 30 min at 4°C. The cells were heat shocked at 42°C for 60 sec and then cooled on ice for 2 min. 700 µl of LB medium was added and the mixture was agitated at 37°C for 1 h. The cells were then plated on LB agar plates containing the appropriate selection antibiotic for the vector used and incubated overnight at 37°C. In the case of blue/white selectable vectors, the LB agar plates were spread with 70 µl of 20 mg/ml X-Gal and 70 µl of 0.1 M IPTG. White colonies were selectively picked for further analysis.

2.15 DNA sequencing and *in silico* analyses

DNA sequencing was performed according to the dideoxy chain termination method of Sanger et al. (1977). The Sequenase Version 2.0 DNA sequencing kit (USB) was used, with vector or gene specific oligonucleotides. The double-stranded DNA was denatured for 10 min with 0.2 M NaOH at 37°C. The DNA was finally precipitated with 3 M ammonium acetate (pH 5.2) and 100% ethanol, then washed with 70% ethanol, dried at 56°C and eventually resuspended with distilled water in order to prepare it for the sequence reaction. Samples were heat-denatured at 95°C for 2 min and loaded on an 8% polyacrylamide gel. Following electrophoresis at 80 W constant power for 2-4 h, the gel was dried at 80°C under vacuum and autoradiographed overnight. Alternatively, automated nucleotide sequencing was applied using an ABI 377 automatic sequencer (Applied Biosystems). Sequence analysis was performed using GENERUNNER programme. The National Center for Biotechnology and Information (NCBI) and worm base databases (www.wormbase.org.db/seq/searches) were used for homology comparisons.

2.16 Recombinant expression of *C. elegans* GS (*Ce*GS)

2.16.1 Cloning of *Ce*GS

The open reading frame (ORF) of the *Ce*GS was amplified from *C. elegans* cDNA with the high fidelity PCR system using the sense oligonucleotide (*Ce*GSs) 5'-ACCCCATATGGCTCAAAAAGATGACCGGATTTTGC-3', containing a *Nde* I restriction site, and the antisense oligonucleotide (*Ce*GSas) 5'ATCGCGGATCCTTATC AAATAAATTCGTAGAGGAATGGGGT-3', containing a *Bam*H I restriction site. The PCR program was 2 min at 95°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 52°C, 1 min 30 sec at 68°C, followed by a final extension step at 72°C for 8 min. The resulting PCR fragment was digested with *Nde* I and *Bam*H I and cloned into the pJC40 vector that had been previously cut with the same endonucleases. The plasmid contains the T7 promoter that drives expression of the recombinant protein. The *Ce*GS was cloned in frame with the N-terminal His-tag that enables the

purification on nickel chelating chromatography. The sequence of the *Ce*GS insert in pJC40 vector was verified by sequencing. The *Ce*GSpJC40 positive construct was then transformed into the competent cells *E. coli* BL21 (DE3).

2.16.2 Induction of protein expression

A single bacterial colony containing the plasmid was picked, grown overnight at 37°C and diluted 1:50 in fresh Luria-Bertani medium containing 50 ug/ml ampicillin. The bacterial culture was grown at 37°C until the OD_{600} value reached 0.5. The expression of the recombinant protein was started by induction with 1 mM IPTG and the temperature was reduced to 25°C overnight.

2.16.3 Purification of re*Ce*GS

The purification of recombinant His-tagged proteins on nickel chelating affinity chromatography was done in batch-wise according to the manufacturer instructions (Qiagen). All purification steps were carried out at 4°C.

Stock solutions for nickel chelating affinity chromatography

Binding buffer	50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole
Wash buffer	50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole
Elution buffer	50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 300 mM imidazole

The bacteria culture from 2.16.2 was harvested by centrifugation at 4.000 xg for 10 min and the pellet was resuspended in binding buffer containing 1 mM DTT, before the cells were sonified using a Branson sonifier. The homogenate was centrifuged at 100,000 xg for 1 h at 4°C using a TFT 80.4 rotor (Kontron). 2 ml of His-bind resin were packed by centrifugation at 500 xg for 5 min and washed twice with binding buffer. The 100 000 xg supernatant was loaded onto the Ni^{2+}-nitrilotriacetate affinity matrix for 3 h. The resin was subsequently washed twice with wash buffer to remove

non-specifically bound contaminating proteins. The protein was eluted in 2 ml of elution buffer containing 1 mM DTT. The eluted protein was applied to an FPLC Sephadex S-200 gel-sizing column (2.6 cm x 60 cm) equilibrated previously with 50 mM Tris-HCl/1 mM DTT, pH 7.2. The Sephadex S-200 column was calibrated with a molcecular mass marker kit (Sigma) with the range 18-2000 kDa to determine the molecular size of the active enzyme.

2.16.4 Enzyme assay and kinetic analyses of recombinant *Ce*GS

All steady-state kinetic analyses were performed with purified recombinant *Ce*GS. To determine *Ce*GS activity, amino acid dependent ADP formation was monitored using a pyruvate kinase and lactate dehydrogenase-coupled assay at 30°C as previously described (Huang et al., 1995, Meierjohann et al., 2002) with minor modifications. Briefly, a standard reaction mixture (final volume 1 ml) contained 100 mM Tris-HCl buffer, pH 7.2, 1 mM EDTA, 75 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 30 mM glycine, 2 mM γ-Glu-Abu, 0.27 mM NADH, 5 U pyruvate kinase and 10 U lactate dehydrogenase. Background rates were determined in the absence of γ-Glu-Abu and subtracted. The rate of ADP formation was assumed to be equal to the rate of NADH oxidation as monitored at 340 nm (ε= 6.2 $\mu mol^{-1}cm^{-2}$). Constant attention was taken to ensure that pH of γ-Glu-Abu was monitored before being used in the assay. One unit of GS is the amount of enzyme forming 1 µmol of product per minute. For the determination of K_m values, the respective dilutions of ATP, γ-Glu-Abu and glycine were prepared in 100 mM Tris-HCl, pH 7.2. Two of the substrates were made saturating while the third was used at various concentrations. The K_m ranges were glycine (1-30 mM), γ-Glu-Abu (0.1-2 mM) and ATP (0.1-4 mM).

2.17 Recombinant expression of *C. elegans* GR (*Ce*GR)

2.17.1 Cloning of *Ce*GR

In order to amplify the *C. elegans* GR coding region, the sense oligonucleotide (*Ce*GRs) 5'-GAGACATATGTCTGGCGTCAAGGAGTTCG-3' containing a *Nde* I

restriction site and the antisense oligonucleotide (*Ce*GRas) 5'-GAGACTCGAGTTATTCCGGCTCACACCTCC-3' containing *Xho* I restriction site were used in a PCR with high fidelity PCR system and *C. elegans* cDNA. The PCR conditions were: an initial denaturing step for 2 min at 95°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 52°C, 1.5 min at 68°C with a final extension time of 10 min at 72°C. The resulting fragment was cloned into the pJC40 vector previously cut with the respective endonucleases. The sequence of the GR insert in pJC40 vector was verified using manual sequencing. The construct was subsequently transformed into competent BL21 (DE3) cells.

2.17.2 Induction of protein expression.

The induction of expression of the recombinant protein was carried out as described for *Ce*GS under 2.16.2.

2.17.3 Purification of r*Ce*GR

His-tagged recombinant protein was purified batch-wise by nickel-chelating affinity chromatography (Qiagen) according to the manufacturer's recommendations. All solutions were used at the concentrations stated and all steps were done at 4°C. 700 µl of His-Bind resin (Qiagen) was centrifuged at 500 xg for 4 min. The overlying solution was discarded and protein supernatant samples were applied and incubated for 3 h at 4°C. Non-specifically bound contaminating proteins were washed from the resin twice with 50 ml of wash buffer which consisted of 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 20 mM imidazole. The bound *Ce*GR protein was eluted with 2 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl and 300 mM imidazole). The elutant was applied to a FPLC Sephadex S-200 gel sizing column (Hiload 26/60) from Pharmacia Biotech, previously equilibrated with KH_2PO_4 buffer at pH 7.2. The Sephadex S-200 column was calibrated with the molecular weight marker kit (Sigma), with a range of 29000-2,000,000 Da, to determine the molecular size of the active enzyme. Protein concentration was thereafter determined according to

Bradford (1976) method with BSA as a standard. The purity of the protein fractions was assessed by SDS/PAGE.

2.17.4 Kinetic analysis of *Ce*GR protein

The activity of *Ce*GR was determined spectrophotometrically at 25°C (UVIKON- 932 spectrophotometer, Goebel, Germany) as previously described by Müller et al. (1995). The decrease of absorbance due to the oxidation of NADPH was recorded at 340 nm (ε= 6.2 $\mu mol^{-1}cm^{-2}$) in a total volume of 1 ml. The standard reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.2), 1 mM EDTA, 200 µM NADPH and enzyme solution. The reaction was initiated by adding 400 µM GSSG. Background rates were determined in the absence of GSSG. The activity that catalyzes the consumption of 1 µmol of substrate per minute was defined as 1 U. To determine the steady-state kinetic parameters (K_m values), the enzyme assay was carried by varying the concentration of one substrate (10-500 µM for NADPH and 10-200 µM for GSSG) in the presence of saturating concentration of the other. The pH- and temperature optimum of the reaction was determined.

2.18 Recombinant expression of *C. elegans* γ-GCS (*Ce*GCS)

2.18.1 Cloning of *Ce*GCS

The coding region of *Ce*GCS was amplified from *C. elegans* cDNA by PCR using the sense primer *Ce*GCS(s) 5`GACCCAAGCTTGGTCTTTTGACGAAAGGTAGTCCGTTG-3` containing a *Hind* III restriction site and the antisense primer *Ce*GCS(as) 5`-ATCGCCTCGAGTTACTAATGTGCACGTTTTTGGCTGGACAC-3' containing a *Xho* I site. The PCR product was isolated, digested and cloned into the expression vector pJC40. The construct was verified by DNA sequencing before it was transformed in BL21-star (DE3) competent cells.

2.18.2 Recombinant expression of *Ce*GCS

An overnight culture initiated from one bacterial colony was grown in LB-amp medium containing 34 µg/ml of chloramphenicol. The culture was allowed to grow at

37°C to OD_{600}=0.5. IPTG was added to induce expression of the recombinant protein. Many expression strategies were carried out with γ-GCS-pJC40, as such IPTG was used at concentrations between 0.1 mM and 1 mM and the induction temperature was varied between 14°C and 37°C. Cells were then harvested and the purification method described above was applied.

Furthermore, the nickel-chelating chromatography steps were performed with buffers containing 6 M urea or 4 M guanidine/HCl, in order to investigate, whether the protein is found in inclusion bodies. The elute was dialysed overnight at 4°C against 1000 volumes of 100 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM EDTA.

In addition, the bacteria pellet obtained after ultracentrifugation was resuspended and sonicated in 100 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM EDTA, 10% or 20% glycerol, before it was loaded onto the Ni^{2+} matrix.

Additional trials were done after the ultracentrifugation by resuspending the bacteria pellet in 100 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM EDTA, 10% or 20% glycerol in solution before it was loaded on Ni^{2+}matrix. Another alternative method was cloning and expressing the protein in pMalp2x vector as described by Ryazanova et al. (2001). 100 ml of a solution containing rich broth (20 g tryptone, 10 g yeast extract, 10 g NaCl, 4 g glucose) and ampicillin was inoculated with 1 ml overnight culture of GCS pmalp2x. The culture was grown at 37°C until OD_{600}=0.5. Expression of recombinant protein was started by the addition of IPTG to a final concentration of 0.3 mM. Cells were grown for additional 4 h. The bacterial cells were harvested at 4000 xg for 10 min and part of the pellet resuspended in 5 ml column buffer (20 mM Tris/HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA 10 mM β-mercaptoethanol) and lysed by sonication. The homogenised sample was centrifuged at 9000 xg for 20 min and the supernatant was loaded onto an amylose column equilibrated with column buffer and allowed to bind overnight. Elution was performed by a step gradient using the same buffer plus 10 mM maltose. The other part of the pellet was resuspended in 10 ml (30 mM Tris HCl, 20% sucrose pH 8.0, 1 mM EDTA) and incubated 7 min at RT shaking. The cells were centrifuged at 8000 xg at 4°C for 10 min. The pellet was resuspended in 10 ml ice cold 5 mM $MgSO_4$, and stirred for 10 min in ice water bath

then centrifuged once again at 8000 xg for 10 min. The supernatant (osmotic shock fluid) was mixed with sample buffer and analysed by SDS PAGE.

2.19 Protein determination methods

2.19.1 Determination of protein concentration

Soluble protein concentrations were determined as described by Bradford (1976), using BSA as standard.

2.19.2 SDS-PAGE under denaturing conditions

For analytical purposes, protein samples were resolved by discontinuous polyacrylamide gel electrophoresis under denaturing conditions, according to the method of Laemmli (1970).

Stock solutions

Monomer stock solutions: 29.1% acrylamide, 0.9% bis-acrylamide.

Running buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS (w/v), 40% glycerine,

7.5% separating gel 1.5 ml separating gel buffer, 3.0 ml H_2O, 1.5 ml 30% acryl amide, 60 µl SDS, 25 µl 10% ammonium persulfate (APS), 5 µl TEMED.

4% stacking gel 1.0 ml stacking gel buffer, 2.44 ml H_2O, 534 µl acrylamide, 40 µl 10% SDS, 20 µl 10% APS, 5 µl TEMED.

Samples were mixed with sample buffer and denatured for 5 min at 100°C. Following centrifugation at 10,000 xg for 2 min, samples were loaded onto a 4% stacking and a 7.5% separating polyacrylamide minigel (Biometra). Protein molecular weight

standard markers were also loaded on the gel with the samples. Electrophoresis was carried out in SDS running buffer at 30 mA for 2 h. The gel was then recovered and proteins were visualized by Coomassie blue or silver staining.

2.19.3 Protein staining gels

2.19.3.1 Coomassie blue staining

Using the method of Mayer and Walker (1987), proteins were visualized on polyacrylamide gels. Following electrophoresis, the gel was fixed for 30 min in 20% trichloroacetic acid (TCA) and stained for 2 h in Coomassie stain solution. The gel was then placed in destaining solution until the background was cleared and the protein bands became visible on the gel.

2.19.3.2 Silver staining

In order to visualize minute amounts of protein, the polyacrylamide gel was stained using the method of Heukeshoven and Dernick (1986). The gel was first incubated for 15 min in solution 1 (30% ethanol, 10% glacial acetic acid), and then for 15 min in solution 2 (30% ethanol, 0.5 M sodium acetate, 0.5% glyceraldehyde, 10.5 mM $Na_2S_2O_3$). After thoroughly rinsing in water for 60 min, the gel was stained for 15 min in solution 3 (6 mM $AgNO_3$, 0.02% formaldehyde) and developed for 5-15 min in solution 4 (0.24 M Na_2CO_3, 0.01% formaldehyde). The development was terminated in a 0.05 M EDTA solution.

2.19.4 Western blotting

Protein was blotted on to a nitrocellulose membrane following SDS-PAGE by the method of Sambrook et al. (1989) using the electrophoresis transfer units 21172-250 NOVABLOT (Pharmacia, Freiburg). The 7.5% gel recovered after SDS-PAGE was equilibrated for 15 minutes in cathode solution (40 mM 6-aminocaproic acid, pH 7.6, 20% methanol). A piece of nitrocellulose membrane and several pieces of Whatman filter paper were cut to the same size as the gel. Six pieces of filter paper were

soaked in anode solution 1 (300 mM Tris-HCl, pH 10.4, 20% methanol) and placed on the moist anode plate of the transfer unit. Three pieces of filter soaked in anode solution 2 (25 mM Tris-HCl, pH 10.4, 20% methanol), followed by the nitrocellulose membrane, which was soaked in the same solution, were placed in the set up. The gel was then placed in direct contact with the membrane followed by 9 pieces of filter paper soaked in cathode solution. The cathode plate was set into place and then transfer at 0.8mA cm^{-2} constant current for 1 h. Following transfer, the nitrocellulose membrane was recovered, air-dried and blocked overnight at 4°C or 1 h at RT, in 5% powdered whole milk (w/v) in PBS, and washed in PBS, 0.05% Tween 20 for 15 minutes and stored at 4°C for subsequent use.

2.19.5 Immunodetection

The nitrocellulose membrane was incubated for 1-2 h in anti-His-AP-conjugated antibody, appropriately diluted 1.5000 in PBS, 1% BSA .The membranes were then washed 3 x 5 min in wash buffer and once in ddH_2O for 5 min. The bound antibody was detected by incubating the membrane in freshly prepared alkaline phosphatase substrate solution (0.1 M Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.033 % NBT, and 0.0165 % BCIP, and 0.83% dimethyl formamide) and developing to the desired intensity. The development was terminated in 20 mM Tris-HCl, pH 2.9, 1 mM EDTA solution. The membrane was rinsed in ddH_2O and air-dried.

2.20 GFP reporter gene constructs

To analyse the *Ce*GS and *Ce*GCS expression pattern in *C. elegans,* several fragments of the promoter regions were amplified by the expand high fidelity PCR system, using *C. elega*ns genomic DNA as a template. The primers are listed in **Table 3**. The PCR conditions were: an initial denaturing step for 2 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 52°C, 3.5 min at 72°C with a final extension time of 10 minutes at 72°C.

The resulting fragment was subcloned after having digested with the appropriate endonucleases into the multiple cloning sites of the pPD95.77 vector provided by Prof. A. Fire (Carnegie Institute, Baltimore, MD, USA). The constructs were transformed into *E. coli* DH5α cells. The sequence of the gene insert in pPD95.77 vector was verified using manual sequencing.

Table 3. Oligonucleotides used for the cloning of *Ce*GS and *Ce*GCS promoter sequences. Introduced restriction sites are underlined.

Primer no.	**Primer (5`…….3`)**	**Restriction enzyme**	**Product (bp)**
GS 9538	CCCAAGCTTGGTTGGCTCCGGATCCTGTGAGC ACGTG	*Hind* III (s)	3036
GS 9539	CCCAAGCTTAGTTTAAACAGACACGAGA	*Hind* III (s)	1415
GS 9540	CCCAAGCTTCAAATAATTCAAGTGGGTTAG	*Hind* III (s)	1061
GS 9541	CCCAAGCTTCAAATAATTCAAGTGGGTTAG	*Hind* III (s)	660
GS 9543	TCCCCCGGGGTTGATAGACGCATGACAAGCCC ATT.	*Sma* I (as)	
GCS 9545	ACGCGTCGACTATTAAATTCCATAAGGTATGGG CTTTCA	*Sal* I (s)	2174
GCS 9533	ACGCGTCGACCGCGTCGAGACCGGGTACCG	*Sal* I (s)	1048
GCS 9532	ACGCGTCGACTATTGATCCGCTCGTG	*Sal* I (s)	813
GCS 9546	ACGCGTCGACGGGAGAATAGACATAGAGTT	*Sal* I (s)	536
GCS 9547	ACGCGTCGACCCAATTAGGACTACGGTAGG	*Sal* I (s)	277
GCS 9544	CGCGGATCCGAGATTGATGAATTGAGCAATTC CGTG	*Bam*HI (as)	

2.20.1 Microinjection

2.20.1.1 DNA preparation for microinjection

All plasmid DNA used for the microinjection of *C. elegans* was prepared using the endo-free plasmid Midi kit. They were diluted in *C. elegans* injection buffer (2% PEG 6000-8000, 20 mM potassium phosphate, 3 mM potassium citrate, pH 7.5) to a final

concentration of 80 µg/ml. Before loading the microinjection needles, the DNA solution was spin in a micro centrifuge to reduce clogging.

2.20.1.2 Transfection of *C. elegans*

Transformation of *C. elegans* was accomplished by microinjecting the DNA solution from 2.20.1.1 into the distal arm of the gonadal syncytium of young adult hermophrodites (Mello and Fire, 1995). The *C. elegans pha*-I (e2123) mutant strain was used for the germline transformation. The pha-1/pBX system (a gift from R. Schnabel Technical University of Braunchweig, Germany) is based on the thermosensitive embryonic lethal mutation of the *pha*-I gene. The worms grow normally at 15°C, but do not develop at 25°C. Following co-injection with the GFP fusion constructs and the pBX plasmid that carried a wild type copy of the *pha-1* gene, animals were recovered and transferred to fresh culture plates and maintained at 25°C (Granato et al., 1994). The embryo lethality caused by the *pha-1* mutation is complemented in transgenic animals with pBX plasmid. As such transgenic animal can be selected by shifting the F1 larvae of injected hermaphrodites from 15°C to 25°C.

2.20.1.3 Preparation of worms for fluorescence microscopy

Worm cultures from which pictures are to be taken, were suspended in M9 buffer and put on a slide together with an equal volume of 1 M sodium azide After 5-10 min the worms were not longer movable and pictures could be taken under fluorescence microscope (Leica DMLS, FITC-filter) with magnification of 20x or 40x.

2.20.2 Stress assays of transgenic *C. elegans* expressing GFP reporter gene

Transgenic *C. elegans* that express the GFP reporter gene under the control of the *Ce*GS and *Ce*GCS promoter, respectively, were transferred to fresh NGM agar plates and incubated overnight at 25°C, prior to treatment with various oxidative stressors and heavy metals as indicated in **Table 4.1** or with UV radiation and heat

shock at 32°C. Worms were harvested the next day and the GFP expression pattern was examined under the fluorescence microscope.

Table 4.1. Oxidative and heavy metal stressors used against transgenic *C. elegans* that express the GFP reporter gene under the control of the *Ce*GS and *Ce*GCS promoter, respectively.

Substances	**Concentrations**
Cummene hydrogenperoxide	1 mM, 2 mM,
Juglone	0.01 mM, 0.02 mM, 0.03 mM
Paraquat	20 mM, 25 mM
Copper sulphate	100 µM
Cadmium chloride	100 µM
Iron (II) chloride	5 mM, 10 mM
Lead chloride	25 µM, 50 µM
Mercury sulfate	50 µM
Zinc chloride	5 mM,10 mM

2.21 Stress experiment with the GCS inhibitor BSO

To investigate the effect of BSO on the survival of *C. elegans*, axenic worm cultures were supplemented with different BSO concentrations (10-30 mM) for 48 h and stress experiments were carried out both, in axenic liquid culture and on NGM agar plates in the presence of OP50 cells. Untreated control worms and worms pretreated with BSO were stressed with juglone (0.03 mM) and paraquat (25 mM). After 18 h the animals were examined and the survival rates calculated.

2.22 Double stranded RNA (dsRNA) mediated interference (RNAi)

2.22.1 Cloning of the *Ce*GS, *Ce*GCS and *Ce*GR RNA*i*-constructs

Fragment of the *Ce*GS, *Ce*GCS, *Ce*GR genes were PCR amplified from *C. elegans* cDNA using the oligonucleotides shown in **Table 4.2**. The PCR conditions were: 95°C for 2 min, 95°C for 45 sec, 48°C for 45 sec, 72°C for 90 sec, and a final step at

72°C for 8 min. The PCR fragments were cut with the appropriate restriction enzymes and cloned into the L4440 vector. The L4440 constructs were checked by sequencing, before being transformed in *E. coli* HT115 cells.

Table 4.2. Oligonucleotides used to amplify the fragments of *Ce*GS, *Ce*GCS and *Ce*GR for cloning into the L4440 plasmid.

Primer	**Primer (5`…….3`)**	**Restriction site**	**Product (bp)**
GS RNAi(s)	GCTCTAGACTGATTATATGTGTCATAAGG	*Xba* I	399
GS RNAi(as)	CGCTCGAGAGCTTCTATCATTCAATGATAATTG	*Xho* I	
GCS RNAi (s)	CCCAAGCTTGGTCTTTTGACGAAAGGTAGT	*Hin*d III	513
GCS RNAi(as)	CCGCTCGAGATTCTTTCTATCAGCTGCTA	*Xho* I	
GR RNAi(s)	GCTCTAGAATGTCTGGCGTCAAGGAGTTCG	*Xba* I	398
GR RNAi(as)	CCGCTCGAGCCACGATACTTCGCTCCGTTG	*Xho* I	

The RNAi procedure was essentially carried out as described by Timmons et al. (2001). A 2 ml LB culture containing 50 µg/ml tetracycline and 50 µg/ml ampicillin was inoculated with a single colony of host cell plus plasmid and cultured overnight at 37°C. The culture was diluted 100 fold and allowed to grow to OD_{600} = 0.4. The induction of the transcription of the dsRNA was initiated by adding IPTG to a final concentration of 0.4 mM, and the culture was incubated with shaking for 2-4 h at 37°C. The cells were either directly applied onto agar plates or were concentrated by centrifugation and then applied. Unless otherwise noted, plates were composed of standard NGM/agar media supplemented with 50 µg/ml ampicillin and 0.4 mM IPTG. Worms were added to plates individually. RNAi phenotypes were observable within 1-5 days, depending on the target gene. Phenotypes were in the F1 progeny of the animals placed on the plate. Plates contained sufficient quantities of bacteria to support the nematode growth during the course of the experiment, which lasted from

1-5 days. Freshly seeded plates were used in all the experiments. However, seeded plates that were maintained for as long as two weeks at 4℃ also produced RNAi phenotypes. For long maintenance of animals on dsRNA expressing food, worms from successive generation were transferred to fresh plates and the animal were constantly supplied with food.

2.22.2 Stress experiments on RNA*i* treated *C. elegans*

C. elegans worms were treated by the RNAi feeding method introduced in 2.22.1 Bacteria carrying an empty L4440 vector were used as controls. Animals were cultivated at different stress concentrations on agar plates for 18 h at 25℃ with each plate containing 10 selected worms, before the survival rates of the worms were estimated. The mean values were calculated from four independent experiments each with at least three survival assays using worms of different generations.

