From the Division of Biochemistry, Department of Medical Biochemistry
and Biophysics, Karolinska Institutet, Stockholm, Sweden

DEVELOPMENT AND BIOMEDICAL APPLICATIONS OF HIGHLY SENSITIVE ENZYME ACTIVITY ASSAYS OF THIOREDOXIN, THIOREDOXIN REDUCTASE AND GLUTAREDOXIN

Sergio J. Montano

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7549-208-7

“Surprises and reverses can serve as an incentive for great accomplishment. There are no rules here, we're just trying to accomplish something.”

- Thomas A. Edison

ABSTRACT

Oxidative stress is implicated in the pathogenesis of many human diseases, thus it is interesting to study the relationship between antioxidants, the development and progression of diseases. The thioredoxin and glutaredoxin systems are ubiquitous redox-active proteins known to be induced in chronic inflammatory related processes, such as cancer and diabetes, to protect against oxidative stress. The thioredoxin system is composed of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. The glutaredoxin system comprises glutathione reductase (GR), glutathione (GSSG/GSH), glutaredoxin (Grx) and NADPH. Trx is an important antioxidant and a redox regulator, crucial for the activation of transcription factors and modulation of intra- and extracellular signaling pathways. While, Grx is another crucial redox regulator with an important role in maintaining intracellular GSH-disulfide-dithiol exchange, apoptosis and cell differentiation. In fact, Trx, Grx and TrxR have been suggested biomarkers for disease monitoring. Thus, there is a current need for new techniques to detect and monitor Trx, Grx and TrxR activities in human patient samples; since the traditional methods showed several limitations related to background, specificity and sensitivity. The aim of this thesis was to develop highly sensitive and reproducible assays to enable analysis of Trx, TrxR and Grx activities in clinical patient samples. Thus, we optimized the synthesis of two fluorescent substrates, dieosin-diglutathione (Di-E-GSSG) and fluorescein labelled insulin (FITC-insulin), which both gave higher fluorescence upon disulfide reduction. The latter, FITC-insulin, was used to develop highly sensitive microplate assays for Trx ($\geq$ 0.4 picomoles) and TrxR ($\geq$ 40 femtomole). Moreover, this method allowed reproducible measurements of re-activated Trx, commonly present in frozen or over-oxidized samples from cell, tissue (biopsies) and blood plasma origin (*manuscript I*). The former Di-E-GSSG, was an excellent substrate of Grx and could be used to glutathionylate proteins (such as BSA, yielding E-GS-BSA), thus becoming a useful fluorescent marker for glutathionylated proteins in gel electrophoresis. This mixed disulfide substrate, E-GS-BSA, allowed measurements of Grx1 and Grx2 activities in picomole concentrations (*manuscript II*). E-GS-BSA was further a key substrate for reverse-S-glutathionylation catalysis studies, which facilitated the characterization and study of the catalytic properties of human recombinant Grx5 (*paper IV*). In addition, we applied these optimized methods to the study of relevant clinical samples from patients showing a mutation in the selenocysteine insertion sequence–binding protein 2 gene, which lead to a multisystem selenoprotein deficiency disorder. Our measurements of TrxR activity in skin biopsies and PBMCs from these patients, showed significant decreased TrxR activity and concomitant increased of ROS levels when compared to healthy controls (*paper III*). In conclusion, we present novel sensitive tools to study Trx, TrxR and Grx activities in complex samples from biological origin. Since these redox enzymes have been suggested as potential biomarkers for several diseases; the actual relevance of our newly developed methodologies, goes beyond the enzymatic measurements performed, as these methods might assist in detecting and/or analyzing disease progression with clear biomedical applications.

LIST OF PUBLICATIONS

I. **Montano SJ**, Lu J, Gustafsson TN, Holmgren A. Sensitive activity assays of mammalian thioredoxin and thioredoxin reductase: Fluorescent disulfide substrates, mechanisms and use with tissue samples. *Manuscript*

II. **Montano SJ**, Coppo L, Padilla A, Holmgren A. Determination of glutaredoxin enzyme activity and S-glutathionylation using fluorescent glutathione. *Manuscript*

III. Schoenmakers E, Agostini M, Mitchell C, Schoenmakers N, Papp L, Rajanayagam O, Padidela R, Ceron-Gutierrez L, Doffinger R, Prevosto C, Luan J, **Montano S**, Lu J, Castanet M, Clemons N, Groeneveld M, Castets P, Karbaschi M, Aitken S, Dixon A, Williams J, Campi I, Blount M, Burton H, Muntoni F, O'Donovan D, Dean A, Warren A, Brierley C, Baguley D, Guicheney P, Fitzgerald R, Coles A, Gaston H, Todd P, Holmgren A, Khanna KK, Cooke M, Semple R, Halsall D, Wareham N, Schwabe J, Grasso L, Beck-Peccoz P, Ogunko A, Dattani M, Gurnell M, Chatterjee K. Mutations in the selenocysteine insertion sequence–binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans. *The Journal of Clinical Investigation*. 2010 Dec;120(12):4220-35.