Table 4.3. Oxidative and metal induced stress substances

Substances	**Concentrations (mM)**
Cumene hydrogenperoxide	2, 4
Juglone	0.01, 0.02, 0.03, 0.05, 0.07, 0.08
Paraquat	20, 25 30
tert-Butyl hydrogenperoxide	2, 5

To assay sensitivity to oxidative stress, young adult RNA*i* treated worms were transferred from NGM plates into small NGM plates (10 worms per plate) that contain the oxidative stress substance at different concentrations (**Table 4.3**) above, the same procedure was applied to wild type worms. Worms were incubated at 20℃ for 18 h prior to analysis. The number of dead animals was counted by continuous absence of pharyngeal pumping and movements after repeated prodding with a pick. Lifespan assays were performed essentially as described by Kampkötter et al. (2003). The survival rate of the RNAi treated worms was then compared to the WT.

The mean values were calculated from four independent experiments each with at least three survival assays using worms of different generations. Juglone at different concentrations was used for the lethality test to compare the significant difference in resistance between the enzymes of the glutathione synthesis (*Ce*GS, *Ce*GCS, *Ce*GR).

CHAPTER 3 RESULTS

3.1 Determination of GSH concentration in *C. elegans*

The total GSH concentration in *C. elegans* was determined spectrophotometrically using a DTNB/GR recycling assay, which couples TNB formation to the actual GSH concentration. The amount of GSH was then determined from the standard curve in which GSH equivalents were plotted against the rate of change of absorbance at 412 nm. The concentration of GSH in *C. elegans* was determined to be 27 ± 1.4 nmol mg^{-1} protein (n = 3).

3.2 Characterisation of *Ce*GCS

3.2.1 Gene organisation of *cegcs*

The *cegcs* gene is composed of seven exons and six introns and is located on chromosome II. The corresponding mRNA includes an ORF of 1941 nucleotides and carries a SL1. The deduced polypeptide of 654 amino acids has a molecular mass of 74 kDa.

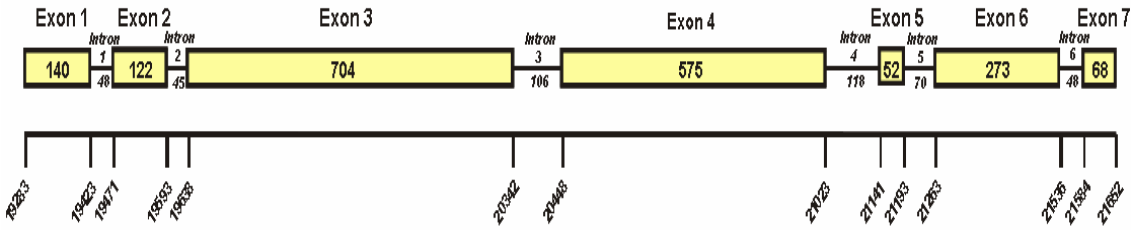


Figure 10. Genomic organisation of *cegcs*.

3.2.2 Alignments of *Ce*GCS with orthologues from other organisms

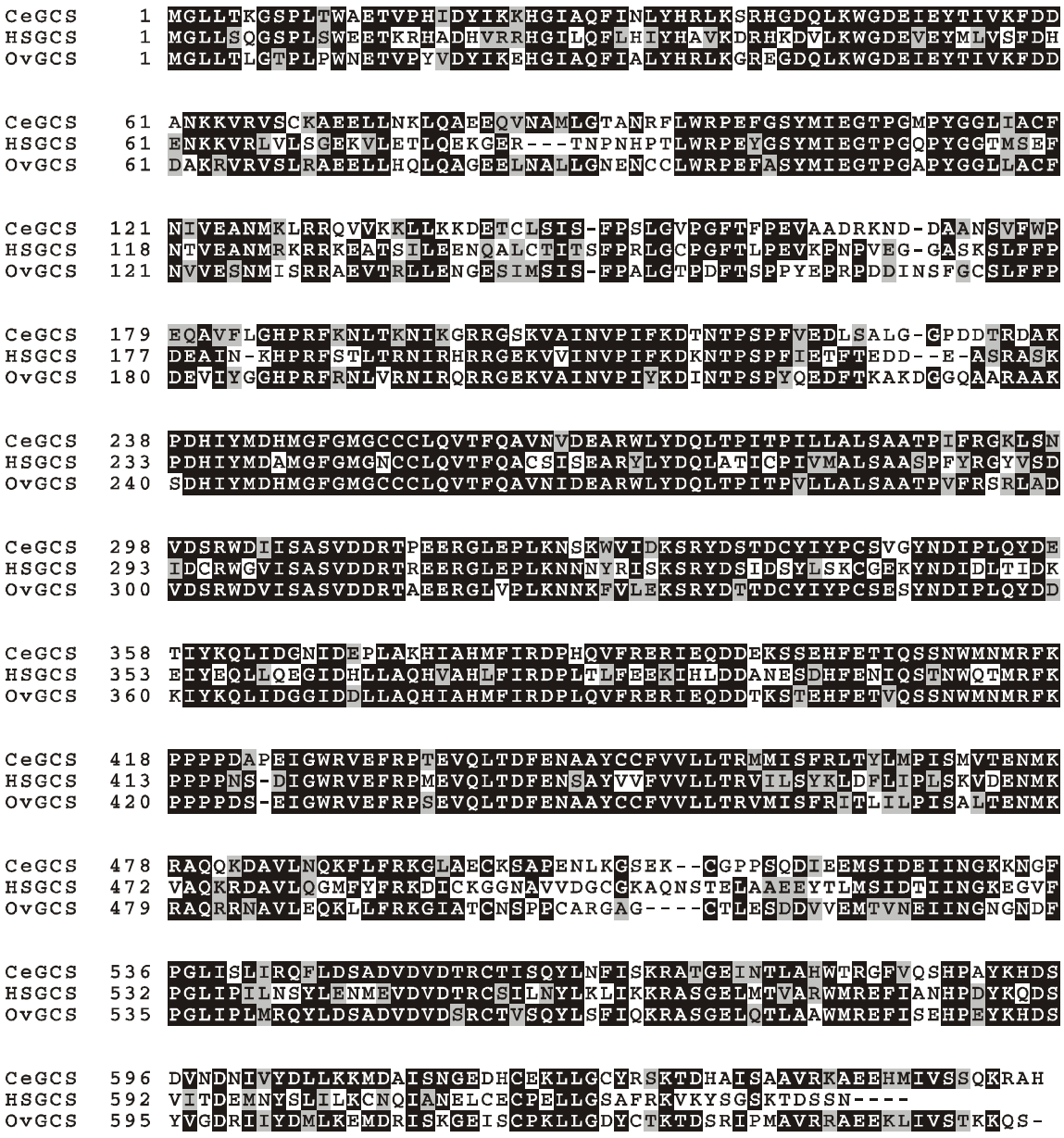


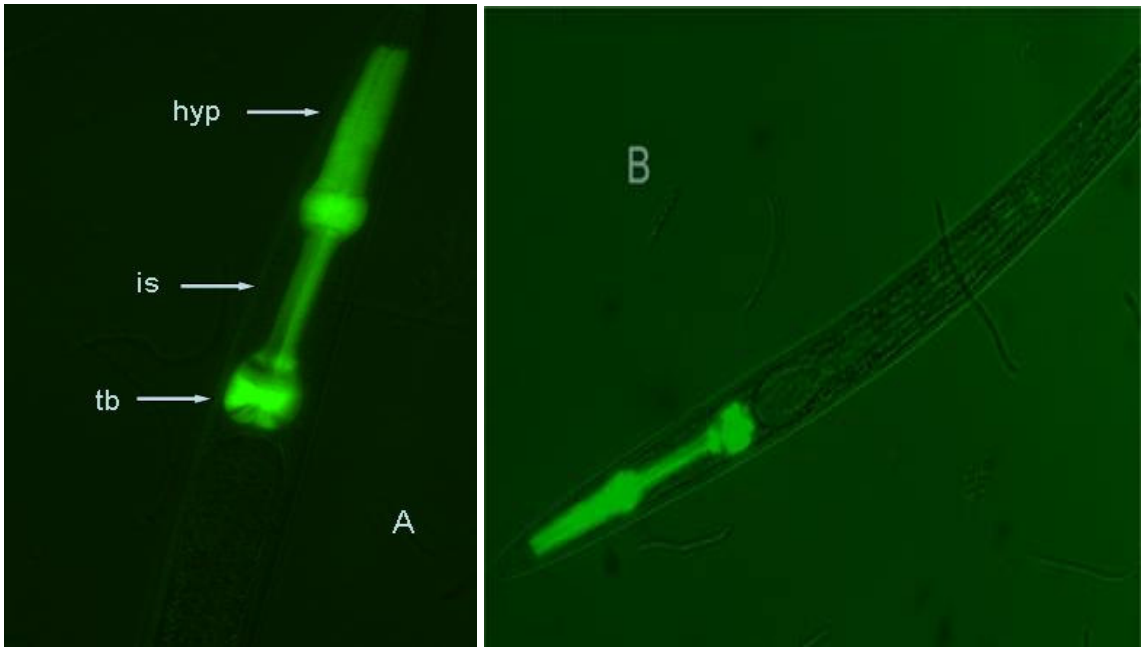
Figure 11. Alignments of *Ce*GCS with orthologues from human and *O. volvulus* GCS. Pair wise analysis using http://searchlauncher.bcm.tmc.edu/ revealed that *Ce*GCS shows 70% homology to the GCS of *O. volvulus* and 54% homology to that of human.

3.2.3 Cloning and recombinant expression of *Ce*GCS

The ORF of *cegcs* was cloned into the expression vectors pJC40 and pMalp2x, respectively. These constructs were transformed into different *E. coli* expression cells (see Material and Methods). Several expression strategies were carried out, including induction by different IPTG concentrations or expression at varying temperatures for different time spans. However, all attempts failed to produce recombinant *Ce*GCS.

3.2.4 Expression pattern of *Ce*GCS in *C. elegans*

The expression pattern of *Ce*GCS in *C. elegans* was analysed by GFP reporter gene expression under the control of the *cecgs* promoter. The *cegcs*::GFP-1 construct that includes 2644 bp of the 3' upstream region, the first exon, the first intron and part of the second exon of the *cegcs* gene (**Figure 12a**) results in a tissue specific expression pattern in the worm. At all larval stages and in adult hermaphrodites, fluorescence was detectable in the pharynx and the AS1 neuron **(Figure 12a).**



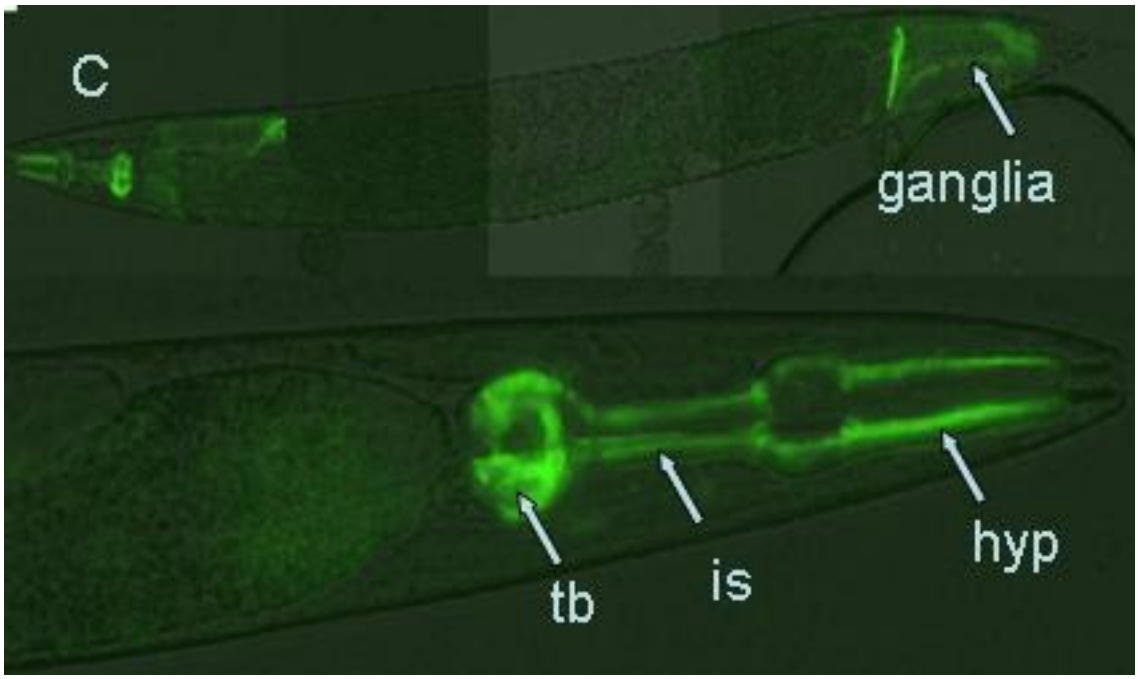
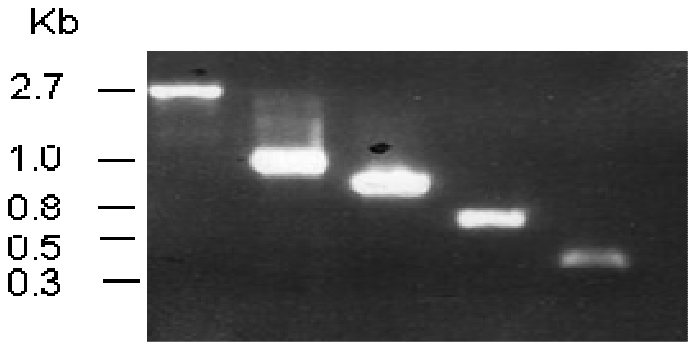


Figure 12a. Expression pattern of GCS::GFP reporter gene construct in the pharynx of *C. elegans.* The patterns of GFP expression in the pharynx of transgenic worms were photographed at magnification of x400 using fluorescence microscopy. (A) L2 stage (B) L3 stage and (C) adult. Strongest staining is observed in the middle portion of the posterior terminal bulbus (tb), isthmus (is) and hypodermis (hyp). Moderate staining is also observed in the ganglia around the anus of the adult.

3.2.5 Determination of the minimal promoter region of *cegcs*

The minimal promoter region of *cegcs* was determined by generating promoter deletion mutants of the *cegcs*::GFP-1 construct (**Figure 12b**) and analysing their GFP expression pattern in transgenic worms. Shortening the 5' upstream region to 207 or 466 bp had no effect on the intensity or the tissue-specific fluorescence pattern. However, 500 bp of the 5' upstream region were found to be not sufficient to promote *cegcs* expression in *C. elegans* (**Figure 12b**), indicating that the minimal promoter region of *cegcs* consists of about 743 bp.

A)



B)

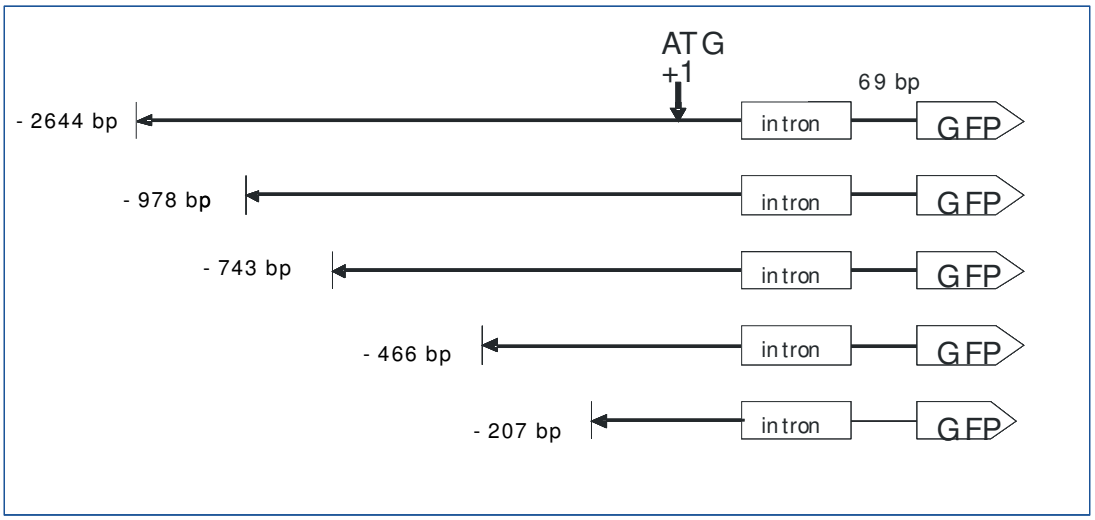
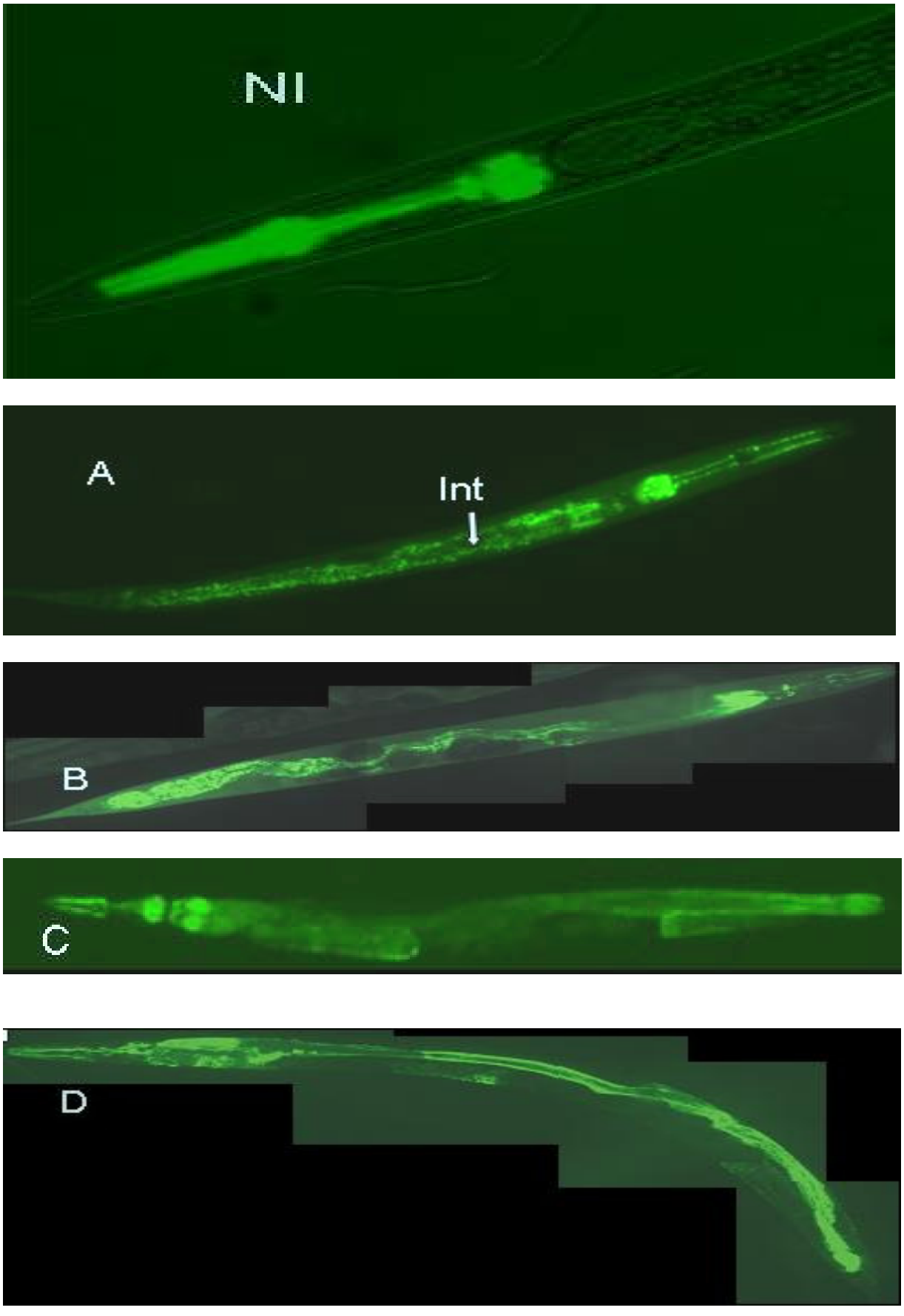


Figure 12b. The minimal promoter region determined by deletion constructs.
A) The agarose gel of the PCR constructs, B) deletion constructs of the promoter starting from the ATG. *gcs*::GFP reporter constructs were constructed from a fragment that contained from 2644 bp, 978 bp, 743 bp, 466 bp, and 207 bp upstream of the initiation ATG along with the sequences encoding the N-terminus *cegcs* sequences were amplified by PCR and cloned into a GFP vector.

3.2.6 Regulation of *Ce*GCS expression

In order to examine, whether the *cegcs* promoter is stress responsive, transgenic worms that carry the *cegcs*::GFP-1 construct were incubated under various stress conditions, followed by analysis of GFP reporter gene expression. All oxidative stressors and heavy metals as well as UV radiation and heat shock treatment led to an enhanced fluorescence in the pharynx and the AS1 neurons. In addition, bright signals, not found under standard culture conditions, appeared in the 20 intestinal cells of *C. elegans*.


NI
A
Int
B
C
D

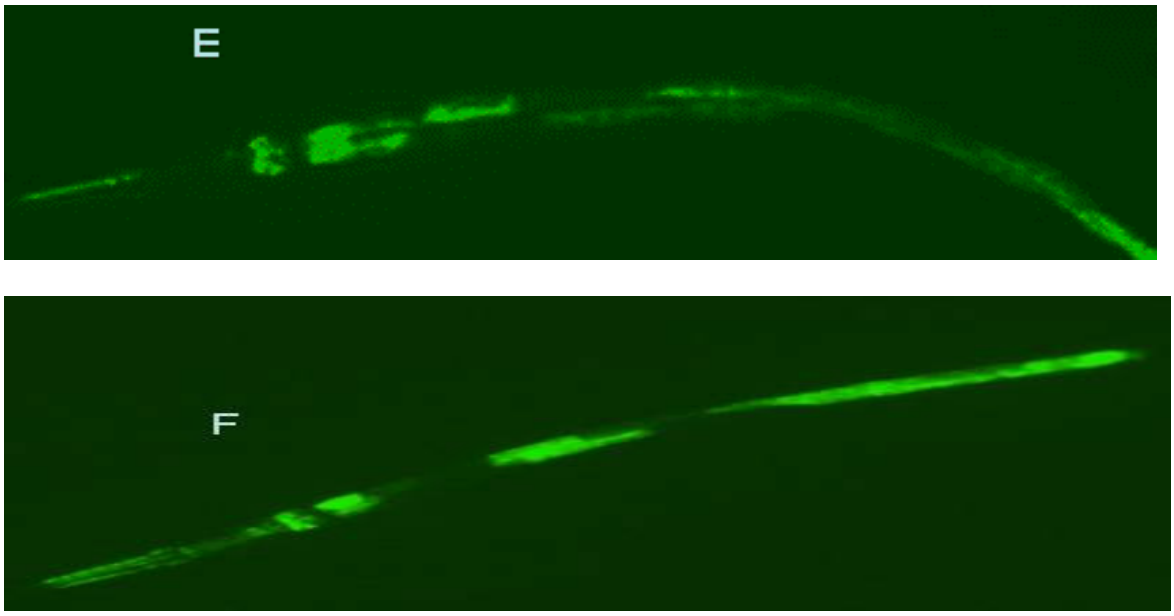


Figure 12c. Induction of *gcs*::GFP expression. (NI) there is no GFP expression at the anterior portion of the pharynx of the untreated animals. Animal were exposed to oxidative stress (AB), heavy metals (CD), heat (E) and UV radiation (F). GFP expression was induced to high levels in the intestine (Int), posterior pharynx and AS1 neurons. Induction of GCS-GFP expression was comparable among different developmental stages of the worm. Over all there is an apparent increase in the pharyngeal and intestinal glowing in the treated animals relative to the untreated worms.

3.2.7 The effect of BSO on stress resistance in *C. elegans*

The role of GSH synthesis of *C. elegans* in stress resistance was tested by using the GCS specific inhibitor BSO. Adult hermaphrodites cultured under axenic conditions were pre-treated with 25 mM BSO for 48 h, before the worms were stressed with 0.05 mM juglone for an additional 18 h and the survival rates were estimated. **Figure 13a** shows that BSO-treated worms with a survival rate of 70% were more sensitive towards the oxidative stressor than control worms with a survival rate of 80%.

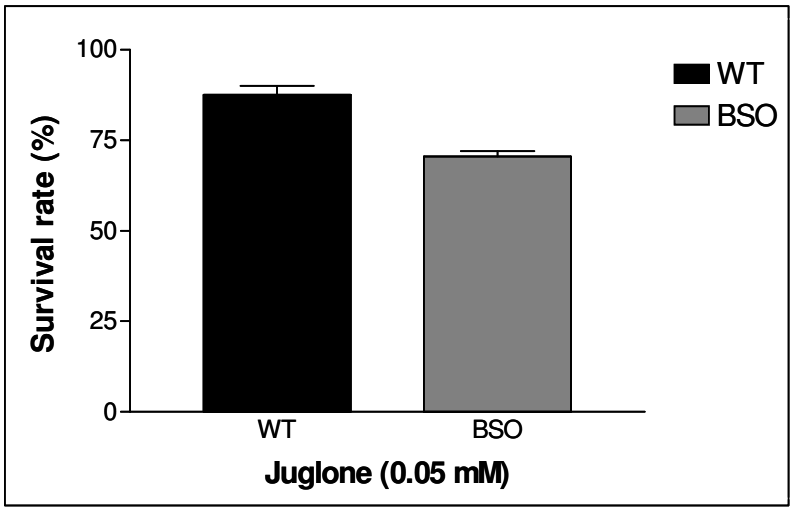


Figure 13a. The effect of Axenic worms were tested on both NGM plates and liquid culture with 0.05 mM juglone. The survival rate was calculated over a period of 24 h. 4 independent experiments were done with total of 40 worms.

3.2.8 Stress assays with CeGCS RNA*i* worms

The first 513 bp of *cegcs* ORF were cloned into the RNA*i* vector L4440. The construct was transformed into *E. coli* HT115 cells and the cells were used in RNA*i* feeding experiments. Under standard culture conditions the knock-down of *Ce*GCS did not result in an obvious phenotype. To investigate the physiological role of *Ce*GCS, RNA*i* treated worms were exposed to the oxidative stressor juglone at various concentrations for 18 h. **Figure 13b** shows that there was a significant difference in the survival rate of the *Ce*GCS RNA*i* compared to the wild type (p-Value <0, 0021).

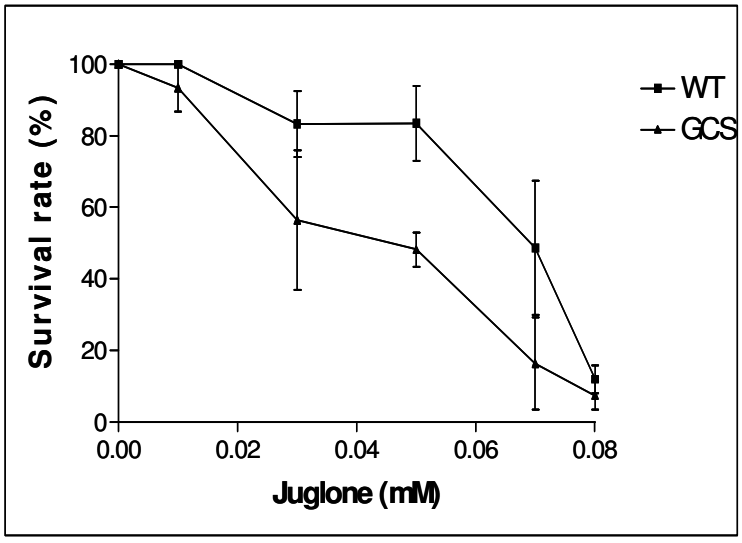


Figure 13b. The effect of oxidative stressors on *Ce*GCS RNA*i* worms. *C. elegans* GCS knock down and control worms were exposed to juglone at various concentrations. The survival rate was estimated after 18 h. The mean values were calculated from 3 independent experiments. Standard error bars are shown. The significance was calculated using Jump test (p-Value <0, 0021

3.2.9 Stress assays with *Ce*GCS RNA*i* subunit (ssu) worms

The last 478 bp of *cegcs* ORF (GCSssu) were cloned into the RNA*i* vector L4440. The construct was transformed into *E. coli* HT115 cells. The knock-down of *Ce*GCSssu did not result in an obvious phenotype under standard culture conditions. In order to investigate the physiological role of *Ce*GCSssu, RNA*i* treated worms were exposed to various oxidative stressors (cumene hydroperoxide and juglone) for 18 h. **Figure 13c** shows that there were not significant differences in the survival rate of the *Ce*GCSssu compared to the wild type.

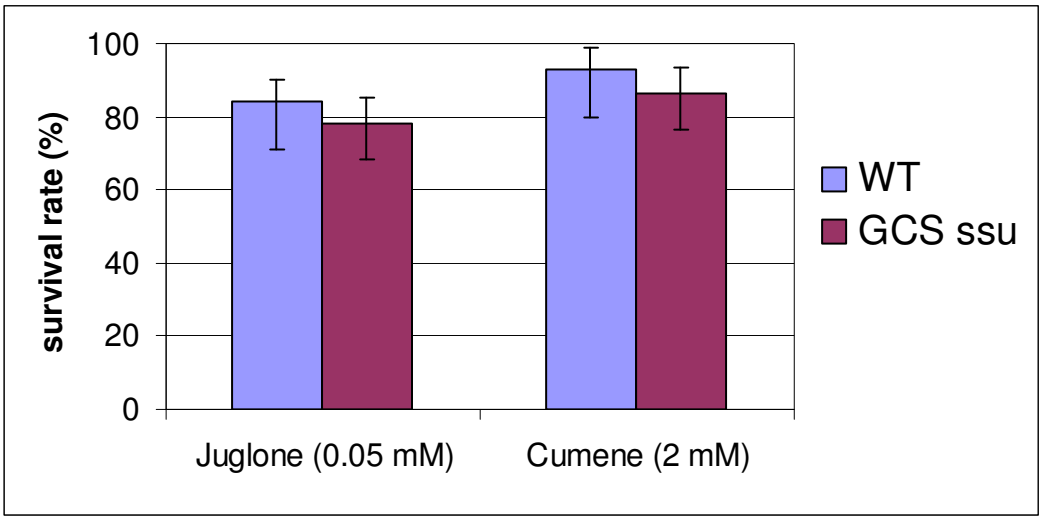


Figure 13c. The effect of oxidative stressors on *Ce*GCSssu RNA*i* worms. *C. elegans* *Ce*GCSssu knock down and control worms were exposed to 2 mM cumene hydroperoxide and 0.05 mM juglone. The survival rate was monitored over a period of 18 h. The mean values were calculated from 3 independent experiments. Standard error bars are shown.

3.3 Characterization of *Ce*GS

3.3.1 Gene Structure of *cegs*

The *cegs* gene is located on chromosome II and consists of 5 exons and 4 introns **(Figure 14**) leading to a transcript with an open reading frame of 1473 bp. The mRNA carries a spliced leader 1 (SL1) and encodes for a deduced polypeptide of 490 amino acids with a molecular weight of 55,000.

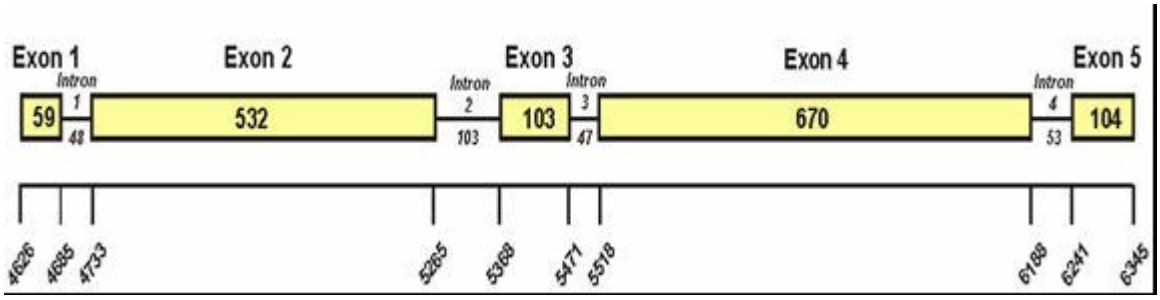


Figure 14 Gene structure of ce*gs*

3.3.2 Analysis of the deduced amino acid sequence of *Ce*GS

The deduced amino acid sequence of *Ce*GS was compared to the GS of other organisms using BCM search launcher (http://searchlauncher.bcm.tmc.edu/) (**Figure 15**). The *Ce*GS is moderately similar to other eukaryotic GS. The sequence identity to the GS of *H. sapiens*, A. *thaliana* and *S. cerevisiae* was 39.4%, 32.9% and 28.4%, respectively.

In comparison to the human GS, the amino acid residues involved in the ATP, Mg^{2+}, GSH, γ-GC and sulphate-binding sites are almost all conserved. However, Asn373 of the ATP binding site and Glu214 of the GSH binding site in human GS are replaced by Leu388 and Asn225 in the *C. elegans* sequence, respectively (**Figure 15**). Furthermore, Cys422 that was shown to play a significant structural or functional role in the human enzyme (Gali et al., 1997) is substituted by an Ala residue in *Ce*GS

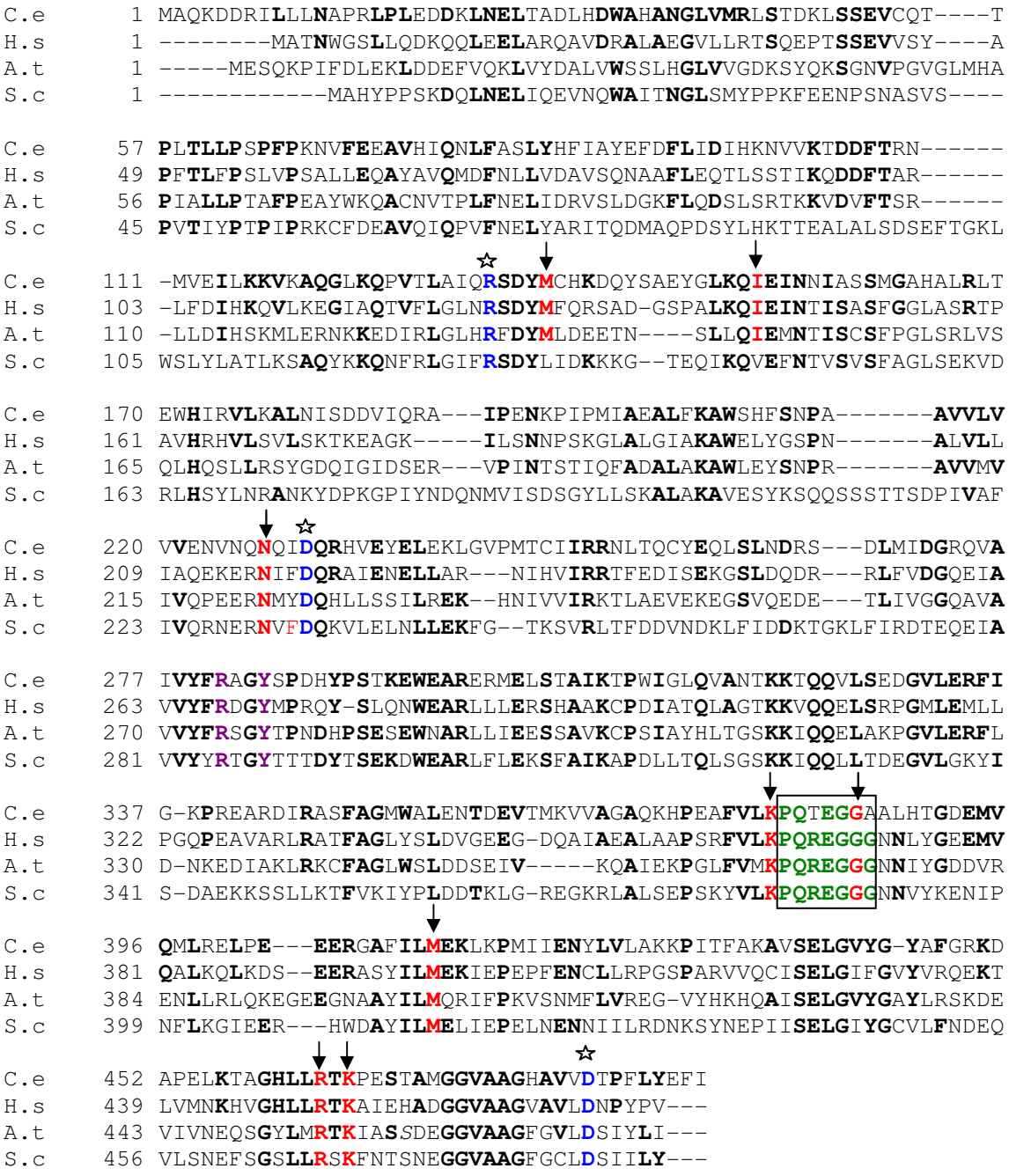


Figure 15 multiple alignment of the deduced amino acid sequence of CeGS with other known eukaryotic GS sequences. The deduced amino acid sequence shows a moderate degree of identity with amino acids sequences of human, *Arabidopsis thaliana* and the yeast *S. cerevisiae*. The boxed region with green letters indicates the conserved glycine-rich loop that is discussed to be a flexible cover of the active site of GS (Wang et al., 1997a, b). Blue letters marked by stars, red letters marked by arrows and violet letters indicate amino acid residues responsible for γGC-, ATP- and GSH-binding, respectively (Meierjohann et al., 2002).

.

3.3.3 Recombinant expression of *Ce*GS

The *Ce*GS was recombinantly expressed as a His-tag fusion protein in *E. coli* BL21 (DE3) cells and purified by nickel chelating chromatography. SDS/PAGE analysis revealed that the His_6-tagged *Ce*GS was purified to apparent homogeneity (**Figure 16**). In accordance with the deduced molecular mass, the protein runs at approximately 57 kDa.

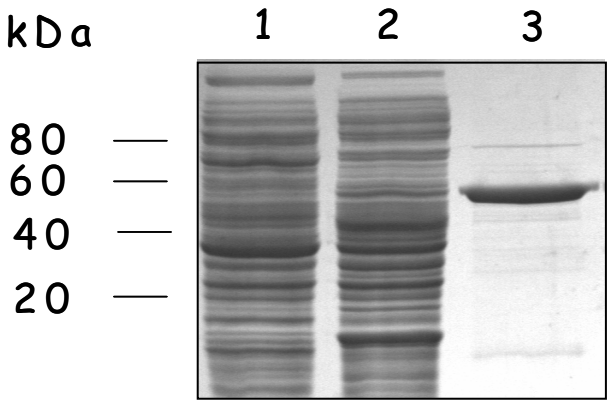


Figure 16. SDS&PAGE of recombinantly expressed GS. Cell extract of *E. coli* BL21 (DE3) cells carrying GS-pJC40 without (Lane 1) and after IPTG induction (Lane 2) and subsequent Ni^{2+} chelating purification (Lane 3). The recombinant *Ce*GS was separated by 7.5% PAGE and visualized with Coomassie brilliant blue. The apparent molecular mass of the His-tagged monomeric protein was approximately 57 kDa. Protein standards are shown on the left in kDa.

The *Ce*GS isolated by affinity chromatography was applied to size exclusive FPLC (SE-FPLC), where the recombinant protein elutes in a single peak corresponding to a molecular mass of about 110 kDa (**Figure. 17**), indicating a dimeric structure under native conditions.

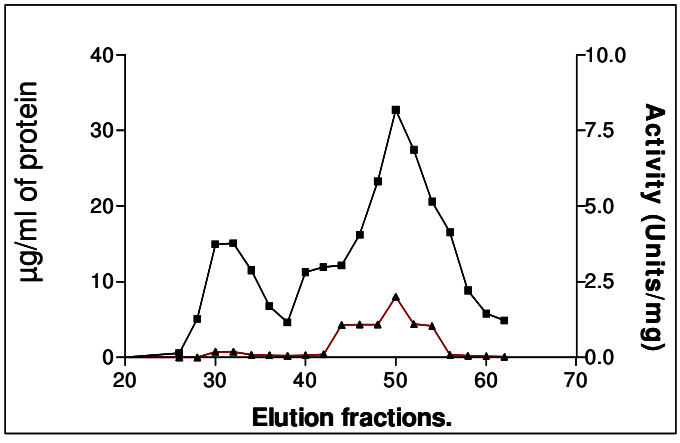


Figure 17. Elution and activity profile of *Ce*GS. Gel filtration was on a Sephadex S-200 26/60 column. The protein concentration is shown on the left (■) and the specific activity of each fration is shown the right (▲).

3.3.4 Kinetic analyses of recombinant *Ce*GS

The recombinant *Ce*GS enzyme was found to be very unstable. Activity was lost upon freezing and declined to about 50 % after two days at 4°C. Supplementing the buffer with 1 mM DTT and 1 mM EDTA did not significantly affect enzyme stability. The enzymatic assay for *Ce*GS was carried out at an optimium pH of 7.0 (**Figure 18**). The specific activity determined under V_{max} conditions was determined to be 1.86 ± 0.30 µmol min^{-1} mg^{-1} protein (n = 3). Using Michealis-Menten and double reciprocal Lineweaver Burk plots, the apparent K_m values for ATP, glycine, and γ-GluAbu were calculated to be 250 ± 23 µM (n = 3), 2.04 ± 0.78 mM (n = 3) and 196 ± 8 µM (n = 3), respectively (**Figure 19**).

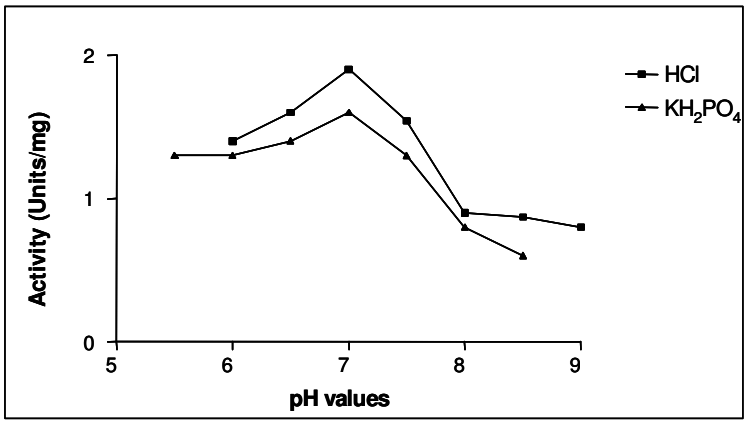


Figure 18. pH optimum determination of *Ce*GS, using two buffer systems.
Tris HCl and KH_2PO_4 .

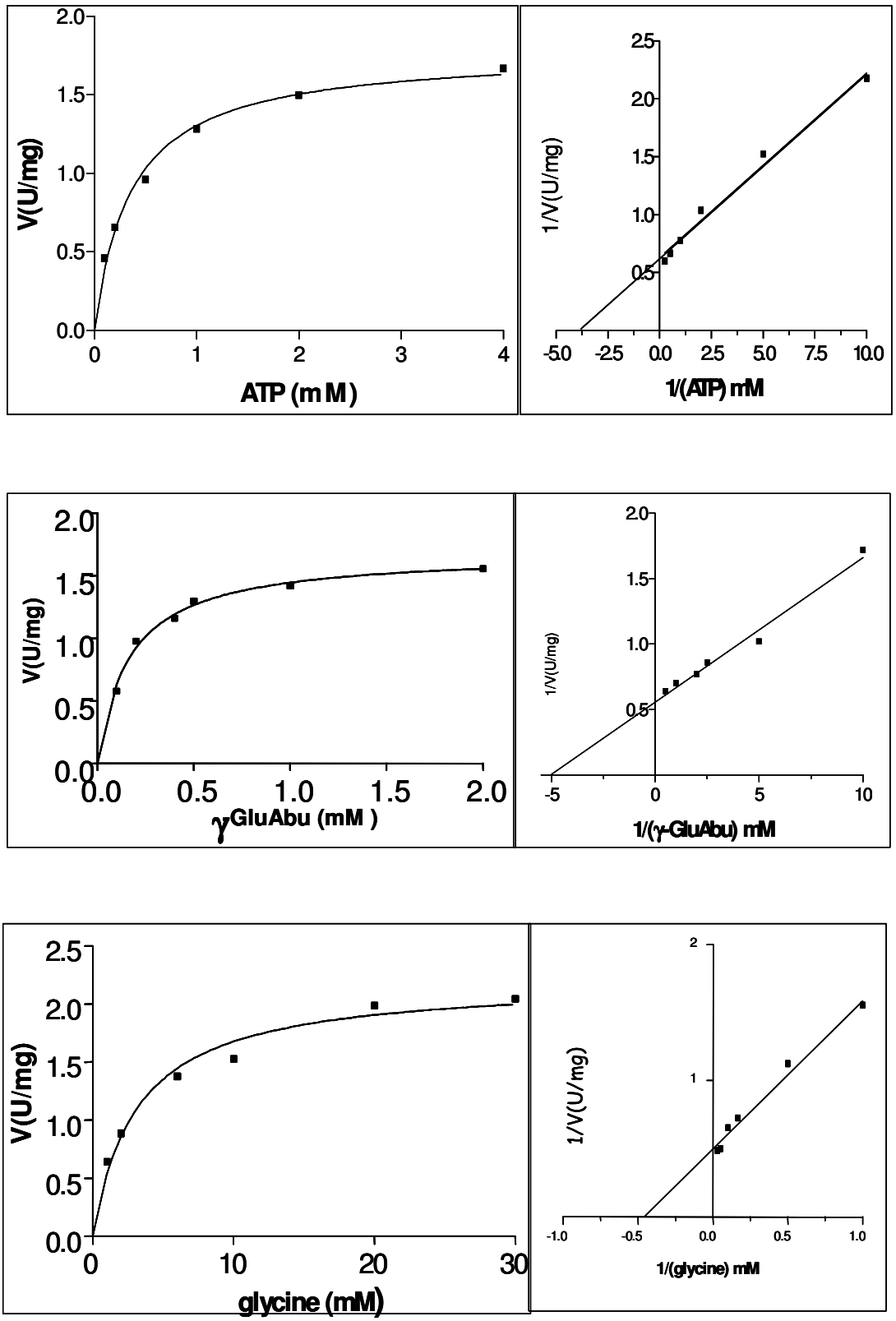


Figure 19. Michaelis-Menten and Lineweaver Burk double reciprocal plots. The following substrates were tested a) ATP, b) γ-GluAbu and c) glycine. The enzyme kinetics were carried out by varying one substrate concentration in the presence of the saturating condition of the other two substrates.

3.3.5 Expression pattern of *Ce*GS-GFP fusion constructs

To determine the expression pattern in *C. elegans*, 2930 bp of the potential promoter region, the first exon, the first intron and part of exon II of the *cegs* were cloned in fusion with GFP reporter gene of the pPD95.77 vector leading to the *cegs*::GFP-1 construct. *C. elegans pha*-1 mutants were microinjected with this construct together with the selection marker pBX. Transgenic animals achieved by thermo-selection at 25°C were analysed by fluorescence microscopy. The pattern of the GFP signal indicates that *Ce*GS is moderately expressed mainly in the posterior and anterior intestine, the AS1 neurones and part of the pharynx of larvae, adults and embryos (**Figure 20a**). To determine the minimal promoter region of *cegs*, promoter deletion mutants consisting of 2930 bp, 1309 bp, 955 bp and 555 bp upstream of the initiation ATG were generated (**Figure 20b**) and introduced into *C. elegans*. The GFP expression of these constructs was analysed in the respective transgenic worms. Fluorescence pattern were not altered with 1309 bp and 955 bp constructs, however there was no GFP expression with the 555 bp construct (**Figure 20b**). Hence, the minimal promoter region of *cegs* - i.e. the shortest region of DNA upstream from the transcriptional start site that mediates cell-specific transcription in *C. elegans* - is suggested to be about 955 bp.

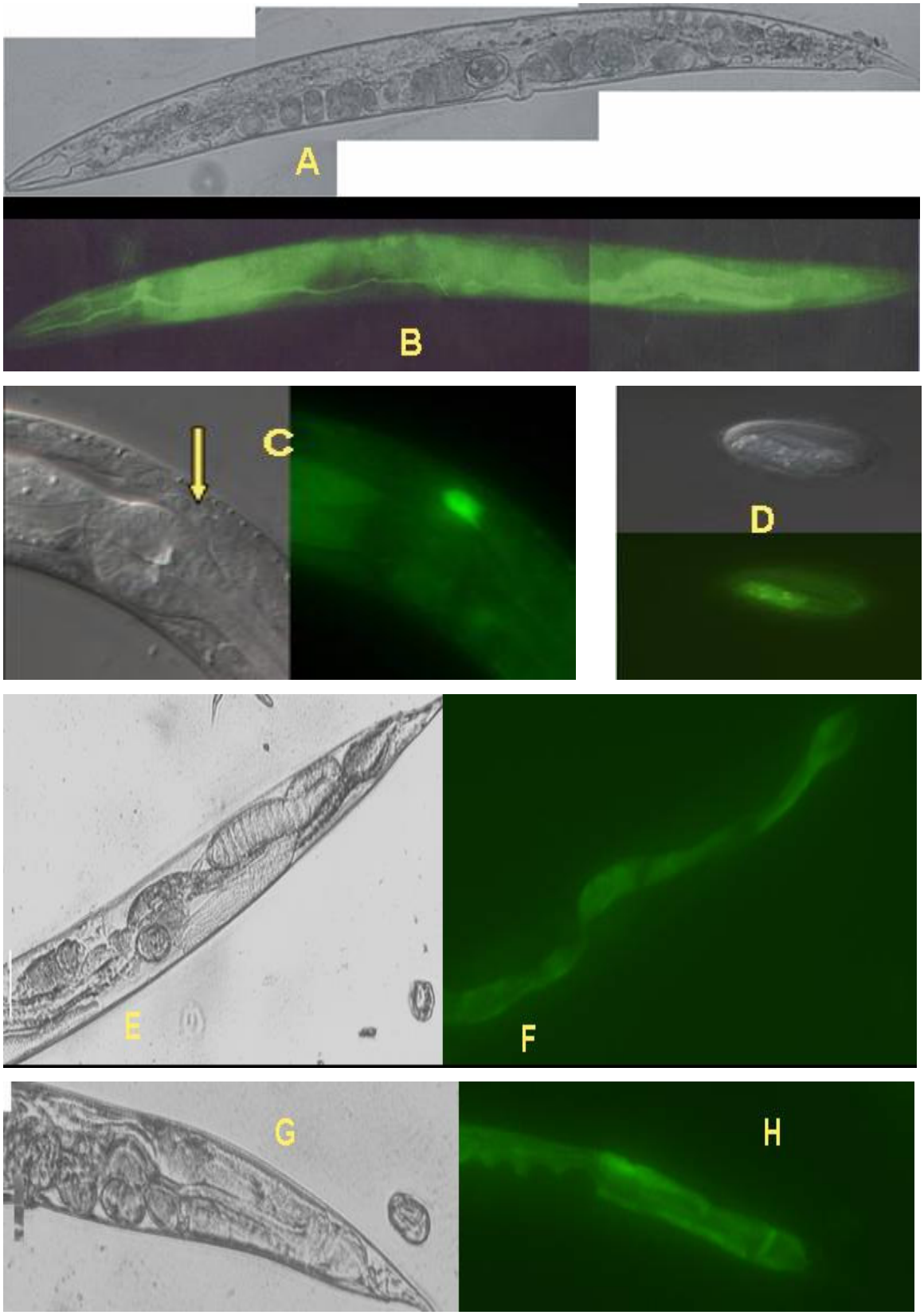
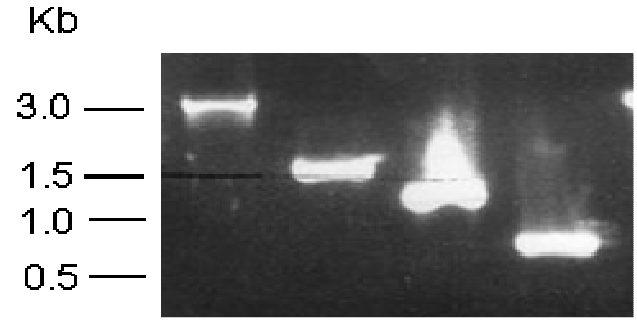


Figure 20a. The expression pattern of *Cegs*::GFP-1 fusion protein in transgenic C. elegans. GFP expression is present in part of the pharynx and intestine (**A,B**), the AS1 neuron indicated by an arrow (**C,D)**,) the intestine of a L1 larvae prior to hatching (**E,F)** and the posterior region of the intestine of an adult hermaphrodite worm (**G,H**).

i).



ii)

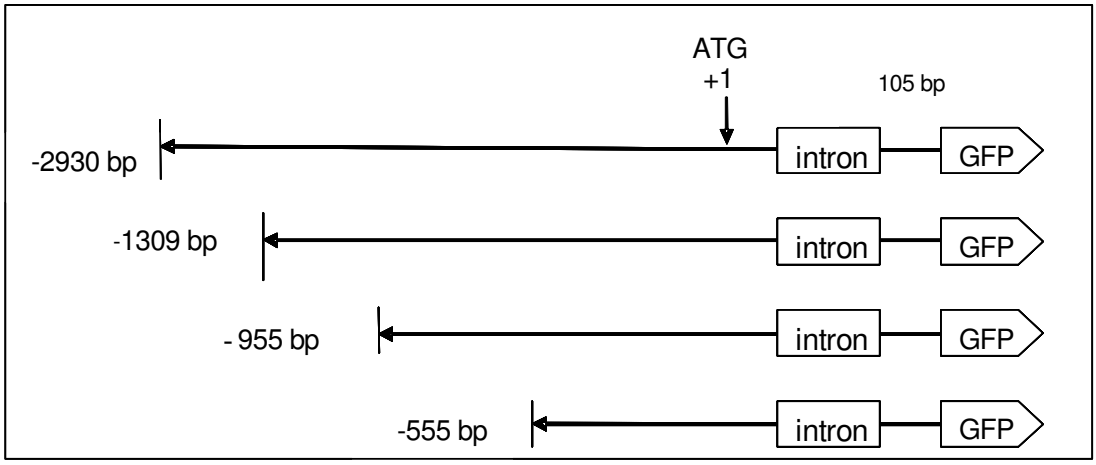


Figure 20b. Determination of the *cegs* minimal promoter region. (i) Agarose gel of the *cegs* promoter PCR fragments that were cloned into pPD95.77 to generate promoter deletion constructs used for the determination of the minimal promoter of the *Ce*GS.
(ii) Schematic organisation of the deletion constructs used to transfect *C. elegans*.

3.3.6 Regulation of the *Ce*GS promoter

In order to determine whether the *cegs* promoter is inducible by oxidative or heavy metal stress, transgenic *C. elegans* carrying the *cegs*::GFP-1 construct were transferred onto NGM plates supplemented with heavy metals (cadmium, copper, zinc, iron) or oxidative stressors (cumene hydrogenperoxide, *tert*-butyl hydroperoxide, juglone, paraquat) at different concentrations (see Material and Methods) for 18 h, before the fluorescence pattern were analyzed. There was no noticeable difference of the GFP expression pattern or fluorescence signal intensity in animals cultured under either normal or stress conditions (**Figure 20c)**. This implies that the *cegs* promoter is not inducible by these stressors.

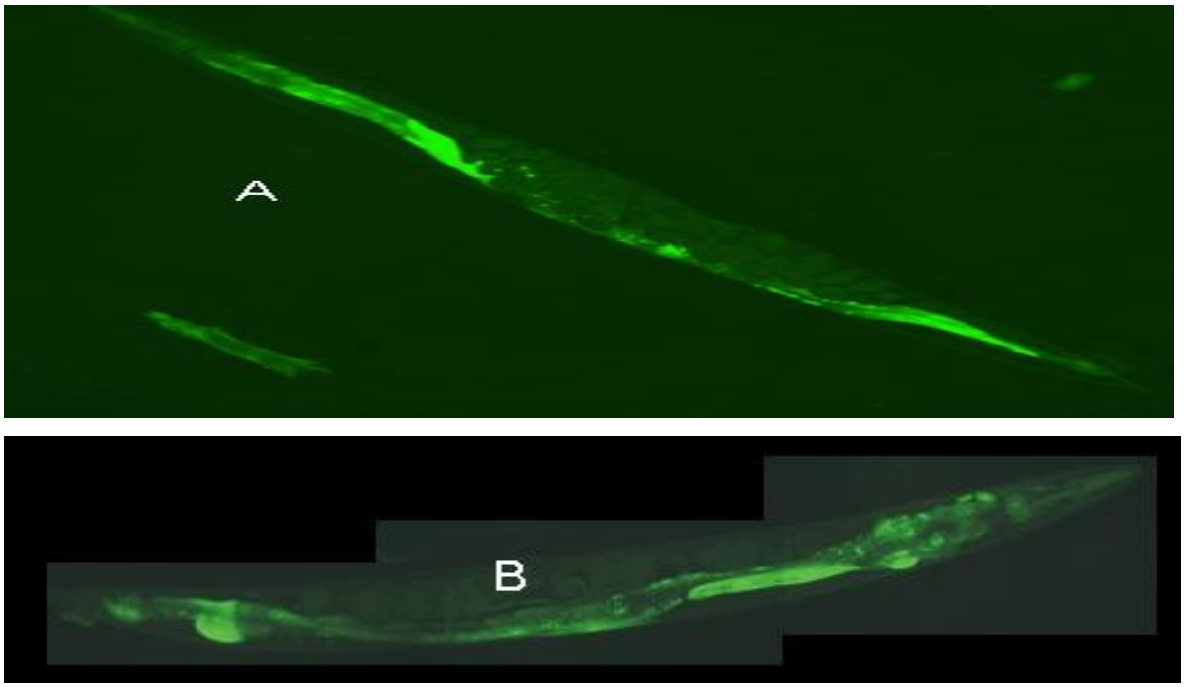


Figure 20c. Exemplary analysis of stress-induced regulation of the *Cegs* promoter. (A) Transgenic *C. elegans* expressing the GFP reporter gene under the control of the *cegs* promoter including 1309 bp of the 5' upstream region were exposed to 2 mM cumene for 18 h. (B) GFP expression pattern of control worm maintained under standard culture conditions.

3.3.7 RNA*i* experiments

The first 399 bp of the *cegs* ORF were cloned into the L4440 vector and transformed into HT115 cells. These bacteria produce dsRNA corresponding to CeGS and were used in RNA*i* feeding experiments. There was no obvious change in phenotype observed after the RNA*i* treatment under standard culture conditions. Furthermore, *Ce*GS-RNA*i* treated worms were used in stress assays. The resistance against oxidative stress of *C. elegans* was not affected by the knock down of the *Ce*GS (**Figure 21).** After exposure to different oxidative stressors for 18 h, the survival rates were not significantly altered, when compared with worms fed with bacteria carrying an empty L4440 vector.

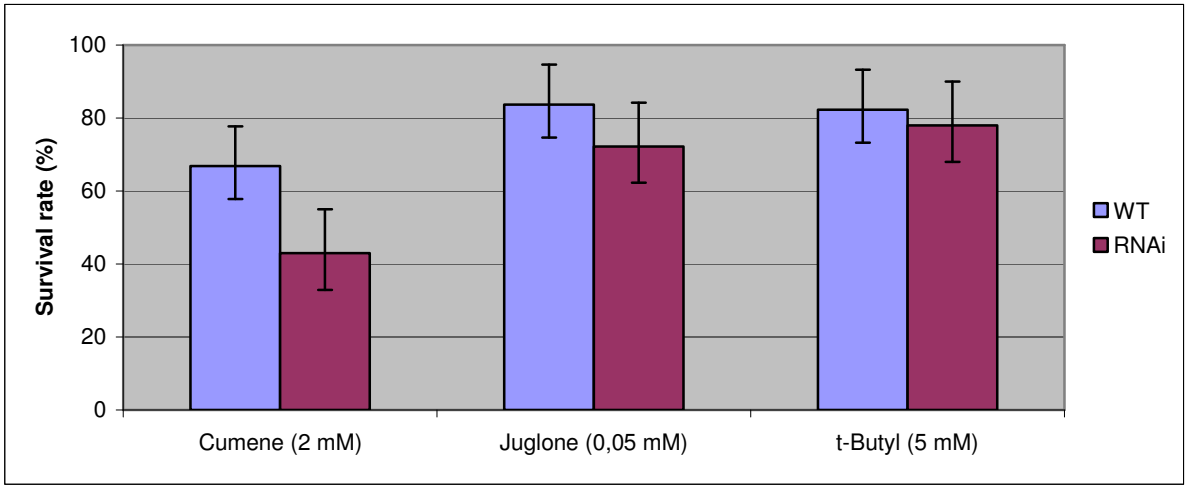


Figure 21. Effect of cumene hydrogenperoxide, juglone and tert-butyl hydrogenperoxide on *Ce*GS RNA*i*-treated *C. elegans* worms. Adult hermaphrodites (F1) pre-treated by RNA*i* feeding were incubated in the presence of the respective drug for 18 h. Results representing the percentage of survival are the mean of three independent experiments.

3.4 Characterization of *Ce*GR

3.4.1 *Cegr* gene and transcript structure

The *cegr* gene is composed of 4 exons interrupted by 3 introns of 55 bp, 50 bp and 56 bp and is located on chromosome III. The mRNA that carries a SL1 contains an ORF consisting of 1380 nucleotides and encodes a polypeptide of 459 amino acids with a deduced molecular mass of 49.8 kDa.

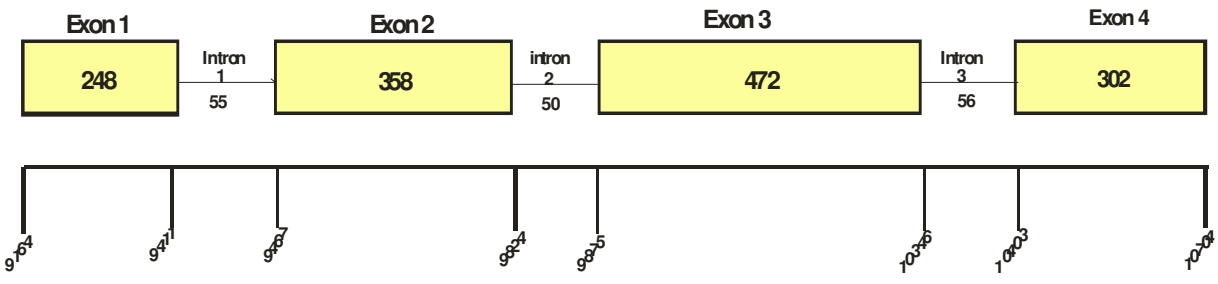


Figure 22. Gene structure of *cegr*.

3.4.2 Amino acid sequence comparison of *Ce*GR with GR from other organisms

The deduced amino acid sequence of *Ce*GR was compared with GR from other organisms using BCM search launcher. Using pairwise sequence analyses, there is a 58.3% and 65% identity with human and the *Onchocerca volvulus* GR, respectively.

Figure 23. Alignment of the deduced amino acid sequence of *Ce*GR with the *O. volvulus* (*Ov*GR) and human enzyme (*Hs*GR). Sequences with specific functional roles are in pink letters. Amino acid residues involved in reductase activity are indicated in boxes. The residues forming the FAD binding domain (62-202 in *Hs*GR; 4-142 in *Ce*GR and 7-145 in *Ov*GR), the NADPH-binding domain (203-337 in *Hs*GR; 143-268 in *Ce*GR; and 146-269 in *Ov*GR), the central domain (338-408 in *Hs*GR; 269-339 in *Ce*GR; and 270-340 in *Ov*GR) and the subunit interface domain (409-519 in *Hs*GR; 340-450 in *Ce*GR; and 341-451 in *Ov*GR) are shown. Arrows indicate the start sites of functional domains.

The residues involved in GSSG binding (Aboagye-Kwarteng et al., 1992) are almost all conserved in *Ce*GR. (**Table 5**) The only difference involves in conservation is the substitution of Pro512 in *Hs*GR with Thr444 in *Ce*GR and Asn161 in *Hs*GR with Gly103 in *Ce*GR. The C- terminal parts of the protein are almost all conserved with that of HsGR and OvGR (residue 415-459 in CeGR, 416-460 in OvGR and 484-520 in HsGR). The nucleotide binding for FAD and NADPH as well as specific binding motif for FAD are well conserved (**Figure 23**).

Table 5 Residues involved in binding the substrate GSSG.

Glutathione group	Residue in *Hs*GR	Residue in *Ov*GR	Residue in *Ce*GR
	T-383	T-315	T-314
	I-387	I-319	I-318
Glu I	R-391	R-323	R-322
	H- 510	H-443	H-442
	T-520	T-452	T-451
	S-74	S-19	S-16
	V-103	V-48	V-45
Cys I	V-108	V-53	V-50
	Y-158	Y-103	Y-100
	H-510	H-443	H-442
	A-78	A-23	A-20
Glu I	R-81	R-26	R-23
	Y-158	Y-103	Y-100
	M-450	**L-382**	M-381
	H-510	H-443	H-442
	P-511	P-444	P-443
Glu II	P-512	**T-445**	**T-444**
	E-515	E-448	E-447
	E-516	E-449	E-448
	L-154	L-99	L-96
Cys II	Y-158	Y-103	Y-100
	H-510	H-443	H-442
	I-157	I-102	I-99
Gly II	Y-158	Y-103	Y-100
	N-161	N-106	**G-103**

Residues that are replaced within *Ov*GR or *Ce*GR with respect to *Hs*GR are indicated in bold

3.4.3 Recombinant expression and characterization of *Ce*GR

The 1380 bp ORF of *CeGR* was amplified by PCR from *C. elegans* cDNA with sequence-specific oligonucleotides and cloned into the expression vector pJC40. The resulting *Ce*GR:pJC40 plasmid was transformed into *E. coli* BL21 (DE3) cells, which

were used to express the recombinant protein. The soluble fraction of the protein was obtained after centrifugation of the sonified resuspended bacteria at 100 000 x g and was purified using Ni^{2+} chelating chromatography. The yield of purified *Ce*GR was 400-600 µg protein per litre of bacteria culture. The *Ce*GR protein was purified to apparent homogeneity as determined by SDS/PAGE analysis (**Figure 24a**) with a subunit molecular mass of approximately 52 kDa. Gelfiltration on a calibrated Sephadex S-200 revealed that the His-tagged fusion protein forms a homodimer of about 100 kDa under native conditions (**Figure. 24b**). Enzyme activity of the *Ce*GR was determined at 25°C using a spectrophotometric assay which coupled NADP production to NADPH oxidation.

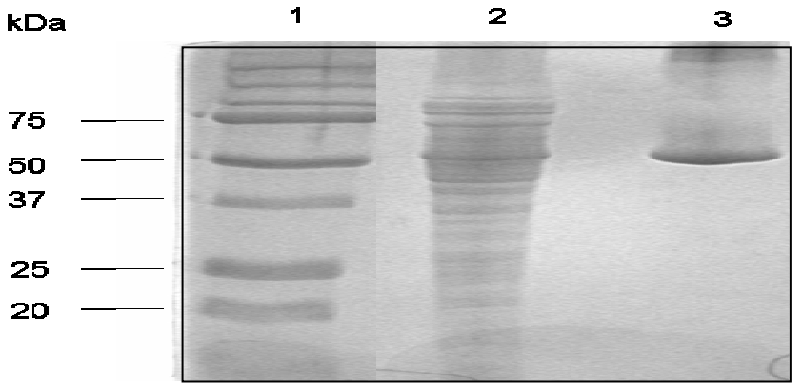


Figure 24a. SDS/PAGE analysis of recombinantly expressed *Ce*GR.
Lane 1, Molecular mass marker; lane 2, *E. coli* BL21 (DE3) lysate containing pJC40-*Ce*GR after IPTG induction; lane 3, purified recombinant *Ce*GR after Ni^{2+} chelating chromatography. Protein was separated by SDS/PAGE on a 7.5% polyacrylamide separating gel and stained with Coomassie Blue.

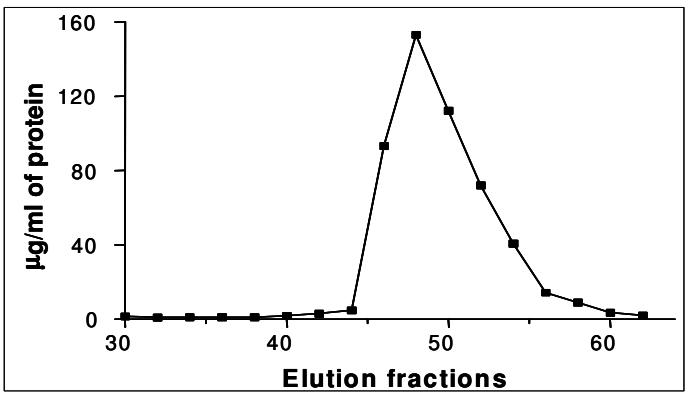


Figure 24b. Elution profile of recombinant *Ce*GR protein on a calibrated Sephadex S-200 FPLC column. The protein elutes in a fraction corresponding to a molecular mass of about 100 kDa.

3.4.3.1 Western blot

Western blot analysis using an anti-His antibody demonstrated successful expression as shown in **Figure 25**. The antiserum recognized the recombinant *C. elegans* GR fusion protein that carries the N-terminal His_6-tag (2.2 kDa).

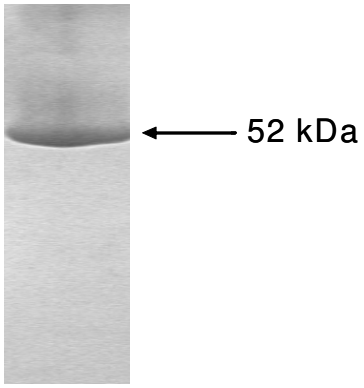


Figure 25. Western blot analysis of *C. elegans* GR. *Ce*GR that carries the His6-tag of 2.2 kDa was subjected to Western blot analysis. The blot was probed with polyclonal anti-HisG-alkaline phosphatase conjugated antibody 1:5000 against the recombinant fusion protein.

3.4.3.2 Determination of the optimum pH value of *Ce*GR

In order to determine the optimum pH for the *Ce*GR reaction, Tris-HCl and potassium phosphate buffer systems were used. **Figure 26** shows that the optimum pH was 7.0 for both buffer systems but the highest activity was observed with phosphate buffer.

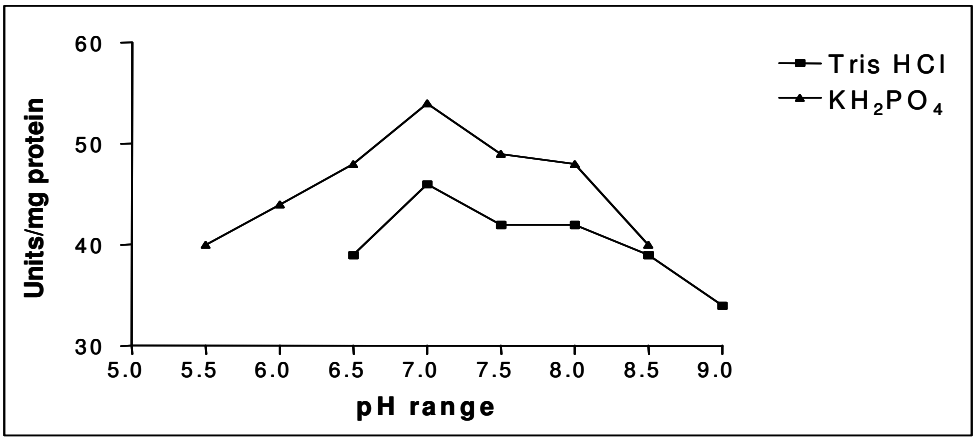


Figure 26. Optimum pH of the *Ce*GR activity. The enzyme activity was determined at different pH ranges (5.5-8.5) with the KH_2PO_4 buffer and Tris-HCl buffer (6.5-9.0), respectively.

3.4.3.3 Kinetic analyses

The specific activity of the *Ce*GR was determined to be 68 ± 3 µmol min^{-1} mg^{-1} protein (n=4). The apparent K_m values of NADPH and GSSG were calculated to be 12.9 ± 1.2 µM (n=4) and 34.1 ± 4.0 µM (n=4), respectively (**Figure 27**).

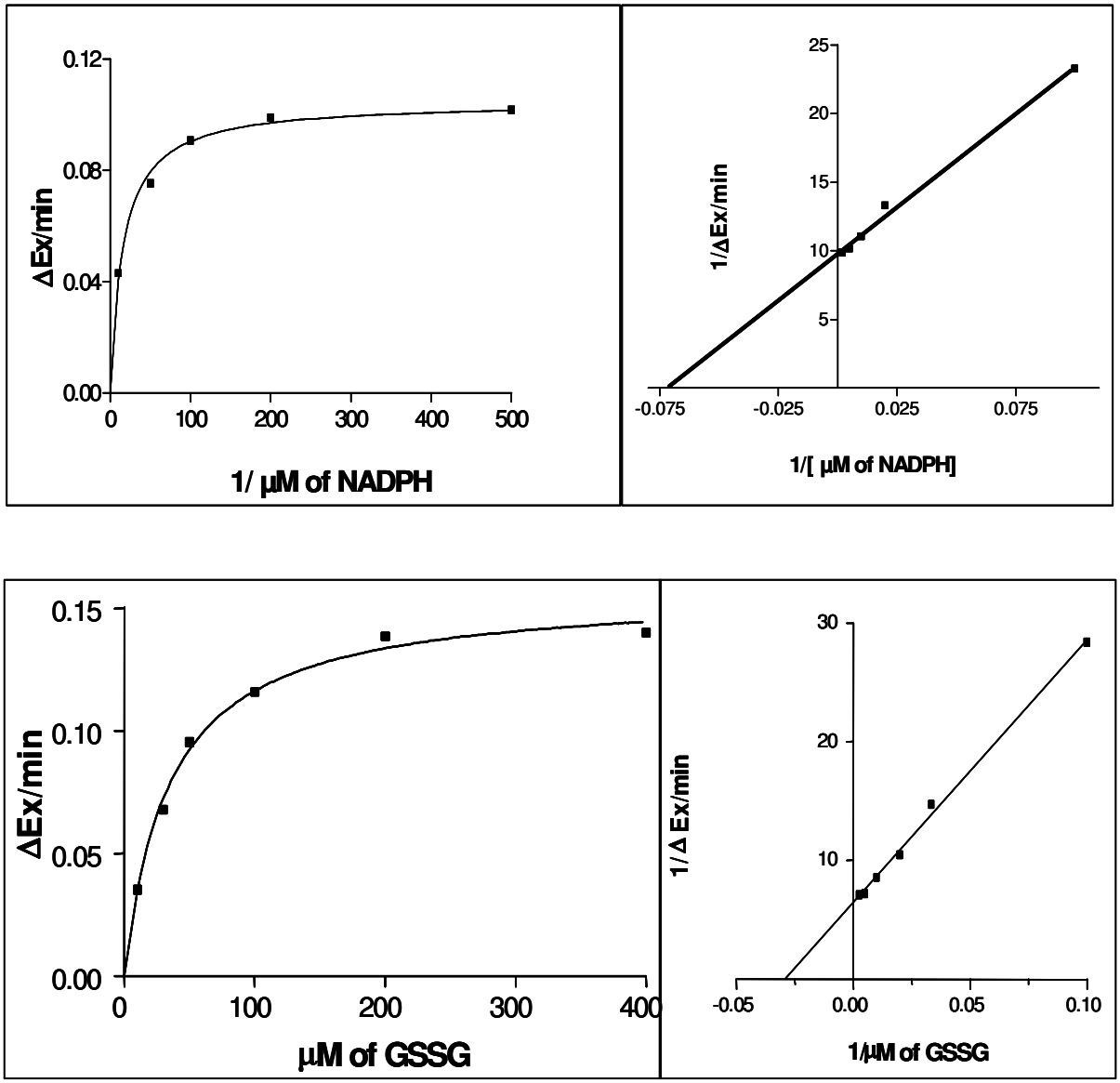


Figure 27. Michaelis-Menten and Lineweaver Burk double reciprocal plots for NADPH and GSSG. The enzyme kinetics were carried out by varying one substrate concentration in the presence of the saturating condition of the other substrates.

3.4.5 Stress assays with *Ce*GR knock down worms

Double stranded RNA corresponding to the first 399 bp of the *Cegr* ORF was produced in HT115 *E. coli* cells. *C. elegans* wildtype hermaphrodites were fed with these bacteria, before RNA*i*-treated adult F1 animals were exposed to the oxidative stressors cumene hydroperoxide, tert-butylhydroperoxide, paraquat and juglone as well as to heat shock at 32°C. The survival rate was monitored over 5 h or estimated after 18 h, respectively (**Figure 28a**/**b**/**c**).

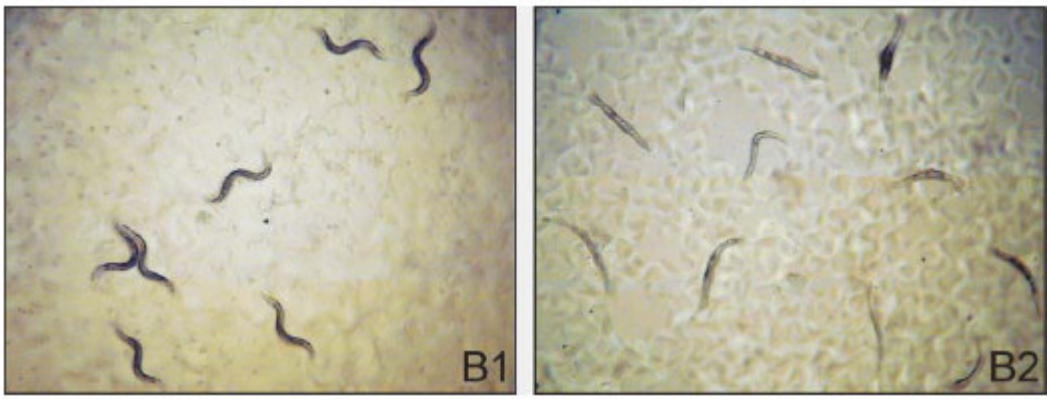
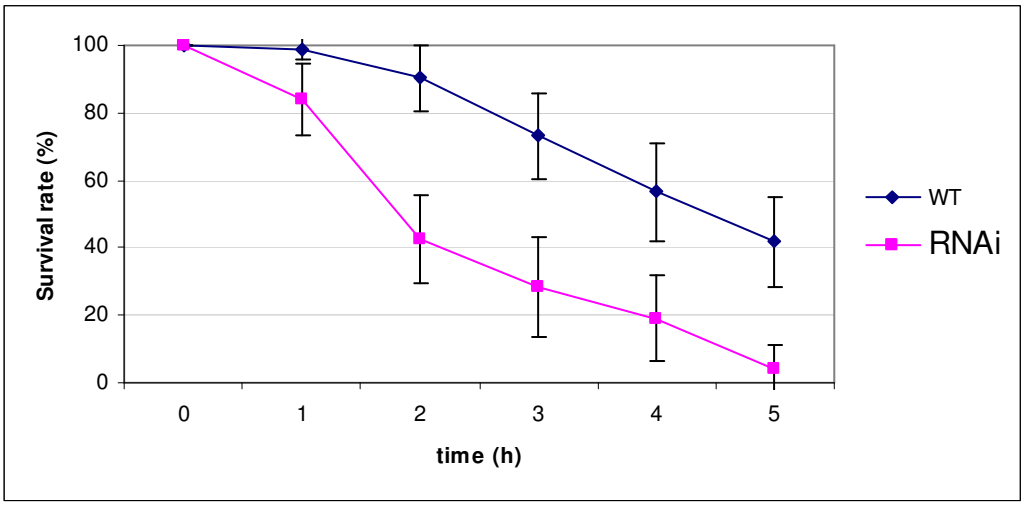


Figure 28a. *Ce*GR RNAi worms on NGM plates under stress conditions.
*Ce*GR RNAi treated worms were very sensitive to oxidative stressors compared to the control worms (B1), the worms were incubated with different oxidative stressors for 18 hours in NGM plates.

In general, *Ce*GR RNA*i* worms were more sensitive to oxidative stress and heat shock than control worms that were fed with bacteria carrying an empty L4440 vector **(Figure 28a**). Exposure to 4 mM cumene hydroperoxide led to a time dependent decrease in the survival rate of both stressed worm populations. However, more than 90% of the control worms were still alive after 2 h incubation, whereas 60% of the *Ce*GR knock down worms were dead ($P<0.0001$). Almost all *Ce*GR RNA*i*-treated worms died within 5 h. At that time point the survival rate of the control worms was 40%. Similarly, the knock down of *Ce*GR resulted in reduced survival rates of 49.3% with tert-butylhydroperoxide ($P<0.002$), 25.6% with juglone ($P<0.001$), 42.2% with paraquat ($P<0.001$) and 62.5% with heat shock at 32°C ($P=0.004$), after 18 h incubation period. (**Figure 28b**). There was no difference in morphology, lifespan, pharynx pumping and in movements as compared to control worms.

b)



c)

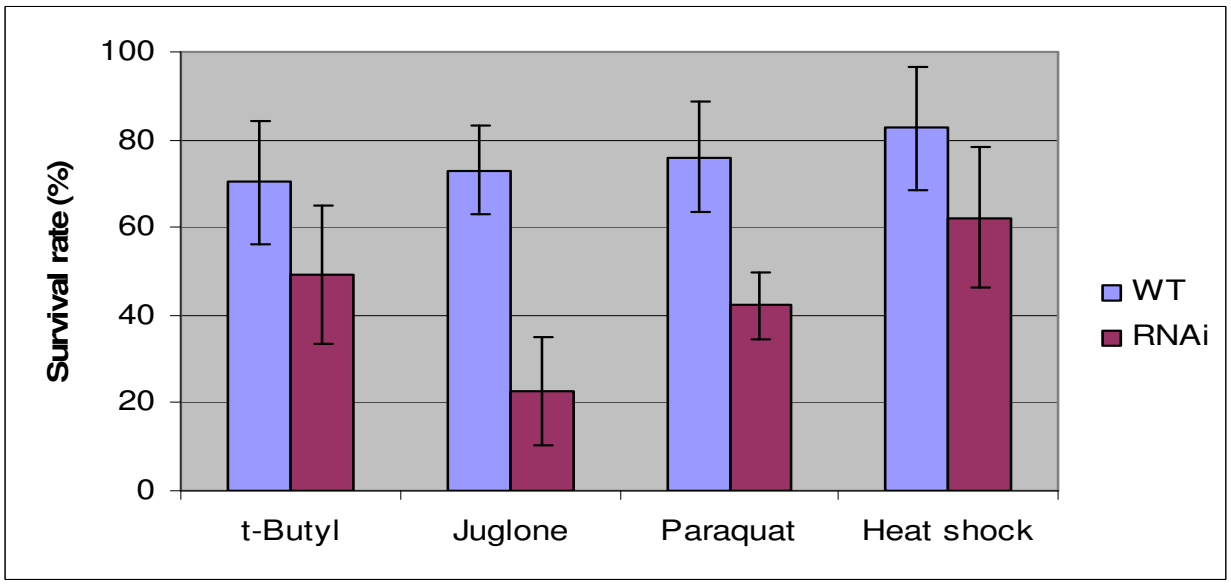


Figure 28b.The effect of oxidative stressors on *Ce*GR RNAi worms. b) RNA*i C. elegans Ce*GR knock down and control worms were exposed to 4 mM cumene hydroperoxide. The survival rate was monitored over a period of 5 h. c) *C. elegans Ce*GR knock down and control worms were exposed to 5 mM *tert*-butylhydroperoxide, 0,05 mM juglone, 25 mM paraquat and heat shock at 32°C. The survival rate was estimated after 18 h. The mean values were calculated from 5 independent experiments. Standard error bars are shown. The significance was calculated using Jump test (p-Value ≤0, 001).

3.5 Comparison of the effect of juglone on *Ce*GS, *Ce*GR and *Ce*GCS RNA*i*-treated worms

In order to determine the differences in sensitivity of *Ce*GS, *Ce*GR and *Ce*GCS knock downs to oxidative stressors, the respective RNA*i*-treated worms were exposed to various juglone concentrations for 18 h, before the survival rates were estimated (**Figure 29**). The drug had a dose-dependent effect on the survival of all worm cultures. At 0.01 mM juglone, control and all knock down animals were not significantly affected by the drug. However, the *Ce*GR and *Ce*GCS knock down animals exhibited a clearly reduced resistance when 0.03 mM and 0.05 mM juglone were added. At 0.05 mM juglone the survival rate of control worms was found to be 80%, whereas only 40% and 52% of the *Ce*GR and *Ce*GCS knock worms survived. The *Ce*GS knock down animals, when compared to the control cultures, showed a drastically enhanced sensitivity towards the oxidative stressors only at higher juglone concentrations (0.07 mM). Almost all animals died in the presence of 0.08 mM juglone. Some of the surviving worms were transferred to normal growth plates without juglone; none of these worms resumed growth, indicating that the effect of juglone is irreversible. Taken together, the *Ce*GR and *Ce*GCS RNA*i* worms were found to be more sensitive towards oxidative stress than *Ce*GS RNA*i* worms.

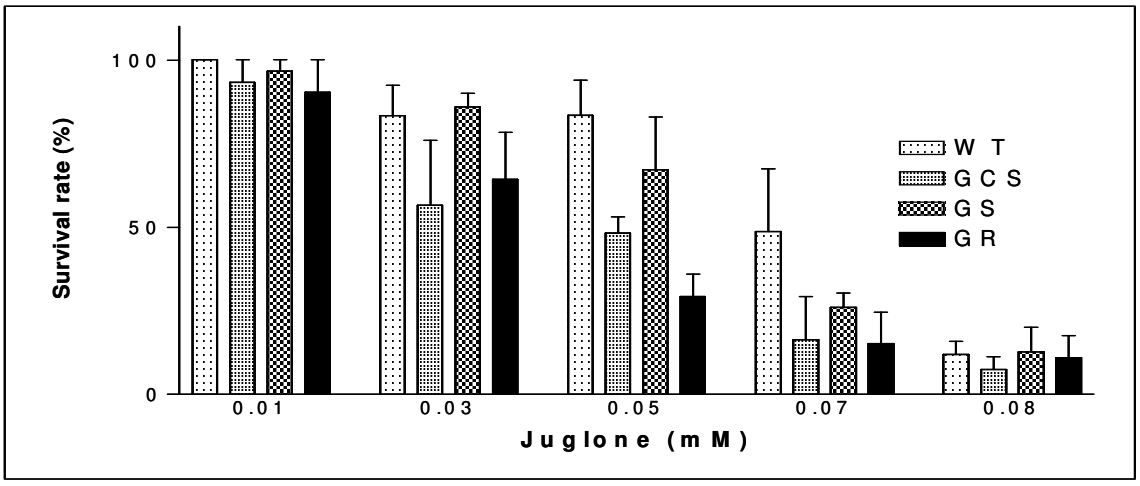


Figure 29. The effect of juglone on *Ce*GS, *Ce*GR and *Ce*GCS RNA*i*-treated worms.
*Ce*GS, *Ce*GR and *Ce*GCS RNA*i* worms were incubated with different concentrations of juglone for 18 h and counted for death and live worms. Data are the mean of three replicates. The significance was calculated using Jump test (p-Value <0, 0003).

Chapter 4 Discussion

Parasitic nematodes have been identified as the major source of chronic ill-health in many parts of the world (Gillespie and Pearson, 2001, Awasthi et al., 2003). For successful intervention of diseases caused by nematodes a strategy based on rational drug development should be favoured, since massive screening of non target-synthesized or randomly designed compounds has failed until now to discover potent and safe drugs against various pathogenic helminthes.

The GSH metabolism of helminthes has been proposed to be a promising drug target, because it plays an important role in antioxidant defense, nutrient metabolism, and regulation of cellular events. GSH is also necessary for the binding and detoxification of electrophiles (Jochmann et al., 1994). It protects the cell against oxidative damage by non-enzymatic scavenging of free radicals and by enzymatic neutralization of toxic hydrogen peroxide, lipid hydroperoxides, and derivatives by glutathione-dependent peroxidases and GSTs.

GSTs are presently the only phase II detoxification enzyme that have been identified and characterized in parasitic nematodes (Brophy et al., 1994, Liebau et al., 1996). In order to interfere with filarial infection, Liebau et al. (1997) proposed that blocking the glutathione conjugation in the filarial worms could be a possible way. Furthermore, some evidence also suggests other enzymes of the glutathione metabolism to be good drug targets since structural differences exist between host and parasite enzyme that might be exploited for the design of specific inhibitors (Karplus et al., 1988).

GSH depletion contributes to oxidative stress which plays a key role in aging and the pathogenesis of many diseases. GSH is maintained in its reduced form by GR. It is postulated that GSH has additional protective functions by promoting heme catabolism (Zhang et al., 1988, Müller et al., 1997, Davioud-Charvet et al., 2001). Furthermore in addition to its protective role and detoxification, GSH is a cellular reductant, catalyst and reactant involved in many biological processes of metabolism, transport and storage (Anderson et al., 1985). Despite the high output of the

enzymatic synthesis, GSH is still lost from the cell. Thus there is a continuous turnover of GSH in all cells at rates that can vary widely, even in different tissues of the same organism (Li et al., 2004, Gupta and Srivastava, 2006). In order to maintain adequate GSH level, it is synthesized by a two step reaction involving two ATP-dependent synthetases, the γGCS and the GS.

The generation of reactive oxygen species and the resulting potential for oxidative stress is a common problem shared by all aerobically growing organisms (Toone et al., 2001) and many features of the resulting transcriptional response appear to be evolutionarily conserved. Consequently, the investigation of model organisms such as *C. elegans* under varying stress conditions is ideal. The free-living nematode *C. elegans,* just like any aerobic organisms, utilizes GSH related enzymes, including GR to maintain cellular redox balance and for protection against reactive oxygen species formed as by-products of aerobic metabolism (Newton et al., 1995, Huynh et al., 2003). Therefore, in the present research GS, GCS and GR from *C. elegans* have been cloned, expressed and the recombinant proteins have been characterized. Furthermore, the expression pattern of *Ce*GCS and *Ce*GS have been analysed by microinjection of respective promoter GFP-fusion constructs in the worms and the minimal promoter regions have been determined. To evaluate the essential function of these enzymes in the survival of the nematodes, RNA mediated interference (RNA*i*) methods were utilized to knock down gene expression.

4.1 Glutathione concentration in *C. elegans*

GSH is considered to be the most prevalent, important and abundant thiol in aerobic cells (Meister and Anderson, 1983). It is ubiquitous in animals, including *C. elegans* and, being water-soluble, is found mainly in the cytosol (85-90%) with remainder in many organelles (Lomaestro and Malone, 1995, Lu, 2000). The concentrations of GSH in animal cells range from 0.05-10 mM (Griffith, 1999, Jones, 2002, Wu et al., 2004), with a significant amount of around 15% being bound to proteins (Sies, 1999). This study shows that the amount of GSH present in *C. elegans* is 27 nmol mg^{-1}

protein which is within the range found in hepatic cells of 20-30 nmol mg^{-1} protein (Brigelius et al., 1983). Similarly, GSH content of the cytoplasmic and mitochondrial fractions of rat liver is 31 ± 5 nmol mg^{-1} protein and 7.0- 0.5 nmol mg^{-1} protein, respectively (Griffith and Meister, 1985), supportive of GSH synthesis only taking place in the cytoplasm. Knowledge regarding in vivo synthesis of glutathione is limited due to the complex compartmentalization of substrates and their metabolism at both the organ and subcellular levels. For example, liver GSH occurs predominantly in perivenous hepatocytes and to a lesser extend in periportal cells (Bella et al., 2002). Changes in plasma GSH level may not reflect changes in GSH synthesis found in specific cell types (Wu et al., 2003).

4.2 GCS from *C. elegans*

GCS is the rate limiting enzyme of GSH biosynthesis which catalyses the ligation of the L-glutamate and L-cysteine to form L-γ-glutamyl-L-cysteine (Plummer et al., 1981). GCS sequences can be divided in three groups: sequences primarily from gamma-proteobacteria, sequences from non-plant eukaryotes, and sequences primarily from alpha-proteobacteria and plants. Although pair wise sequence identities between groups are insignificant, conserved sequence motifs are found, suggesting that the proteins are distantly related (Copley and Dhillon, 2002). In mammals, GCS is a heterodimer comprising of a catalytic subunit (73 kDa, GCS (h)) and a regulatory light subunit (30 kDa, GCS (l) (Hayes and Mclellan, 1999). The predicted gene, *gcs-1* is the *C. elegans* ortholog of GCS (h). *Ce*GCS belongs to the group of non-plant eukaryotes. Its expression is under the control of the SKN-1 CNC transcription factor. *Ce*GCS is predicted to function, in a conserved oxidative stress response pathway, as a phase II detoxification enzyme (An and Blackwell, 2003). In diverse organisms, a common mesendodermal tissue field gives rise to the endoderm and a mesoderm subset that forms the heart and blood in vertebrates (Reiter et al., 1999, Warga and Nusslein-Volhard, 1999, Rodaway and Patient, 2001). In *C. elegans*, mesendodermal development is initiated by the transcription factor SKN-1, which was identified in a screen for genes that are required maternally

for formation of pharyngeal tissue (Bowerman et al., 1992). SKN-1 functions similarly to bZIP proteins that regulate Phase II detoxification genes in vertebrates (Nrf1, Nrf2) and yeast (Yap1p, Pap1p) (Itoh et al., 1999, Toone et al., 2001, Delaunay et al., 2002).

The goal of this work was to characterize the enzyme *Ce*GCS, and to assess its role in oxidative stress. The complete *cegcs* coding region consists of 7 exons and 6 introns. The size of the entire gene including the 5`and 3`UTR (2396 bp), is shorter than the *ovgcs* counterpart, which is 5762 bp and composed of 14 exons and 12 introns (Lüersen et al., 2000). The cDNA encodes 654 amino acid with a predicted molecular mass of 74 kDa. The start of the mature peptide was predicted by comparison with the GCS from human (77% identity) and *O. volvulus* (89 % identity) (**Figure 12**).

4.2.1 Recombinant expression of *Ce*GCS

Several *E. coli* expression systems were used to recombinantly express *Ce*GCS. The pJC40 vector is designed for the expression of recombinant proteins under the control of the T7 RNA polymerase (Clos and Brandau, 1994). This vector encodes a fusion protein with a cleavable amino-terminal histidine tail of 10 residues, thus allowing its purification by metal chelating chromatography. The vector pMAL-p2X is designed to produce maltose binding protein (MBP) fusions, where the protein of interest can be cleaved after purification from MBP with the specific protease factor Xa. The fusion protein is purified by one-step affinity purification specific for MBP (Bach et al., 2001). Furthermore, expression of the *Ce*GCS was performed in different *E. coli* cells but the protein precipitated in inclusion bodies and was highly insoluble in all expression systems used. Several strategies were adapted in an effort to express the recombinant protein. One strategy was to reduce the level of expression by varying the concentrations of IPTG (0-1mM). However, even in the absence of IPTG expression levels remained high and resulted in the formation of inclusion bodies. This implies that expression could not be controlled due to the presence of a leaky promoter

encoding the T7 RNA polymerase. This phenomenon has also been observed with several other recombinant protein expression systems (Uhlen and Moks, 1990, Strandberg and Enfors, 1991, Rudolph and Lilie, 1996, Nomine et al., 2001). Moreover, different temperatures were applied for the recombinant protein expression: 37°C, room temperature and 18°C, however the expression levels remained high and the formation of inclusion bodies appeared. Unfortunately also the *Ce*GCS fused to MBP was found only in inclusion bodies and not in the supernatant.

4.2.2 Promoter analyses of *Ce*GCS

GCS expression pattern in *C. elegans* was investigated by GFP reporter gene expression under the control of the *cegcs* promoter. The *ce*gcs::GFP-1 construct results in a tissue specific expression pattern. The promoter segment contained three consensus SKN-1-binding sites and corresponded to the intervening sequence between *gcs-1* and the nearest upstream gene (An and Blackwell, 2003). At all larval stages and in adult hermaphrodites, GCS::GFP fluorescence was readily detectable in the pharynx and the AS1 neurons. Promoter deletion analyses enabled the identification of the minimal promoter region of *cegcs* to be approximately 744 bp.

It was interesting to determine whether the promoter of *cegcs* is responsive to oxidative stress and heavy metals. Upon exposure to various oxidative stress inducers (paraquat, juglone, cumene hydroperoxide), heavy metals (cadmium, iron, copper) at different concentrations and conditions, heat and UV radiation, the expression increased dramatically across the intestine. Recently it has been shown that *gcs-1* expression in the intestine of *C. elegans* is induced by the transcription factor SKN-1 in response to paraquat-induced oxidative stress (An and Blackwell, 2003). Moreover, experiments using transgenic *C. elegans* demonstrated that cadmium is a potent activator of the transcription of the *gcs* gene (Freedman et al., 1992). Stephen and Jamieson (1997) reported that in yeast *gcs* gene expression was also inducible by the heavy metal cadmium and was depending on the transcription factor Yap1. Since GCS expression is inducible by oxidative stress, it is assumed

that it is involved in the defence against ROS resulting from cellular metabolism. Here it was found that the GCS has a functional focus in the intestinal tract. The pharynx and intestine are directly in contact with the outside world and could provide the first line of defence against deleterious factors (Koga et al., 2000). The digestive system handles energy intake and processing, and responds to external stress. Mesodermal tissues that are developmentally linked to the endoderm are found to be involved in nutrient uptake, transfer and responses to exogenous agents. SKN-1 represents an extreme example of a connection between development and function because it initiates the formation of multiple organs, including the entire feeding and digestive system. A number of genes in *C. elegans* that are thought to be involved in various types of stress response are expressed in organs that have a guardian function, such as the pharynx, or the intestine or both. For example, the expression of the metal-binding metallothionein genes, mtl-l and mtl-2, is induced in the intestine upon exposure to cadmium or heat. In addition, mtl-1 is constitutively expressed in the pharynx (Freedman et al., 1993). The multidrug resistance gene mrp-l which is involved in protection against cadmium and arsenite is expressed in the pharynx, the pharyngeal intestinal valve, the two most anterior cells of the intestine and recto intestinal valve (Broeks et al., 1996). The *Ov*GST1, which function in detoxification of xenobiotics and the protection against oxidative stress, is also expressed in the pharynx (Krause et al., 2001). It has been reported that the ROS defences mobilized by human Nrf proteins are thought to be beneficial in chronic disease states. This gene activation pathway is also important for drug detoxification, and therefore for chemotherapeutic agent tolerance and it may provide a widely applicable means of cancer prevention (Chan et al., 2001, Hayes and McMahon, 2001, Wolf, 2001). During postembryonic stages, SKN-1 functions similarly to Nrf proteins in responses to oxidative stress. The oxidative stress response mediated by SKN-1 appears to be conserved among *C. elegans*, vertebrates, and single-celled eukaryotes, implying that this is an ancient pathway, and that the role of SKN-1 in initiating mesendodermal development may have arisen from its role in general detoxification mechanism (An and Blackwell, 2003).

4.2.3 RNA mediated interference (RNA*i*)

RNA*i* analysis is widely used in *C. elegans* to identify gene function and has been adapted as a high throughput screening method to identify genes that are involved in essential processes (Timmons et al., 2001, Kittler et al., 2004, May and Plasterk, 2005, Wang and Barr, 2005, Kotze and Bagnall, 2006). RNA*i* was conducted to knock down or silence gene expression of the *Ce*GCS to confirm its essential role in *C. elegans.* The result of the PCR clearly indicated that the transcript of the gene was down regulated. It is worthy to note that no abnormal change in phenotype was observed. Feeding and growth were normal after RNA*i* application, this might be because the worm is able to compensate the loss of GSH synthesis. In order to maintain redox homeostasis in the cell and to cope with the excess of ROS produced during oxidative stress (Liao and Yu, 2005), the expression of GCS in *C. elegans* might be inducible. Since oxidative stress is known to generate reactive oxygen species *in vivo*, the response of the *Ce*GCS RNA*i* worms to oxidative stress was investigated. RNA*i* treated worms and wild type worms were exposed to different stressors at different concentrations for 18 h. On average, 50-60% of the RNA*i* treated worms survived compared to wild type survival of 75-90%. This observation indicates that following exposure to stressors, RNA*i* treated worms were more sensitive to stressors than the normal worms. The obtained result is similar to that obtained by An and Blackwell (2003), where loss of GCS-1 activity via RNA*i* led to a low percentage of larval lethality and intestinal abnormalities, suggesting that the enzyme plays a role in postembryonic development. The importance of GCS in the synthesis of GSH in *C. elegans* was also shown by Liao and Yu (2005), where GSH was found to play a critical role in protection from arsenic induced oxidative stress. The vital role of GCS has also been described in other organisms. For example, in *T. brucei*, knock down of GCS leads to cell death demonstrating that GCS is essential for survival of the parasite (Huynh et al., 2003). In yeast, deletion of the *gcs* gene leads to growth arrest, which can be restored by either GSH or reducing agents such as dithiothreitol (Sipos et al., 2002).

4.2.4 Inhibition of *Ce*GCS with buthionine sulfoximine (BSO)

BSO is a specific transition-state inhibitor of GCS. It shows low toxicity to human tissues and has little intrinsic chemical reactivity (Bailey, 1998). BSO acts solely through inhibition of GSH synthesis and does not directly affect other cellular thiols (Griffith, 1981). BSO had been demonstrated previously to have antifilarial activities in *O. volvulus* and anthelminthic effects on *A. suum* (Lüersen et al., 2000, Hussein and Walter, 1995). To investigate the effect of BSO administration on *C. elegans*, it was applied at a concentration of 20 mM for 24 h. Similar to GCS RNA*i* there was no visible change in the phenotype observed between the worms with and without BSO. Pretreatment with oxidative stressors for 9 h resulted in a progressive decrease in the survival rate of BSO treated worms compared to the wild type. When the incubation time was continued for 18 h, the BSO treated worms contained 70% survival rate as compared to wild type (87%). These findings demonstrate the potential value of GSH depletion for the treatment of parasitic diseases. It is worthy to note that BSO depletes intracellular thiols to a similar extent as GCS RNA*i* (Huynh et al., 2003). BSO inhibits synthesis of GSH not only by affecting the GCS reaction but also by preventing transport of the γ-Glu-conjugates making it even more cytotoxic. Chemotherapeutic effects of BSO as an inhibitor of GSH synthesis has also been reported in experimental trypanosomiasis (Arrick, 1981, Karp et al., 2001, Huynh et al., 2003).

4.3 GS from *C. elegans*

4.3.1 Analysis of nucleotide sequence

Glutathione synthetase catalyses the ATP-dependent ligation of gamma-glutamylcysteine with glycine to form glutathione. GS sequences can be divided in two distinct groups corresponding to bacteria and eukaryotes. Proteins in both groups have a common structural fold, but pairwise sequence identities showed that they have no significant relationship to each other (Copley and Dhillon, 2002). The structure of human GS indicates that it belongs to the recently identified ATP-grasp superfamily, although it displays no detectable sequence identity with other family

members including its bacterial counterpart, *E. coli* GS. *Ce*GS, similar to human GS, is difficult to identify as a member of this family due to a rare gene permutation which has resulted in a circular shift of the conserved secondary structure elements in these enzymes with respect to the other known ATP-grasp proteins (Polekhina et al., 1999). By sequence alignment the *Ce*GS was found to be the ortholog to the human GS (Kuwabara and Oneil, 2001). The coding region of the *Ce*GS gene consists of 5 exons and 4 introns leading to a transcript with an open reading frame of 1473 bp and encodes a polypeptide of 490 amino acids. It contains a spliced leader (SL1) sequence.

4.3.2 Analysis of the deduced amino acid sequence of the *Ce*GS

The amino acid sequence of GS is moderately identical to that of human (39.4%), *Arabidopsis thaliana* (32.9%) and yeast *S. cerevisiae* (28.4%) with several regions at the C-terminus being highly conserved. Unlike the *Plasmodium falciparum* GS sequence that is larger and has several insertions which have effects on the stability of the protein (Meierjohann et al., 2002), *C. elegans* GS is almost similar with respect to molecular weight and amino acid residues to other organisms. Despite the high identity between the amino acids of *C. elegans* with those of other organisms, some variations in the local structure and substrate binding still exists. It is not possible to exactly explain why the *Ce*GS behaves differently to the human or the rat enzyme. Possibly non-conserved or semi-conserved residues involved in substrate binding are responsible for the differences in kinetics (Njalsson et al., 2001, Phlippen et al., 2003). In order to find out which amino acids are involved in substrate binding and product binding during catalysis, the *Ce*GS was compared to the resolved structure of human GS, which contains ADP, GSH, a SO_4^{2-} ion and two Mg^{2+} ions (Polekhina et al., 1999). Most of the residues implicated in catalysis and substrate binding are conserved in *Ce*GS (**Table 1**). Furthermore, the cysteine residue (Cys422) plays a significant structural or functional role and could be involved in catalytic activity of human enzyme (Gali et al., 1997). This is substituted by an alanine in *Ce*GS. This alone could be responsible for the extremely low activity of *Ce*GS compared to the

human enzyme. On the other hand, Lys367 in the *Arabidopsis* sequence is equivalent to Lys379 in the *C elegans* sequence and this residue has been implicated in the binding of the adenine ring and α-phosphate of ATP (Wang and Oliver, 1997a). The *C. elegans* sequence from Glu383 to Ala 386 aligns well with the *Arabidopsis* sequence from Glu371 to Gly374 with a similar flexible loop that has been suggested to fold over the substrate binding site and protect the γ-glutamyl cysteinylphosphate intermediate from the reaction with bulk water (Wang and Oliver, 1997b). Wang and Oliver (1997a) confirmed that the glycine-rich loop found in *Arabidopsis* and *E. coli* GS might serve the same function in covering the active site of the enzyme.

Table 6. Differences in co-factor binding between *Ce*GS and human GS

Substrates	Human GS	*Ce*GS
ATP	Asn373	Leu388
	Tyr375	Thr390
	Ile401	Leu415
	Leu188	Ile199
GSH	Gly214	Asn225
γ-GluCys	Leu286	Met301

4.3.3 Kinetic mechanism of the *Ce*GS

*Ce*GS was expressed recombinantly in *E. coli* BL21 (DE3) and the purified protein is active as a dimer with a subunit size of 55 kDa. The specific activity of the protein (1.86 µmol min^{-1} mg^{-1}protein), is 3 times lower than that of the human enzyme. The lower activity of *Ce*GS might be due to the substitution of the bulky valine residue for Ala386 as suggested by Njalsson et al. (2001). Compared with the bacterial GS, the kinetic properties of the eukaryotic enzymes are not well characterized**.** *Ce*GS shares the dimeric structure with the GS isolated from other eukaryotes (Gogos and Shapiro, 2002). Similar to earlier efforts (Wang and Oliver, 1997, Meierjohann et al., 2002), degradation of *Ce*GS was observed during heterologous expression and subsequent purification of the highly unstable protein. In other studies it was observed that the choice of *E. coli* cells had an effect on stability and activity of the protein (Jez and Cahoon, 2004). The steady-state kinetic parameters determined for *Ce*GS (**Table 2**)

were in close range to those reported for the GS from other eukaryotes (Njalsson et al., 2000, Luo et al., 2000, Meierjohann et al., 2002). The K_m value for ATP of 250 µM was found to be similar to the K_m for human enzyme and 4 times higher than the values determined for the *Plasmodium* and rat enzymes. The K_m for glycine of 2.04 mM was in the same range as reported for the human enzyme and 2.5 times lower than that of *Plasmodium*. The K_m for γ-GluAbu of 196 µM was 3 times lower compared to human and 4 times higher than that found for the rat enzyme (**Table 7**).

Table 7. Comparison of molecular masses and kinetic parameters of *Ce*GS with *H. sapiens*, *R norvegicus*, and *P. falciparum* enzymes

	C. elegans	*H. sapiens*[1]	*R. norvegicus*[2]	*P. falciparum.*[3]
Native molecular mass (kDa)	110	105	105	154 ± 8
Vmax (U/mg[1])	1.86 ± 3	6.01 ± 1.4	11.3	5.24 ± 0.7
K_m γGluAbu (µM)	196 ± 8	650 ± 220	42	107.1 ± 23
K_m ATP (µM)	250 ± 23	220 ± 30	37	59.1 ± 10
K_m Glycine (mM)	2.04 ± 0.78	1.34 ± 0.34	0.913	5.04 ± 0.82
K_{cat} (S^1)	ND	5.25	13.0	6.71 ± 0.9

ND- Not determined; mean ± S.D. are given.
1. Njalsson et al., (2000)
2. Luo et al., (2000)
3. Meierjohann et al., (2002)

The negative cooperative binding effect of γ-GluAbu observed for the rat enzyme (Luo et al., (2000) was not found in *C. elegans*. The low activity of *Ce*GS compared to others might be due to some amino acids present in the enzyme that affect enzyme activity. Amino acids like L-cysteine caused a significant enzyme inhibition of 11% in filarial worm (Gupta et al., 2002). The N-terminal amino acid residues of *Ce*GS which have an essential function in determining the activity of the protein are not highly conserved with that of other eukaryotes (Wang and Oliver, 1997b).

4.3.4 Promoter analysis of *Ce*GS and RNA*i*

The functional analysis of the promoter regions of the *Ce*GS genes is reported in this study. The constructs of *cegs* gene promoter regions fused to the GFP were microinjected into the *C. elegans* (Fire et al., 1990). The GFP expression was observed using fluorescence microscope in all larval stages and adults. The pattern of the GFP signal indicates the expression of *cegs* promoter mainly in the anterior and posterior intestine, the ASI neuron and part of the pharynx. By means of promoter deletion, the minimal promoter region of *cegs* was found to be approximately 955 bp The expression of *Ce*GS in the intestine and part of the pharynx points to an essential role in stress response in this organism. Similarly, in *C. elegans* the mek-1 reporter fusion proteins are expressed in pharyngeal muscles, a portion of the intestine and neurons and is thought to be involved in stress response (Koga et al., 2000). The P-glycoprotein gene *pgp-1*, which mediates resistance to cadmium and arsenite, is expressed exclusively in the intestine (Lincke et al., 1993, Broeks et al., 1996). The P-glycoprotein gene *pgp-3* which confers resistance to chloroquine and colchicine, is expressed in the intestine and in the H-shaped excretory cell (Broeks et al., 1995). Furthermore, the *Ce*GSTO-1 which confers resistance to oxidative stress is exclusively expressed in the intestine (Burmeister, 2006). *Ce*GS::GFP worms were exposed to oxidative stress and heavy metals at various concentrations and conditions, but no induction of expression was observed. In contrast, heat-shock proteins, like HSP-16, which are expressed in intestine, and other somatic cells in response to heat stress, readily respond upon exposure to heavy metals including copper, cadmium and certain biological active compounds (Stringham, et al., 1992, Stringham and Candido, 1993, Jones et al., 1996). The RNA*i* experiment was conducted to knock down or silence part of the gene of the *Ce*GS. RNA*i* was induced using bacteria to deliver double stranded RNA in order to further elucidate the protective function of *Ce*GS. Similar to *Ce*GCS there was no change in phenotype. The resistance of *Ce*GS RNA*i* worms to artificially generated ROS such as cumene hydroperoxide, paraquat and juglone was

examined. As a result of exposure to these stressors, the knock-down of the *Ce*GS did not significantly affect the resistance of the worms against oxidative stress.

4.4 GR from *C. elegans*

4.4.1 Analysis of nucleotide and deduced amino acid sequence

GR is a flavoprotein found in both cytosol and mitochondria whose activity is NADPH dependent (Sun, 1990). GR catalyzes the reduction of oxidized glutathione according to: GSSG + NADPH + H^+ → 2GSH + $NADP^+$. The enzyme is important in maintaining a reducing environment within the cell (Akerboom et al., 1982, Mittl and Schulz, 1994). The *Ce*GR gene is composed of 4 exons and 3 introns of short length (55 bp) and spans 1451 bp. The identified gene (1380 bp) encodes a 5`UTR and carries a specific spliced leader at its 5`end, 403 nucleotides upstream of the first in-frame methionine. Similar to *O. volulus* GR, the 5`UTR is unusually long when compared with other *C. elegans* cDNAs, such as omega-GST1 (Burmeister, 2006), glutathione synthetase, possibly indicating a specific function of this region. It is well established that the formation of secondary structures within the 5´UTR as is found in the S-adenosylmethionine decarboxylase, a highly regulated gene in eukaryotes, is involved in mRNA stability and translational control (Shantz et al., 1994). The deduced amino acid sequence of *Ce*GR cDNA encodes a polypeptide of 459 amino acids. The amino acid sequence has 58% identity with human GR, and 65% identity with *O. volvulus GR*. This is much higher than the similarity of the putative *P. falciparum* GR to human with only 35% sequence identity (Müller et al., 1995). As indicated in **Table 3**, similar to *Ov*GR, 17 out of 19 amino acid residues involved in GSSG binding are identical in *Ce*GR compared with the *Hs*GR. The only difference is the substitution of Pro512 in *Hs*GR with Thr444 in *Ce*GR and Asn161 in *Hs*GR with Gly103 in *Ce*GR. Since these amino acids are not polar and are almost of comparable sizes, this exchange might not be of particular structural or functional significance (Färber et al., 1996). These results suggest that the *Ce*GR belongs to the same flavoprotein oxidoreductase which also includes lipoamide dehydrogenase (Mattevi et al., 1991) and trypanothione reductase (Kuriyan et al., 1991a). On the

contrary, the amino acid sequences of the human and *P. falciparum* thioredoxin reductase proteins show a high degree of homology to the *Ce*GR deduced amino acid sequence (Gasdaska et al., 1995, Müller et al., 1996). It is suggested that *Ce*GR might also belongs to the family of large thioredoxin reductases (Kuriyan et al., 1991b, Müller et al., 1996). The residues around the catalytically active cysteines (Cys44 and Cys49 in *Ce*GR) are identical with those found in the human GR. The motifs of FAD-binding are present in all known species of GR (Schulz, 1992) including the *Ce*GR.

4.4.2 The recombinant protein of *Ce*GR is active and stable

Recombinant *Ce*GR was expressed in the *E. coli* system. The expression level was between 400 and 600 µg of protein per litre of bacterial culture. The protein was expressed as a histidine fusion protein and was purified subsequently using Ni^{2+} chelating affinity chromatography. The purified protein is a homodimer of two subunits that have an apparent molecular mass of 50 kDa as confirmed by SDS-PAGE. These results correspond well with data for purified GR from human erythrocytes (Tutic et al., 1990, Müller et al., 1995), mouse liver (Lopez-Barea and Lee, 1979), calf brain (Gutterer et al., 1999), fish (Speers-Roesch and Ballantyne, 2005), *S. digitata*, (Müller et al., 1995), as well as *O. volvulus* (Müller et al., 1997). The protein is active and stable even at -70°C. The specific activity of the protein is 68 µmol min^{-1} mg^{-1} protein (**Table 3**). It appears to be in the same range with that of *Ov*GR (78 µmol min^{-1} mg^{-1}) and 4 times lower when compared to *Hs*GR (240 µmol min^{-1} mg^{-1}), indicating a lower efficiency of catalysis for the nematode enzyme (Müller et al., 1997). The *Ce*GR activity is also 3 times higher than the purified native protein of *S. digitata* and the *Ascaris suum* GR which have specific activities of 24 and 32 µmol min^{-1} mg^{-1}protein, respectively. Regarding its substrates GSSG and NADPH, the purified GR displays Michaelis-Menten behaviour. Compared with *Hs*GR, the affinity of *Ce*GR to the substrate GSSG is slightly higher (34 µM compared to 58 µM) and much higher than *Ov*GR (130 µm). With respect to substrate binding, the K_m value of the *Ce*GR domain for NADPH is within the range of values seen with K_m

values of other GRs: 12.9 µM for *Ce*GR, 9.6 µM for *Hs*GR and 10.9 µM for *Ov*GR. These results indicate that the physical and kinetic properties of *Ce*GR are very similar to all other known GRs. A heterogeneity among these K_m values could be attributed to differences in the solutions used during measurement, as the K_m values of GR for its substrates depend on the ion strength of the buffer used (Williams, 1976, Gutterer et al., 1999). It remains to be investigated whether there is any difference in susceptibility to known inhibitors, eg. trivalent arsenical melarsen oxide, with respect to other known GRs

Table 8. Comparison of properties of glutathione reductase from human, *S. digitata, O. volvulus* and *C.elegans*

	Human*	***S. digitata****	***O. volvulus****	***C. elegans***
Subunit molecular mass (kDa)	50	51	54	50
Native molecular mass (kDa)	98	97	110	100
Specific activity (U/mg)	246	27.3	78 ± 3	68 ± 3 Ω
K_m GSSG (µM)	58 ± 2.1	24.0 ± 0.24	130.7 ± 13.0	34.1 ± 4 Ω
K_m NADPH (µM)	9.6 ± 0.2	n.d.	10.9 ± 2.4	12.9 ± 1.2 Ω

n.d. not determined
* Data obtained from Müller et al., 1995
Ω Data shown represent means ± SE of four independent experiments.

4.4.3 *Ce*GR is sensitive to oxidative stressors

GSH/GSSG is the most important redox couple and plays a crucial role in antioxidant defense, nutrient metabolism and even the regulation of pathways essential for the whole body homeostasis (Sies, 1999, Wu et al., 2004). As GR is responsible for the regeneration of GSH during detoxification of peroxides and radicals, this enzyme is involved in the defense against oxidative stress that appears to be associated with several neurodegenerative diseases (Halliwell, 1992; Bowling and Beal, 1995; Bains and Shaw, 1997). The role of GR as an antioxidant in *C. elegans* was assessed in this study using RNA interference. Similar to *Ce*GS and *Ce*GCS RNA*i*, there was no visible phenotype detected in *Ce*GR RNA*i*. Several chemical agents (cumene

hydroperoxide, paraquat and juglone) at different concentrations were used to exert oxidative stress in this study but differences in the effects of different reagents have received little attention. *Ce*GR RNA*i* responded differently to the various stressors used. The *Ce*GR RNA*i* worms were more sensitive to oxidative stress and to heat shock than the control worms. Exposure to 4 mM cumene hydroperoxide led to a time dependent decrease in the survival rate of stressed RNA*i* and control worms. However, more than 40% of the control worms were still alive after 5 h incubation, whereas only 5% of the *Ce*GR knock down worms survived. Similarly, the knock down of *Ce*GR resulted in a significant reduction in survival rates when worms were stressed by tert-butylhydroperoxide, juglone, paraquat or heat shock. Almost all *Ce*GR RNA*i*-treated worms died with juglone. These findings suggest that the *Ce*GR is essential for the survival of *C. elegans* under stress conditions. RNA*i* induced depletion of *Ce*GR causes an increase in GSSG levels and the loss of cellular thiol pools can lead to cell death. Other studies have also pointed out to the importance of GR in organisms. In the filarial nematode, *Brugia malayi*, higher level of glutathione peroxidase and reductase, possibly protect the worms against the injurious effect of H_2O_2 (Gupta et al., 2004). In plants, over expression of GR in the chloroplast increases the antioxidant capacity of leaves to withstand oxidative stress (Foyer et al., 1995). Similar studies carried out in rat also indicated the importance of GR as an antioxidant, (Özmen et al., 2004).

4.4. *Ce*GS-, *Ce*GR- and *Ce*GCS-RNA*i* treated worms respond differently to juglone

*Ce*GS-, *Ce*GR- and *Ce*GCS-RNA*i* treated worms responded differently to juglone due to their different roles in the organism. In order to investigate possible differences in sensitivity, the knock down worms, were exposed to various juglone (5-hydroxy-1,4-naphthoquinone) concentrations and the dead worms were scored over an 18 h period. The general survival rate was dose dependent. At 0.01 mM juglone, control and all knock down animals were not significantly affected by the drug. For control, the survival did not show a significant decrease until exposure to 0.03 mM and 0.07

mM juglone. On the contrary, the survival rate of *Ce*GR and *Ce*GCS was significantly decreased after exposure to 0.03 mM and 0.05 mM. The survival rate at 0.05 mM was 25% and 50% of *Ce*GR and *Ce*GCS knockdown worms, respectively, as compared to 80% of the control worms. The *Ce*GS knock down animals, when compared to the control cultures, showed a drastically enhanced sensitivity only at higher juglone concentrations (0.07 mM). This suggests that at exposure to low juglone concentrations, GSH biosynthesis through regulation of GCS provides a protective mechanism against juglone toxicity in *C. elegans*. The markable decrease in sensitivity of *Ce*GR knockdown worms to juglone clearly demonstrates that the *Ce*GR gene confers an increased resistance to oxidative stress. This study shows that, while *Ce*GR and other defense systems and enzymes such as superoxide dismutase (SOD) or catalase are available to protect the cells against oxidative stress (Callahan et al., 1988, Kleczkowski et al., 2004, Arkblad et al., 2005), intracellular GSH status is essential for the survival rate of *C. elegans* when subjected to oxidative stress such as juglone. In addition, studies have shown that glutathione related enzymes, including GSTs, are affected by oxidative stress (Liebau et al., 2000, Kampkötter, et al., 2003, Leiers et al., 2003, Ayyadevara et al., 2005). Thus, it is conceivable that in *C. elegans*, oxidative stressors affect intracellular GSH status through regulation of intracellular glutathione biosynthesis and GR. It can therefore be concluded that glutathione metabolism represents an excellent potential target for the design of drug against parasitic nematodes. Nematodes have a potent antioxidant defense system, which is likely to reflect the necessity for self-protection against ROS when produced by the normal metabolic processes or by immune system of the host or exogenous substances.

Chapter 5 Summary

Oxidative stress can be defined as a set of conditions that leads to the chemical or metabolic generation of oxygen-derived species such as the superoxide anion (O_2^-) hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH^\cdot$), lipid peroxides, or related species. Such agents cause various degrees of toxicity in cells and can lead to irreversible damage. In order to protect the cells against oxidative stress and prevent damage, aerobic organisms have a number of antioxidant systems.

Glutathione (GSH) plays a vital role in antioxidant systems in aerobic organisms. Here the glutathione reductase (GR) is crucial in maintaining the correct intracellular redox balance and protecting the cell against oxidative stress. GSH serves as a cofactor for glutathione peroxidases (GPX) and as a co-substrate for glutathione S-transferases (GSTs), mainly involved in detoxification of electrophilic xenobiotics and secondary products of lipid peroxidation. Apart from the GSH redox cycle, the de novo synthesis of GSH ensures the maintenance of intracellular GSH levels. This involves two consecutive enzymatic reactions: The ligation of glutamate and cysteine is the rate limiting step and is catalysed by the γ-glutamylcysteine synthetase (GCS); the addition of glycine is catalysed by the glutathione synthetase (GS). GSH depletion has been discussed as a chemotherapeutic strategy against tumours and infectious diseases and the GS, GCS and GR are proposed as potential drug targets.

This study investigates the role of the GSH synthesis enzymes GCS and GS as well as the redox enzyme GR in the model nematode *Caenorhabditis elegans* focusing on gene structure, characterisation and kinetics of these recombinantly expressed enzymes. In addition, RNA interference (RNA*i*) experiments were conducted in order to investigate whether these proteins are essential for the survival of nematodes and can therefore be proposed as target for the development of novel drugs against parasitic nematodes.

By BLAST search the GCS from *C. elegans* (*cegcs)* was identified. The cDNA corresponds to a polypeptide of 654 amino acids with a predicted molecular mass of 74 kDa. The ORF of *cegcs* was cloned into the prokaryotic expression vectors

pJC40, pMalp2x and the constructs were transformed into different *Escherichia coli* expression cells. However, several strategies to produce the recombinant protein failed preventing the enzyme´s characterization. Therefore the study on *Ce*GCS was focussed on the analysis of the expression pattern, using the reporter gene “green fluorescence protein” (GFP), and on knock down of the *Ce*GCS by RNA*i*. GFP expression was observed using fluorescence microscope in all larval stages and adults of the transgenic *C. elegans*. The pattern of the GFP signal indicates the expression of *Ce*GCS mainly in the pharyngeal region and ASI neuron. Deletion analysis identified the minimal promoter region of *cegcs* to be approximately 744 bp. Further, the stress responsiveness of the *cegcs* promoter was analysed. For this, transgenic worms were treated with heavy metals, various oxidative stressors, UV radiation and heat shock. The results clearly demonstrate that *Ce*GCS is highly upregulated upon stress in all developmental stages. In adult worms enhanced fluorescence was found in the pharynx, intestine and ASI neuron. RNA*i* experiments were conducted to further elucidate the essential role of the *Ce*GCS in a multicellular organism. The knock down worms showed no obvious phenotypic alterations, demonstrating that the enzyme is not essential for normal worm development under laboratory conditions. However, survival of the knock down worms was altered when exposed to oxidative stress conditions, with knock down worms showing a decreased resistance to oxidative stress compared to the control worms. Furthermore, the role of GSH synthesis of *C. elegans* in stress resistance was tested using the GCS specific transition-state inhibitor buthionine sulfoximine (BSO). Pretreatment with oxidative stressors resulted in a progressive decrease of the survival rates of BSO treated worms compared to the wild type worms under same conditions. It is worthy to note that BSO depletes intracellular thiols to a similar extent as *Ce*GCS RNA*i*.

In order to investigate further the synthetic pathway of GSH in *C. elegans*, the ORF of *cegs* was cloned and expressed recombinantly in *E. coli* BL21 (DE3) cells. Most of the residues implicated in catalysis and substrate binding are conserved in the deduced amino acid sequence of *Ce*GS. The only noticeable difference is the substitution of Cys422 in the human enzyme to Ala436 in *Ce*GS, perhaps

responsible for the lower enzymatic activity observed in *Ce*GS. The protein is active as a dimer, with a subunit molecular mass of 55 kDa. The recombinant enzyme was found to be very unstable. The addition of several reducing reagents did not significantly enhance enzyme stability. The enzymatic assay for *Ce*GS was carried out at an optimium pH of 7.0 and at 30°C. The specific activity was determined to be 1.9 µmol min^{-1} mg^{-1}, the K_m value for γ-GluAbu, glycine and ATP were calculated to be 250 µM, 2 mM and 196 µM, respectively. These values are in the same range as those of the mammalian enzymes. To investigate sites of *Ce*GS expression, *C. elegans* worms were transformed by microinjection with the putative promoter region fused to the reporter gene GFP. Analysis of the protein expression pattern revealed expression mainly in the intestine and the excretory system of all post-embryonic stages. By means of promoter deletion, the minimal promoter region of *cegs* was found to be approximately 955 bp. Oxidative stressors and heavy metals did not induce expression of the *cegs* gene. Furthermore, the knock down of the *Ce*GS by RNA*i* did not significantly affect the resistance of the worms against oxidative stress.

The GR is a flavoprotein that catalyses the NADPH-dependent reduction of GSSG to GSH. The mRNA is *trans*-spliced and contains an ORF, consisting of 1380 bp, which encode a polypeptide of 459 amino acids with a deduced molecular mass of 49.8 kDa. The regions involved in substrate- and cofactor-binding are almost conserved, with a total of 17 out of 19 amino acid residues involved in GSH-binding being identical. The *Ce*GR was expressed in *E. coli* BL21 (DE3). The yield of purified *Ce*GR was 400-600 µg protein per litre of bacteria culture. The *C. elegans* protein is active as a dimer and stable with a specific activity of 68 µmol min^{-1} mg^{-1} protein. Gel filtration shows that the protein forms a homodimer of about 100 kDa under native conditions. The K_m values of NADPH and GSSG were calculated to be 12.9 µM and 34 µM, respectively. The role of *Ce*GR as an antioxidant in *C. elegans* was assessed in this study using RNA*i*. RNA*i*-treated worms were exposed to the oxidative stressors as well as to heat shock at 32°C. The survival rate of RNA*i* treated worms decreases significantly as compared to wild type worms under same stress conditions. *Ce*GS, *Ce*GR and *Ce*GCS RNA*i*-treated worms responded differently to

the oxidative stress exerted by the stressor juglone. While there was no remarkable increase in sensitivity towards juglone in the *Ce*GS-RNA*i*-treated worms, the increase in sensitivity observed for the *Ce*GR- and *Ce*GCS-RNA*i* treated worms was highly significant. It can therefore be concluded that glutathione depletion represents a potential target for the drug design against parasitic nematodes.

Future work will include analysis of the expression pattern of *Ce*GR using the reporter gene GFP and the use of specific known inhibitors, such as the trivalent arsenical melarsen oxide, in order to analyse differences in susceptibility compared to the host GR.

References

Aboagye-Kwarteng, T., Smith, K., Fairlamb, A. H. (1992) Molecular characterization of the trypanothione reductase gene from *Crithidia fasciculata* and *Trypanosoma brucei*: comparison with other flavoprotein disulphide oxidoreductases with respect to substrate specificity and catalytic mechanism. Mol Microbiol. 21, 3089-99.

Akerboom, T.P., Bilzer, M., Sies, H. (1982) The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. J Biol Chem. 8, 4248-52.

An, J. H., Blackwell, T. K. (2003) SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. Genes Dev. 15, 1882-93.

Anderson, M. E. (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol. 113, 548-55.

Anderson, M. E, Powrie F, Puri, R. N, Meister, A. (1985) Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione. Arch Biochem Biophys. 239, 538-48.

Anderson, R. M., May, R. M. (1991) Infectious diseases of humans: dynamics and control. Oxford University Press. 122-29.

Arber, N., Shirin, H., Fadila, R., Melamed, E., Pinkhas, J., Sidi, Y. (1990) Pseudotumor cerebri associated with leuprorelin acetate. Lancet. 335, 668.

Argyrou, A., Vetting, M. W., Blanchard, J.S. (2004) Characterization of a new member of the flavoprotein disulfide reductase family of enzymes from *Mycobacterium tuberculosis*. J Biol Chem. 279, 52694-702.

Arkblad, EL., Tuck, S., Pestov, N. B., Dmitriev, R.I., Kostina, M. B., Stenvall, J., Tranberg, M., Rydstrom, J. (2005) A *Caenorhabditis elegans* mutant lacking functional nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative stress. Free Radic Biol Med. 38, 1518-25.

Armstrong, R. N. (1991) Glutathione S-transferases: reaction mechanism, structure, and function. Chem Res Toxicol. 2, 131-40.

Arrick, B. A., Griffith, O. W., Cerami, A. (1981) Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. J Exp Med. 153, 720-5.

Awasthi, S., Bundy, D. A., Savioli, L. (2003) Helminthic infections. BMJ. 327, 431-3.

Ayyadevara S, Dandapat A, Singh SP, Benes H, Zimniak L, Reis RJ, Zimniak P (2005). Lifespan extension in hypomorphic daf-2 mutants of *Caenorhabditis elegans* is partially mediated by glutathione transferase CeGSTP2-2. Aging Cell. 4, 299-307.

Bach, H., Mazor, Y., Shaky, S., Shoham-Lev, A., Berdichevsky, Y., Gutnick, D. L., Benhar, I. (2001) *Escherichia coli* maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies. J Mol Biol. 312, 79-93.

Bailey, H. H., Mulcahy, R. T., Tutsch, K. D., Arzoomanian, R. Z., Alberti, D., Tombes, M. B., Wilding, G., Pomplun, M., Spriggs, D. R. (1994) Phase I clinical trial of intravenous L-Buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. J Clin Oncol. 12, 194-205.

Bailey, H. H. (1998) L-S,R-buthionine sulfoximine: historical development and clinical issues. Chem Biol Interact. 111-112, 239-54.

Bailey, T. A., John, A., Mensah-Brown, E. P., Garner, A., Samour, J., Raza, H. (1998) Drug metabolizing enzyme systems in the houbara bustard (*Chlamydotis undulata*). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 120, 365-72.

Bains, J. S., Shaw, C. A. (1997) Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res Brain Res Rev. 25, 335-58.

Bauer, H., Gromer, S., Urbani, A., Schnolzer M., Schirmer, R. H., Muller, H. M. (2003) Thioredoxin reductase from the malaria mosquito *Anopheles gambiae*. Eur J Biochem. 270, 4272-81.

Bella, D. L., Hirschberger, L. L., Kwon, Y. H., Stipanuk, M. H. (2002) Cysteine metabolism in periportal and perivenous hepatocytes: perivenous cells have greater capacity for glutathione production and taurine synthesis but not for cysteine catabolism. Amino Acids. 23, 453-8.

Bellomo, G., Orrenius, S. (1985) Altered thiol and calcium homeostasis in oxidative hepatocellular injury. Hepatology. 5, 876-82.

Beutler, E., Gelbart, T., Kondo, T., Matsunaga, A. T. (1999) The molecular basis of a case of gamma-glutamylcysteine synthetase deficiency. Blood. 94, 2890-4.

Bhargava, K. K., Le Trang, N., Cerami, A., Eaton, J. W. (1983) Effect of arsenical drugs on glutathione metabolism of *Litomosoides carinii*. Mol Biochem Parasitol. 9, 29-35.

Blaxter, M. L., Page, A. P., Rudin, W., Maizels, R. M. (1992) Nematode surface coats: actively evading immunity. Parasitol Today. 8, 243-7.

Bowerman, B., Eaton, B. A., Priess, J. R. (1992) skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. Cell. 68, 1061-75.

Bowling, A. C., Beal, M. F. (1995) Bioenergetic and oxidative stress in neurodegenerative diseases. Life Sci. 56, 1151-71.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72, 248-54.

Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics. 77, 71-94.

Brigelius, R., Muckel, C., Akerboom, T. P., Sies, H. (1983) Identification and quantitation of glutathione in hepatic protein mixed disulfides and its

relationship to glutathione disulfide. Biochem Pharmacol. 32, 2529-34.

Broeks, A., Janssen, H. W., Calafat, J., Plasterk, R. H. (1995) A P-glycoprotein protects *Caenorhabditis elegans* against natural toxins. EMBO J. 14, 1858-66.

Broeks, A., Gerrard, B., Allikmets, R., Dean, M., Plasterk, R. H. (1996) Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J. 15, 6132-43.

Brophy, P. M., Brown, A., Pritchard, D. I. (1994) A PCR strategy for the isolation of GSTs from gastro-intestinal nematodes. International Journal for Parasitolgy 24, 1059-1061.

Burmeister, C. (2006) Charakterisierung der Omega-Glutathion S-Transferase CeGSTO-1 und des Elongationsfaktors der Translation CeEF-1γ bei Caenorhabditis elegans. Dissertation zur Erlangung des Doktorgrades des Departments Biologie der Fakultät für Mathematik, Informatik und Naturwissenschaften der Universität Hamburg.

Callahan, H. L., Crouch, R. K., James, E. R. (1988) Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? Parasitol Today. 4, 218-25.

Chan, K., Han, X. D., Kan, Y. W. (2001) An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci USA. 98, 4611-6.

Clemens, S. (2006) Evolution and function of phytochelatin synthases. Journal of Plant Physiol. 163, 319–32.

Clos, J., Brandau, S. (1994) pJC20 and pJC40--two high-copy-number vectors for T7 RNA polymerase-dependent expression of recombinant genes in *Escherichia coli.* Protein Expr Purif. 5, 133-7.

Cobbett, C. S. (2000) Phytochelatins and their roles in heavy metal detoxification. Plant Physiol. 12, 3825-32.

Cohen, S. N., Chang, A. C, Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci USA. 69, 2110-4.

Collinson, L. P., Dawes, I. W. (1995) Isolation, characterization and overexpression of the yeast gene, GLR1, encoding glutathione reductase. Gene.156, 123-7.

Copley, S. D., Dhillon, J. K. (2002) Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. Genome Biol. 3, research0025.

Cheng, W. H., Ho, Y. S., Ross, D. A., Valentine, B. A., Combs, G. F., Lei, X. G. (1997) Cellular glutathione peroxidase knockout mice express normal levels of selenium-dependent plasma and phospholipid hydroperoxide glutathione peroxidases in various tissues. J Nutr. 127, 1445-50.

Dahl, N., Pigg, M., Ristof, F. E., Gali, R., Carlsson, B., Mannervik, B., Larsson, A., Board P. (1997) Missense mutations in the human glutathione synthetase gene result in severe metabolic acidosis, 5-oxoprolinuria, hemolytic anemia and neurological dysfunction. Hum Mol Genet. 6, 1147-52.

Dalton, T. P., Dieter, M. Z., Yang, Y., Shertzer, H. G., Nebert, D. W. (2000) Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. Biochem Biophys Res Commun. 279, 324-9.

Davioud-Charvet, E., Delarue, S., Biot, C., Schwobel, B., Boehme, C. C., Mussigbrodt, A., Maes, L., Sergheraert, C., Grellier, P., Schirmer, R. H., Becker, K. (2001) A prodrug form of a *Plasmodium falciparum* glutathione reductase inhibitor conjugated with a 4-anilinoquinoline. J Med Chem. 44, 4268-76.

Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J., Toledano, M. B. (2002) A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell. 11, 1471-81.

DeLeve, L. D., Kaplowitz, N. (1991) Glutathione metabolism and its role in hepatotoxicity. Pharmacol Ther. 52, 287-305.

Del Maestro, R. F. (1980) An approach to free radicals in medicine and biology. Acta Physiol Scand Suppl. 492, 153-68.

Deneke, S. M., Fanburg, B. L. (1989) Regulation of cellular glutathione. Am J Physiol. 257, L163-73.

Deonarain, M. P., Scrutton, N. S., Berry, A., Perham, R. N. (1990) Directed mutagenesis of the redox-active disulphide bridge in glutathione reductase from *Escherichia coli*. Proc Biol Sci. 241, 179-86.

Droge, W. (2002) Free radicals in the physiological control of cell function. Physiol Rev. 82, 47-95.

Dudley, R. W., Khairallah, M., Mohammed, S., Lands, L., Des Rosiers, C., Petrof, B.J. (2006) Dynamic responses of the glutathione system to acute oxidative stress in dystrophic mouse (mdx) muscles. Am J Physiol Regul Integr Comp Physiol. 291,R704-10.

Espey, M. G., Miranda, K. M., Thomas, D. D., Xavier, S., Citrin, D., Vitek, M. P., Wink, D. A. (2002) A chemical perspective on the interplay between NO, reactive oxygen species, and reactive nitrogen oxide species. Ann N Y Acad Sci. 962, 195-206.

Fabian, T. J., Johnson, T. E. (1994) Production of age-synchronous mass cultures of *Caenorhabditis elegans*. J Gerontol. 49, B145-56.

Farber, P. M., Becker, K., Muller, S., Schirmer, R. H., Franklin, R. M. (1996) Molecular cloning and characterization of a putative glutathione reductase gene, the PfGR2 gene, from *Plasmodium falciparum*. Eur J Biochem. 239, 655-61.

Dormer, U. H., Westwater, J., McLaren, N. F., Kent, N. A., Mellor, J., Jamieson, D. J. (2000) Cadmium-inducible expression of the yeast GSH1 gene requires a functional sulfur-amino acid regulatory network. J Biol Chem. 275, 32611-6.

Duke, R. C., Ojcius, D. M., Young, J. D. E. (1996) Cell suicide in health and disease. Sci Am. 275, 80-7.

Fiers, W., Beyaert, R., Declercq, W., Vandenabeele, P. (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene. 18, 7719-30.

Fire, A. (1986) Integrative transformation of *Caenorhabditis elegans.* EMBO J. 5, 673–2680.

Fire, A., Harrison, S. W., Dixon, D. (1990) A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. Gene 93, 189–198.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 39, 1806-11.

Foyer, C. H., Souriau, N., Perret, S., Lelandais, M., Kunert, K. J., Pruvost, C., Jouanin, L. (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. Plant Physiol. 10, 91047-57.

Franklin, C. C., Krejsa, C. M., Pierce, R. H., White, C. C., Fausto, N., Kavanagh, T. J. (2002) caspase-3-dependent cleavage of the glutamate-L-cysteine ligase catalytic subunit during apoptotic cell death. Am J Pathol. 160, 1887-94.

Freedman, A. M*.,* Mak, I. T*.,* Stafford, R. E*.,* Dickens, B. F*.,* Cassidy, M. M*.,* Muesing, R. A*.,* Weglicki, W. B. (1992) Erythrocytes from magnesium-deficient hamsters display an enhanced susceptibility to oxidative stress. Am J Physiol. 262, C1371-5.

Freedman J. H., Slice, L. W., Dixon, D., Fire, A., Rubin, C. S. (1993) The novel metallothionein genes of *Caenorhabditis elegans.* Structural organization and inducible, cell-specific expression. J. Biol. Chem*.,* 268, 2554–2564.

Frendo, P., Jimenez, M. J., Mathieu, C., Duret, L., Gallesi, D., Van de Sype, G., Herouart, D., Puppo, A. (2001) A *Medicago truncatula* homoglutathione

synthetase is derived from glutathione synthetase by gene duplication. Plant Physiol. 126, 1706-15.

Fridovich, I. (1986) Biological effects of the superoxide radical. Arch Biochem Biophys. 247, 1-11.

Fuchs, J. A., Warner, H. R. (1975) Isolation of an *Escherichia coli* mutant deficient in glutathione synthesis. J Bacteriol. 124, 140-8.

Gali, R. R., Board, P. G (1997) Identification of an essential cysteine residue in human glutathione synthase. Biochem J. 321, 207-10.

Gallo, J. M., Brennan, J., Hamilton, T. C., Halbherr, T., Laub, P. B., Ozols, R. F., O'Dwyer, P. J. (1995) Time-dependent pharmacodynamic models in cancer chemotherapy: population pharmacodynamic model for glutathione depletion following modulation by buthionine sulfoximine (BSO) in a phase I trial of melphalan and BSO. Cancer Res. 55, 4507-11.

Gasdaska, P. Y., Gasdaska, J. R., Cochran, S., Powis, G. (1995) Cloning and sequencing of a human thioredoxin reductase. FEBS Lett. 373, 5-9.

Ghisla, S., Massey, V. (1989) Mechanisms of flavoprotein-catalyzed reactions. Eur J Biochem. 181, 1-17.

Gillespie, S. H., Pearson, R. D. (2001) Intestinal nematodes. Principle and practice of clinical parasitology. 21, 560-67.

Gogos, A., Shapiro, L. (2002) Large conformational changes in the catalytic cycle of glutathione synthase. Structure. 10, 1669-76.

Granato, M., Schnabel, H., Schnabel, R. (1994) pha-1, a selectable marker for gene transfer in *C. elegans*. Nucleic Acids Res. 22, 1762-3.

Grant, C. M., MacIver, F. H., Dawes, I. W. (1997) Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. Mol Biol Cell. 8, 1699-707.

Griffith, O. W. (1981) Depletion of glutathione by inhibition of biosynthesis. Methods Enzymol. 77, 59-63.

Griffith, O. W. (1982) Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J Biol Chem. 257, 13704-12.

Griffith, O. W., Meister, A. (1985) Origin and turnover of mitochondrial glutathione. Proc Natl Acad Sci USA. 82, 4668-72.

Griffith, O. W. (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med. 27, 922-35.

Griffith, O. W., Mulcahy, R. T. (1999) The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. Adv Enzymol Relat Areas Mol Biol. 73, 209-67.

Guerrant, R. L., Walker, D. H., Weller, P. F. (2006) Tropical infectious diseases. Principles pathogens and practice. 2, 1152-1160.

Gupta, S., Tripathi, R., Srivastava, A. K., Iqbal, J. (2002) Depletion of intracellular glutathione of *Setaria cervi* by buthionine sulfoximine, carmustine and their analogues. In: Callaos N, He Y, Perez-Perez JA (eds.): Proceedings of IIIS and IFSR, 17, 491–96.

Gupta, R., Bajpai, P., Tripathi, L. M., Srivastava, V. M., Jain, S. K., Misra-Bhattacharya, S. (2004) Macrophages in the development of protective immunity against experimental *Brugia malayi* infection. Parasitology. 129, 311-23.

Gupta, S., Bhandari, Y. P., Reddy, M. V., Harinath, B. C., Rathaur, S. (2005) *Setaria cervi*: immunoprophylactic potential of glutathione-S-transferase against filarial parasite *Brugia malayi*. Exp Parasitol. 109, 252-5.

Gupta, S., Srivastava, A. K. (2006) Glutathione metabolism of filarial worms: A vulnerable target for the design and synthesis of new antifilarial agents. Med Sci Monit, 12, HY1-9.

Gutterer, J. M., Dringen, R., Hirrlinger, J., Hamprecht, B. (1999) Purification of glutathione reductase from bovine brain, generation of an antiserum, and immunocytochemical localization of the enzyme in neural cells. J Neurochem. 73, 1422-30.

Halliwell, B. (1992) Oxygen radicals as key mediators in neurological disease: fact or fiction. Ann Neurol. 32, S10-5.

Hanahan, D. (1985) In: cDNA cloning Vol. 1. A pratical approach, IRL Press, Oxford, 109-114.

Hayes, J. D., Pulford, D. J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 30, 445-600.

Hayes, J. D., Ellis, E. M., Neal, G. E., Harrison, D. J., Manson, M. M. (1999) Cellular response to cancer chemopreventive agents: contribution of the antioxidant responsive element to the adaptive response to oxidative and chemical stress. Biochem Soc Symp. 64, 141-68.

Hayes, J. D., McLellan, J. I. (1999) Glutathione and glutathione-dependent enzymes represent a coordinately regulated defence against oxidative stress. Free Rad Biol Med. 27, 273–300.

Hayes, J. D., McMahon, M. (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. Cancer Lett. 174, 103-13.

Henikoff, S. (1995) Comparative methods for identifying functional domains in protein sequences. Biotechnol Annu Rev. 1, 129-47.

Herman, M. A., Horvitz, H. R. (1994) The *Caenorhabditis elegans* gene lin-44 controls the polarity of asymmetric cell divisions Development. PMID. 120, 1035-1047.

Heukeshoven, J., Dernick, R. (1986) Vereinfachte und universelle Methode zur Silberfärbung von Proteinen in Polyacrylamidgelen: Bemerkung zum Mechanismus der Silberfärbung: LKB-Application note RE-034.

Howden, R., Goldsbrough, P. B., Andersen, C. R., Cobbett, C. S. (1995) Cadmium-sensitive, cad1 mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. 107, 1059-66.

Huang, C. S., Chang, L. S., Anderson, M. E, Meister, A. (1993) Catalytic and regulatory properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. J Biol Chem. 268, 19675-80.

Huang, C. S., He, W., Meister, A., Anderson, M. E. (1995) Amino acid sequence of rat kidney glutathione synthetase. Proc Natl Acad Sci USA. 92, 1232-6.

Hussein, A. S, Walter, R. D. (1995) Purification and characterization of γ-glutamylcysteine synthetase from *Ascaris suum*. Mol Biochem Parasitol, 72, 57-64.

Hussein, A. S., Walter, R. D. (1996) Inhibition of glutathione synthesis of *Ascaris suum* by buthionine sulfoximine. Parasitol Res. 82, 372-4.

Huynh, T. T., Huynh, V. T., Harmon, M. A., Phillips, M. A. (2003) Gene knockdown of gamma-glutamylcysteine synthetase by RNAi in the parasitic protozoa *Trypanosoma brucei* demonstrates that it is an essential enzyme. J Biol Chem. 278, 39794-800.

Inoue, Y., Kimura, A. (1996) Identification of the structural gene for glyoxalase I from *Saccharomyces cerevisiae*. J Biol Chem. 271, 25958-65.

Itoh, K., Yamamoto, M. (1999) Transcriptional regulation of the phase II drug detoxifying enzyme genes. Tanpakushitsu Kakusan Koso. 44, 2370-6.

Izawa, S., Inoue, Y., Kimura, A. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. FEBS Lett. 368, 73-6.

Jez, J. M., Cahoon, R. E. (2004) Kinetic mechanism of glutathione synthetase from *Arabidopsis thaliana*. J Biol Chem. 279, 42726-31.

Jochmann, C., Klee, S., Ungemach, F. R., Younes, M. (1994) The role of glutathione and protein thiols in CBrCl3-induced cytotoxicity in isolated rat hepatocytes. Pharmacol Toxicol. 75, 7-16.

Jones, D. P. (2002) Redox potential of GSH/GSSG couple: assay and biological significance. Methods Enzymol. 348, 93-112.

Jowet, T., Rizki, T. M., Rizki, R. M. (1986) Regulation of synthesis of larval serum proteins after transplantation of larval fat body into adult *Drosophila melanogaster*. Dev Biol. 116, 23-30.

Kampkotter, A., Volkmann, T. E., de Castro, S. H., Leiers, B., Klotz, L. O., Johnson, T. E., Link, C. D., Henkle-Duhrsen, K. (2003) Functional analysis of the glutathione S-transferase 3 from *Onchocerca volvulus* (Ov-GST-3): a parasite GST confers increased resistance to oxidative stress in *Caenorhabditis elegans*. J Mol Biol. 325, 25-37.

Kanzok, S. M., Fechner, A., Bauer, H., Ulschmid, J. K., Muller, H. M., Botella-Munoz, J., Schneuwly, S., Schirmer, R., Becker, K. (2001) Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. Science. 291, 643-6.

Karp, D. R., Shimooku, K., Lipsky, P. E. (2001) Expression of gamma-glutamyl transpeptidase protects ramos B cells from oxidation-induced cell death. J Biol Chem. 276, 3798-804.

Karplus, P. A., Schulz, G. E. (1987) Refined structure of glutathione reductase at 1.54 A resolution. J Mol Biol. 195, 701-29.

Karplus, P. A, Krauth-Siegel RL, Schirmer RH, Schulz GE. (1988) Inhibition of human glutathione reductase by the nitrosourea drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea. A crystallographic analysis. Eur J Biochem. 171, 193-8.

Karplus, P. A., Schulz, G. E. (1989) Substrate binding and catalysis by glutathione reductase as derived from refined enzyme: substrate crystal structures at 2 A resolution. J Mol Biol. 210, 163-80.

Kelly, B. S., Antholine, W. E., Griffith, O. W. (2002) *Escherichia coli* gamma-glutamylcysteine synthetase. Two active site metal ions affect substrate and inhibitor binding. J Biol Chem. 277, 50-8.

Kennerdell, J. R., Carthew, R. W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 95, 1017-1026.

Kerboeuf, D., Aycardi, J. (1999) Unexpected increased thiabendazole tolerance in *Haemonchus contortus* resistant to anthelmintics by modulation of glutathione activity. Parasitol Res. 85, 713-8.

Kimble, J., Hirsh, D. (1979) The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. Dev Biol. 70, 396-417.

Kimble, J., Hodgkin, J., Smith, T., Smith J. (1982) Suppression of an amber mutation by microinjection of suppressor tRNA in *C. elegans*. Nature. 299, 456-8.

Kittler, R., Pelletier, L., Ma, C., Poser, I., Fischer, S., Hyman, A. A., Buchholz, F. (2005) RNA interference rescue by bacterial artificial chromosome transgenesis in mammalian tissue culture cells. Proc Natl Acad Sci USA. 102, 2396-401.

Kleczkowski, M., Klucinski, W., Sikora, J., Zdanowicz, M. (2004) Role of antioxidants in the protection against oxidative stress in cattle--trace elements and enzymatic mechanisms (Part 3). Pol J Vet Sci. 7, 233-40.

Koga, M., Zwaal, R., Guan, K. L., Avery, L., Ohshima, Y. (2000) *A Caenorhabditis elegans* MAP kinase kinase, MEK-1, is involved in stress responses. EMBO J. 19, 5148-56.

Kosower, N. S., Kosower, E. M. (1978) The glutathione status of cells. Int Rev Cytol, 54, 109–60.

Kotze, A. C., Bagnall, N. H. (2006) RNA interference in *Haemonchus contortus*: suppression of beta-tubulin gene expression in L3, L4 and adult worms in vitro. Mol Biochem Parasitol. 145, 101-10.

Krause, S., Sommer, A., Fischer, P., Brophy, P. M., Walter, R. D., Liebau, E. (2001) Gene structure of the extracellular glutathione S-transferase from *Onchocerca volvulus* and its overexpression and promoter analysis in transgenic *Caenorhabditis elegans*. Mol Biochem Parasitol. 117, 145-54.

Krauth-Siegel, R. L., Blatterspiel, R., Saleh, M., Schiltz, E., Schirmer, R. H., Untucht-Grau, R. (1982) Glutathione reductase from human erythrocytes. The sequences of the NADPH domain and of the interface domain. Eur J Biochem. 121, 259-67.

Kunert, K. J., Cresswell, C. F., Schmidt, A., Mullineaux, P. M., Foyer, C. H. (1990) Variations in the activity of glutathione reductase and the cellular glutathione content in relation to sensitivity to methylviologen in *Escherichia coli*. Arch Biochem Biophys. 282, 233-8.

Kuriyan, J., Krishna, T. S., Wong, L., Guenther, B., Pahler, A., Williams, C. H., Jr, Model, P. (1991a) Convergent evolution of similar function in two structurally divergent enzymes. Nature. 352, 104-5.

Kuriyan, J., Kong, X. P., Krishna, T. S., Sweet, R. M., Murgolo, N. J., Field, H., Cerami, A., Henderson, G. B. (1991b) X-ray structure of trypanothione reductase from *Crithidia fasciculata* at 2.4-A resolution. Proc Natl Acad Sci USA. 88, 8764-8.

Kuwabara, P. E., O'Neil, N. (2001) The use of functional genomics in *C. elegans* for studying human development and disease. J Inherit Metab Dis. 24, 127-38.

Kwong, M., Kan, Y. W., Chan, J. Y. (1999) The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. Role for Nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. J Biol Chem. 274, 37491-8.

Kyes, S., Pinches, R., Newbold, C. (2000) A simple RNA analysis method shows var and rif multigene family expression patterns in *Plasmodium falciparum*. Mol Biochem Parasitol. 105, 311-5.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680-5.

Lei, X. G., Evenson, J. K., Thompson, K. M., Sunde, R. A. (1995) Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. J Nutr. 125, 1438-46.

Leiers, B., Kampkotter, A., Grevelding, C. G., Link, C.D., Johnson, T. E., Henkle-Dührsen, K. (2003) A stress-responsive glutathione S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. Free Radic Biol Med. 34, 1405-15.

Leslie, I. G., Leanne, C. S., Bernard, D. L. (2005) Introduction of an additional pathway for lactate oxidation in the treatment of lactic acidosis and mitochondrial dysfunction in *Caenorhabditis elegans*. 18367-72.

Lewis, J. A., Fleming, J. T. (1995) Basic culture methods. Methods Cell Biol. 48, 3-29.

Li, Y., Wei, G., Chen, J. (2005) Glutathione: a review on biotechnological production. Appl Microbiol Biotechnol 66, 233-242.

Liao, V. H., Yu, C. W. (2005) *Caenorhabditis elegans* gcs-1 confers resistance to arsenic-induced oxidative stress. Biometals. 18, 519-28.

Liebau, E., Schonberger, O. L., Walter, R. D., Henkle-Duhrsen, K. (1994a) Molecular cloning and expression of a cDNA encoding glutathione S-transferase from *Ascaris suum*. Mol Biochem Parasitol. 63, 167–70.

Liebau, E., Walter, R. D., Henkle-Duhrsen, K. (1994b) *Onchocerca volvulus*: Isolation and sequence of a second glutathione-S-transferase cDNA. Exp Parasitol. 79, 68–71.

Liebau, E., Wildenburg, G., Brophy, P. M., Walter, R. D., Henkle-Duhrsen, K. (1996) Biochemical analysis, gene structure and localization of the 24 kDa glutathione S-transferase from *Onchocerca volvulus*. Mol Biochem Parasitol. 80, 27–39.

Liebau, E., Spillner, E., Henkle-Duhrsen, K. (1997) pOVEX vector: prokaryotic expression and purification of onchocerciasis vaccine candidate antigens as fusion proteins with the 24 kD *Onchocerca volvulus* glutathione S-transferase. Trop Med Int Health. 2, 691-4.

Liebau, E., Eschbach, M. L., Tawe, W., Sommer A., Fischer, P., Walter, R. D., Henkle-Duhrsen, K., (2000) Identification of a stress-responsive *Onchocerca volvulus* glutathione S-transferase (Ov-GST-3) by RT-PCR differential display. Mol Biochem Parasitol. 109,101-10.

Lincke, C. R., Broeks, A., The, I., Plasterk, R. H., Borst, P. (1993) The expression of two P-glycoprotein (pgp) genes in transgenic *Caenorhabditis elegans* is confined to intestinal cells. EMBO J. 12, 1615-20.

Lomaestro, B. M., Malone, M. (1995) Glutathione in health and disease: pharmacotherapeutic issues. Ann Pharmacother. 29, 1263-73.

Lopez-Barea, J., Lee, C. Y. (1979) Mouse-liver glutathione reductase. Purification, kinetics, and regulation. Eur J Biochem. 98, 487-99.

Lu, S. C. (2000) Regulation of glutathione synthesis. Curr Top Cell Regul. 36, 95-116.

Luersen, K., Muller, S., Hussein, A., Liebau, E., Walter, R. D. (2000) The gamma-glutamylcysteine synthetase of *Onchocerca volvulus*. Mol Biochem Parasitol. 111, 243-51.

Luo, J. L., Huang, C. S., Babaoglu, K., Anderson, M. E. (2000) Novel kinetics of mammalian glutathione synthetase: characterization of gamma-glutamyl substrate cooperative binding. Biochem Biophys Res Commun. 275, 577-81.

Maizels, R. M., Blaxter, M. L., Selkirk, M. E. (1993) Forms and functions of nematode surfaces. Exp Parasitol. 77, 380-4.

Mandell, G., Bennett, J., Dolin, R. (2005) Principle of infectious disease. Pediatric Infectious Disease Journal. 2, 3258-3261.

Mannervik, B. (1985) The isoenzymes of glutathione transferase. Ad Enzymol Relat Areas Mol Biol. 57, 357–417.

Mattevi, A., Schierbeek, A. J., Hol, W. G. (1991) Refined crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* at 2.2 A resolution. A comparison with the structure of glutathione reductase. J Mol Biol. 220, 975-94.

May, R. C., Plasterk, R. H. (2005) RNA interference spreading in *C. elegans*. Methods Enzymol. 392, 308-15.

May, M. J., Vernoux, T., Sanchez-Fernandez, R., Van Montagu, M., Inze, D. (1998) Evidence for postranslational activation of gamma glutamylcysteine synthetase during plant stress responses. Proc. Natl. Acad. Sci. USA 95, 12049-54.

Mayer, R. J, WalkerJ. H. (1987) Immunochemical methods in cell and molecular biology. Academic Press Ltd.

Meierjohann, S., Walter, R. D., Muller, S (2002). Glutathione synthetase from *Plasmodium falciparum*. Biochem J. 363, 833-8.

Meister, A. (1988) Glutathione metabolism and its selective modification. J Biol Chem. 263, 17205-8.

Meister, A., Anderson, M. E. (1983) Glutathione. Annu Rev Biochem. 52, 711-60.

Mello, C. C., Kramer, J. M., Stinchcomb, D., Ambros, V. (1991) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959-70.

Melov, S. Mitochondrial oxidative stress. Physiologic consequences and potential for a role in aging. Ann N Y Acad Sci. 908, 219-25.

Mittl, P. R., Schulz, G. E. (1994) Structure of glutathione reductase from *Escherichia coli* at 1.86 A resolution: comparison with the enzyme from human erythrocytes. Protein Sci. 3, 799-809.

Müller, S., Walter, R. D., Fairlamb, A. H. (1995) Differential susceptibility of filarial and human erythrocyte glutathione reductase to inhibition by the trivalent organic arsenical melarsen oxide. Mol Biochem Parasitol, 7, 211–19.

Müller, S., Gilberger, T. W., Fairlamb, A. H., Walter, R. D. (1997) Molecular characterization and expression of *Onchocerca volvulus* glutathione reductase. Biochem J, 325, 645–51.

Müller, S., Gilberger, T. W., Krnajski, Z., Luersen, K., Meierjohann, S., Walter, R.D. (2001) Thioredoxin and glutathione system of malaria parasite *Plasmodium falciparum.* Protoplasma. 217, 43-9.

Nagel, R. L., Roth, E. F., Jr. (1989) Malaria and red cell genetic defects. Blood. 74, 1213-21.

Newton, G. L., Javor, B. (1985) gamma-Glutamylcysteine and thiosulfate are the major low-molecular-weight thiols in halobacteria. J Bacteriol. 161, 438-41.

Newton, G. L., Fahey, R. C. (1995) Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. Methods Enzymol. 251, 148-66

Nicholas, W. L. (1984) The biologz of free living nematodes. 2nd Ed. Claredon Press, Oxford.

Njalsson, R., Carlsson, K., Olin, B., Carlsson, B., Whitbread, L., Polekhina, G., Parker, M. W., Norgren, S., Mannervik, B., Board, P. G., Larsson, A. (2000) Kinetic properties of missense mutations in patients with glutathione synthetase deficiency. Biochem J. 349, 275-9.

Njalsson, R., Norgren, S., Larsson, A., Huang, C. S., Anderson, M. E., Luo, J. L. (2001) Cooperative binding of gamma-glutamyl substrate to human glutathione synthetase. Biochem Biophys Res Commun. 289, 80-4.

Nomine, Y., Ristriani, T., Laurent, C., Lefevre, J. F., Weiss, E., Trave, G. (2001) A strategy for optimizing the monodispersity of fusion proteins: application to purification of recombinant HPV E6 oncoprotein. Protein Eng. 4, 297-305.

Novina, C. D., Sharp, P. A. (2004) The RNAi revolution. Nature. 430, 161-4.

Novogrodsky, A., Tate, S. S., Meister, A. (1976) gamma-glutamyl transpeptidase, a lymphoid cell-surface marker: relationship to blastogenesis, differentiation, and neoplasia. Proc Natl Acad Sci USA. 73, 2414-8.

Ohtake, Y., Satou, A., and Yabuuchi, S. (1990) Isolation and characterization of glutathione-deficient mutants in *Saccharomyces cerevisiae*. Agric. Biol. Chem. 54, 3145-3150.

Ozmen, I., Kufrevioglu, O. I. (2004) Effects of antiemetic drugs on glucose 6-phosphate dehydrogenase and some antioxidant enzymes. Pharmacol Res. 50, 499-504.

Pai, E. F., Karplus, P. A., Schulz, G. E. (1988) Crystallographic analysis of the binding of NADPH, NADPH fragments, and NADPH analogues to glutathione reductase. Biochemistry. 27, 4465-74.

Perham, R. N., Leistler, B., Solomon, R. G., Guptasarma, P. (1996) Protein engineering of domains in flavoprotein disulphide oxidoreductases: contributions to folding and assembly. Biochem Soc Trans. 24, 61-6.

Phlippen, N., Hoffmann, K., Fischer, R., Wolf, K., Zimmermann, M. (2003) The glutathione synthetase of *Schizosaccharomyces pombe* is synthesized as a homodimer but retains full activity when present as a heterotetramer. J Biol Chem. 278, 40152-61.

Plummer, J. L., Smith, B. R., Sies, H., Bend, J. R. (1981) Chemical depletion of glutathione in vivo. Methods Enzymol. 77, 50-9.

Polekhina, G., Board, P. G., Gali, R. R., Rossjohn, J., Parker, M. W. (1999) Molecular basis of glutathione synthetase deficiency and a rare gene permutation event. EMBO J. 18, 3204-13.

Pu, Y. S., Hour, T. C., Chen, J., Huang, C. Y., Guan, J. Y., Lu, S. H. (2002a) Arsenic trioxide as a novel anticancer agent against human transitional carcinoma--characterizing its apoptotic pathway. Anticancer Drugs. 13, 293-300.

Pu, Y. S., Hour, T. C., Chen, J., Huang, C. Y., Guan, J. Y., Lu, S. H. (2002b) Cytotoxicity of arsenic trioxide to transitional carcinoma cells. Urology. 60, 346- 50.

Rahman, Q., Abidi, P., Afaq, F., Schiffmann, D., Mossman, B. T, Kamp, D. W., Athar, M. (1999) Glutathione redox system in oxidative lung injury. Crit Rev Toxicol. 29, 543-68.

Rangasamy, T., Cho, C. Y., Thimmulappa, R. K., Zhen, L., Srisuma, S. S., Kensler, T. W., Yamamoto, M., Petrache, I., Tuder, R. M., Biswal, S. (2004) Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. J Clin Invest. 114, 1248-59.

Reiter, R. J., Tan, D. X., Cabrera, J., D'Arpa, D., Sainz, R. M., Mayo, J. C., Ramos, S. (1999) The oxidant/antioxidant network: role of melatonin. Biol Signals Recept. 8, 56-63.

Riddle, D. L., Brenner, S. (1978) Indirect suppression in *Caenorhabditis elegans*. Genetics. 89, 299-314.

Rietveld, P., Arscott, L. D., Berry, A., Scrutton, N. S., Deonarain, M. P., Perham, R. N., Williams, C. H. Jr. (1994) Reductive and oxidative half-reactions of glutathione reductase from *Escherichia coli*. Biochemistry. 33, 13888-95.

Ristoff, E., Augustson, C., Geissler, J., de Rijk, T., Carlsson, K., Luo, J. L., Andersson, K., Weening, R. S., van Zwieten, R., Larsson, A., Roos, D. (2000) A missense mutation in the heavy subunit of gamma-glutamylcysteine synthetase gene causes hemolytic anemia. Blood. 95, 2193-6.

Rodaway, A., Patient, R. (2001) Mesendoderm. An ancient germ layer? Cell, 105, 169-172.

Ruwende, C., Hill, A. (1998) Glucose-6-phosphate dehydrogenase deficiency and malaria. J Mol Med. 76, 581-8.

Ryazanova, L. V., Pavur, K. S., Petrov, A. N., Dorovkov, M. V., Ryazanov, A. G. (2001) Novel type of signaling molecules: protein kinases covalently linked with ion channels. Mol Biol. 35, 271-283.

Sadrzadeh, S. M., Graf, E., Panter, S. S., Hallaway, P. E., Eaton, J. W. (1984) Hemoglobin. A biologic fenton reagent. J Biol Chem. 259, 14354-6.

Sahlman, L., Williams, C. H. Jr. (1989) Titration studies on the active sites of pig heart lipoamide dehydrogenase and yeast glutathione reductase as monitored by the charge transfer absorbance. J Biol Chem. 264, 8033-8.

Salzman, L. A., Weissbach, A. (1967) Formation of intermediates in the replication of phage lambda DNA. J Mol Biol. 28, 53-70.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd Edition, Cold Spring HarbourLaboratory Press, Plainview, New York.

Sanger, F., Nicklen, S., Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-54.

Savvides, S. N., Scheiwein, M., Bohme, C. C., Arteel, G. E., Karplus, P. A., Becker, K., Schirmer, R. H. (2002) Crystal structure of the antioxidant enzyme glutathione reductase inactivated by peroxynitrite. J Biol Chem. 277, 2779-84.

Schirmer, R. H., Krauth-Siegel, R. L., Schulz, G. E. (1989) In: Coenzymes and Cofactors (Dophin, D., Poulson, R. And Avramovic, O., Eds) 3, 553-596, John Wiley & Sons, New York.

Schnabel, L. H., Schnabel, R. (1990) An organ-specific differentiation gene, pha-I, from *C. elegans*. Science 250, 686-688.

Schulz, G. E., Zappe, H. A., Worthington, D. J. Rosemeyer, M. A. (1975) Crystals of human erythrocyte glutathione reductase. FEBS Lett. 54, 86-88.

Schulz, G. E., Karplus, P. A. (1988) High resolution structure and catalytic action of human glutathione reductase. Biochem. Soc. Trans. 16, 81-88.

Schulz, G. E. (1992) Binding of nucleotides by proteins. Curr. Opin. Struct. Biol. 2, 61-67.

Shakes, D. C., Epstein, H. F., 1995. *Caenorhabditis elegans* modern biological analysis of an organism. Methods in Cell Biol. 48, 654.

Shantz, L. M., Viswanath, R., Pegg, A. E. (1994) Role of the 5'-untranslated region of mRNA in the synthesis of S-adenosylmethionine decarboxylase and its regulation by spermine. Biochem J. 302, 765-72.

Shi, Z. Z., Habib, G. M., Rhead, W. J, Gahl, W. A., He, X., Sazer, S., Lieberman, M. W. (1996). Mutations in the glutathione synthetase gene cause 5-oxoprolinuria. Nat Genet. 14, 361-5.

Singh, A., Rangasamy, T., Thimmulappa, R. K., Lee, H., Osburn, W. O., Brigelius-Flohe, R., Kensler, T. W., Yamamoto, M., Biswal, S. (2006) Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs is regulated by Nrf2. Am J Respir Cell Mol Biol. 1-8.

Sies, H. (1986) Biochemie des oxidativen Stress. Angew. Chem. 98, 1061-1098

Sies, H. (1989). Biochemistry of thiol groups: the role of glutathione. Naturwissenschaften. 76, 57-64

Sies, H. (1999). Glutathione and its role in cellular functions. Free Radic Biol Med. 27, 916-21

Sipos, K., Lange, H., Fekete, Z., Ullmann, P., Lill, R., Kispal, G. (2002) Maturation of cytosolic iron-sulfur proteins requires glutathione. J Biol Chem. 277, 26944-9

Slater, A. F., Stefan, C., Nobel, I., van den Dobbelsteen, D.J., Orrenius, S. (1995) Signalling mechanisms and oxidative stress in apoptosis. Toxicol Lett. 82-83, 149-53.

Sommer, A., Nimtz, M., Conradt, H. S., Brattig, N., Boettcher, K., Fischer, P., Walter, R. D., Liebau, E. (2001) Structural analysis and antibody response to the extracellular glutathione S-transferases from *Onchocerca volvulus*. Infect Immun. 69, 7718-28.

Speers-Roesch, B., Ballantyne, J. S. (2005) Activities of antioxidant enzymes and cytochrome c oxidase in liver of Arctic and temperate teleosts. Comp Biochem Physiol A Mol Integr Physiol. 140, 487-94.

Steenkamp, D. J. (2000) Thiol metabolism of the trypanosomatids as potential drug targets. IUBMB Life. 53, 243-8.

Stephen, D. W., Jamieson, D. J. (1997) Amino acid-dependent regulation of the *Saccharomyces cerevisiae* GSH1 gene by hydrogen peroxide. Mol. Microbiol. 23, 203–210.

Stevens, R. G., Creissen, G. P., Mullineaux, P.M. (1997) Cloning and characterisation of a cytosolic glutathione reductase cDNA from pea (*Pisum sativum* L.) and its expression in response to stress. Plant Mol Biol. 35, 641-54.

Strandberg, L., Enfors, S. O. (1991) Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. Appl Environ Microbiol. 57, 1669-74.

Stringham, E. G., Jones, D., Candido, E. P. (1992) Expression of the polyubiquitin-encoding gene (ubq-1) in transgenic *Caenorhabditis elegans*. Gene. 113, 165-73.

Stringham, E. G., Candido, E. P. (1993) Targeted single-cell induction of gene products in *Caenorhabditis elegans*: a new tool for developmental studies. J Exp Zool. 266, 227-33.

Stinchcomb, D. T., Shaw, J. E., Carr, S. H., Hirsh, D. (1985) Extrachromosomal DNA transformation of *Caenorhabditis elegans*. Mol Cell Biol. 5, 3484-96.

Studier, F. W., Moffat, B. A. (1986) Use of bacteriophageT7 RNA polymerase to direct selective high level expression of cloned genes. J. Mol. Biol. 189, 113-130.

Sugiyama, K., Izawa, S., Inoue, Y. (2000) The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. J Biol Chem. 275, 15535-40.

Sulston, J. E., Schierenberg, E., White, J. G., Thomson, J. N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev Biol. 100, 64-119.

Sun, Y. (1990) Free radical antioxidant enzymes, and carcinogenesis. Free Rad. Biol. Med. 8, 583-599

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., Mello, C. C. (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell. 99, 123-32.

Tetaud, E., Manai, F., Barrett, M. P., Nadeau, K., Walsh, C. T., Fairlamb, A. H. (1998) Cloning and characterization of the two enzymes responsible for trypanothione biosynthesis in *Crithidia fasciculata*. J Biol Chem. 273, 19383-90.

Thompson, G. A., Meister, A. (1977) Interrelationships between the binding sites for amino acids, dipeptides, and gamma-glutamyl donors in gamma-glutamyl transpeptidase. J Biol Chem. 252, 6792-8.

Timmons, L., Fire, A. (1998) Specific interference by ingested dsRNA. Nature. 395, 854.

Timmons, L., Court, D. L., Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans.* Gene. 263, 103-12.

Toborek, M., Hennig, B. (1994) Fatty acid-mediated effects on the glutathione redox cycle in cultured endothelial cells. Am J Clin Nutr. 59, 60-5.

Toone, W. M., Morgan, B. A., Jones, N. (2001) Redox control of AP-1-like factors in yeast and beyond. Oncogene. 20, 2336-46.

Tutic, M., Lu, X. A., Schirmer, R. H., Werner, D. (1990) Cloning and sequencing of mammalian glutathione reductase cDNA. Eur J Biochem. 188, 523-8.

Uhlen, I. R., Moks, T. (1990) Gene fusions for purpose of expression: an introduction. Methods Enzymol. 185, 129-143.

Usanga, E. A., Luzzatto, L. (1985) Adaptation of *Plasmodium falciparum* to glucose 6-phosphate dehydrogenase-deficient host red cells by production of parasite-encoded enzyme. Nature. 313, 793-5.

van Bladeren, P. J. (2000) Glutathione conjugation as a bioactivation reaction. Chem Biol Interact. 129, 61-76.

Veine, D. M., Arscott, L. D., Williams, C. H., Jr. (1998) Redox potentials for yeast, *Escherichia col*i and human glutathione reductase relative to the NAD+/NADH redox couple: enzyme forms active in catalysis. Biochemistry. 37, 15575-82.

Wang, J., Barr, M. M. (2005) RNA interference in *Caenorhabditis elegans*. Methods Enzymol. 392, 36-55.

Wang, C. L., Oliver, D. J. (1997a) Identification of a putative flexible loop in *Arabidopsis* glutathione synthetase. Biochem J. 322, 241-4.

Wang, C. L., Oliver, D. J. (1997b) Glutathione synthetase: similarities of the proteins from *Schizosaccharomyces pombe* and *Arabidopsis thaliana*. Biochem J. 326, 563-6.

Warga, R. M., Nusslein-Volhard, C. (1999) Origin and development of the zebrafish endoderm. Development. 126, 827-38.

Westwater, J., McLaren, N. F., Dormer, U.H, Jamieson DJ. (2002) The adaptive response of *Saccharomyces cerevisiae* to mercury exposure. Yeast. 19, 233-9.

WHO Expert committee on Onchocerciasis control (1987) Onchocerciasis and its control. WHO technical Report Series 752.

WHO Expert committee on Onchocerciasis (River Blindness) (2000) Fact Sheet No 95: 1–4.

Williams, D. G. (1976) Methods for the estimation of three vitamin dependent red cell enzymes. Clin Biochem. 9, 252-5.

Wolf, G. (2001) Transforming a health care system by transforming professional practice. J Perinat Neonatal Nurs. 15, 1-7.

Worthington, D. J., Rosemeyer, M. A. (1974) Human glutathione reductase: purification of the crystalline enzyme from erythrocytes. Eur J Biochem. 48, 167-77.

Wu, A. L., Moye-Rowley, W. S. (1994) GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. Mol Cell Biol. 14, 5832-9.

Wu, Q., Huang, K., Xu, H. (2003) Effects of long-term selenium deficiency on glutathione peroxidase and thioredoxin reductase activities and expressions in rat aorta. J Inorg Biochem. 94, 301-6.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., Turner, N. D.(2004) Glutathione metabolism and its implications for health. J Nutr. 134, 489-92.

Xia, C., Hu, J., Ketterer, B., Taylor, J. B. (1996) The organization of the human GSTPI gene promoter and its response to retinoic acid and cellular redox status. Biochem J, 313, 155–61.

Xiang, C., Oliver, D. J. (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell. 10, 1539-50.

Yuan, J. (2003) Genetic studies of aging and longevity in model organisms. Bioessays. 25, 134-41.

Zamore, P., Tuschl, T., Sharp, P., Bartel, D. (2000) RNA*i* double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 101, 25-33.

Zhang, Y., Konig, I., Schirmer, R. H. (1988) Glutathione reductase-deficient erythrocytes as host cells of malarial parasites. Biochem Pharmacol. 37, 861-5.

Zhang, L. (1989) Phospholipid hydroperoxide glutathione peroxidase: specific activity in rats of different ages. Biochim Biophys Acta, 1006, 140–43.

Acknowledgements

First, I will express my appreciation to the Deutscher Akademischer Austausch Dienst (DAAD) for the scholarship granted to undertake this research.

I would like to thank Professor Dr. Rolf D. Walter for accepting me to do a PhD in the Department of Biochemistry and for accepting to co-referee the dissertation. My profound gratitude to Professor Dr. Eva Liebau for accepting to supervise this thesis. Most importantly, I would like to thank her for the huge support, guidance and patience throughout the course of this study. Also, I am very much indebted to Dr. Kai Lüersen for the amazing kindness and critical observations in this thesis and during the last phase of this research.

Special thanks to all my friends and colleagues at the Bernhard Nocht Institute. In particular, my deepest appreciation goes to:

- Bärbel Bergmann for the continuous help during the past four years.
- Marie-Luise Eschbach for always being a good and supporting friend.
- Marzena and Silke for the friendliness and frank discussions.
- Cora for the solutions to the frequent computer problems and the honest advice and care.
- Ingrid for the valuable comments and rewarding discussions.
- Robin for the moral support and help in German language and translations.
- Jana, Martin, Rosie and Nashya for the creative ideas, sharing knowledge and experience.
- Carsten and Tim for the cooperation and friendly attitude.

My deepest thanks also goes to my family friends, Siggi and Adri van den Boorgaard for the huge moral support they have given me and my small family in Germany.

My deepest gratitude to my loving parents, Deborah and Jeremiah Ajonina for the great sacrifices and difficulties they had to endure to see me through the university and the encouragement that played a major role in the success of this thesis.

I am also indebted to my mother in-law for the great inspiration, love and encouragement.

I am sincerely thankful to my husband, Christopher Buzie and my two kids, Caris and Kelsie Buzie for being such a wonderful source of inspiration, consolation and encouragement. With you as my family I feel blessed, motivated and proud.

To all my brothers and sisters especially Irene, Humphrey, Gordon, Stanley and Gildas I say God bless you all for the endless love, support and care.

To the Almighty God be the glory.