IV. Johansson C, Roos AK, **Montano SJ**, Sengupta R, Filippakopoulos P, Guo K, von Delft F, Holmgren A, Oppermann U, Kavanagh KL. The crystal structure of human GLRX5: iron−sulfur cluster co-ordination, tetrameric assembly and monomer activity. *Biochemical Journal*. 2011 Jan 15;433(2):303-11.

CONTENTS

LIST OF ABBREVIATIONS

[Fe-S]	Iron sulfur
ASK	Apoptosis signaling kinase
CoQ 10	Ubiquinone / Ubiquinol
Cys	Cysteine
Di-E-GSSG	Di-eosin-glutathione disulfide
DTNB	5,5´-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E. coli	Escherichia coli
E-GS-BSA	Eosin-glutathione-albumin
EITC	Eosin 5-isothiocyanate
FAD	Flavin adenine dinucleotide
FiTC	Fluorescein isothiocyanate
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-trandsferase
LDL	Low density lipoproteins
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
PBMC	Peripheral blood mononuclear cell
PDI	Protein disulfide isomerase
P-S_2	Protein disulfide
P-SG	Glutathionylated protein
P-SH	Protein thiol
PTEN	Phosphatase and tensin homologue deleted on chromosome
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SBP2	Selenocysteine insertion sequence–binding protein 2
SOD	Superoxide dismutase
TGR	Thioredoxin-glutathione reductase
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Txnip	Thioredoxin interacting protein

1 INTRODUCTION

Reduction and oxidation reactions, known as redox, are chemical reactions characterized by the transfer of one or more electrons from one atom to another. A reduction takes place when an atom or molecule gains electrons whereas oxidation occurs when an atom or molecule loses electrons. The redox homeostasis is preserved by molecules that induce reduction or oxidation reactions and these are referred to antioxidants or pro-oxidants. Redox state is the balance of oxidized and reduced molecules in biological systems such as cells or organs, which is preserved by an interaction between the anti- and pro-oxidants. The maintenance of the redox state is important for living organism in order to sustain crucial biological processes such as growth, metabolism, gene expression, etc. The intracellular milieu is always maintained in a reduced state while the environment outside cells is prone to be more oxidized. The delicate redox state of a cell can however be perturbed as a result of low antioxidant protection or exposure to high amounts of oxidants resulting in oxidative stress. Redox state imbalance consequentially alters cellular processes, causing damage to cellular components such as DNA, lipids, and proteins among others. The formation of free radicals (atoms or molecules with unpaired number of electrons) and peroxide ions (O_2^{2-}) are common sources that perturb the redox homeostasis. These types of atoms or molecules are commonly known as reactive oxygen species (ROS).

1.1 REACTIVE OXYGEN SPECIES

Aerobic organisms require oxygen for the cellular respiration in order to produce energy. During this process derivatives of oxygen may produce ROS (1,2). Low concentrations of ROS are required for certain cellular processes such as regulation of protein functions, apoptosis, activation of transcription factors and regulation of genes (3-8). However, excess of ROS may disturb important cellular processes and cause damage to DNA, proteins and lipids (9-12). The most common type of ROS are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). Superoxide anions are formed when oxygen gains a single electron. This free radical lacks the ability to penetrate membranes and is not highly reactive *per se*, but it is the precursors of other reactive species (13). Hydrogen peroxide is not a free radical, but an intermediate for production of the very reactive hydroxyl radical. When hydrogen peroxide is reduced by either Cu^{+} or Fe^{2+} (Fenton reaction), hydroxyl radicals are formed and these free radicals may be deleterious to a wide range of molecules including lipids, DNA, amino acids and carbohydrates (*Figure 1*).

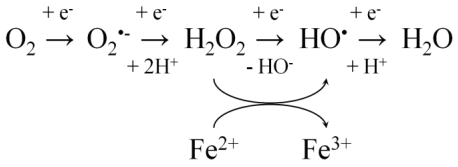

Figure 1. **The formation of reactive oxygen species**.

1.1.1 Redox imbalance and diseases

ROS may cause damage to a variety of molecules, thereby affecting many physiological processes resulting in the development of various diseases such as cancer, neurodegenerative and metabolic disorders (14-21).

1.1.1.1 Cancer

Redox imbalance has been found in cancer cells and it is therefore believed that oxidative stress is closely linked to oncogenic stimulation (22). For instance, when DNA is damaged the tumor suppressor proteins p53 and PTEN (phosphatase and tensin homologue deleted on chromosome 10) prevent the cell from dividing until either the damage is repaired or the cell undergoes apoptosis (23,24). However, both p53 and PTEN are very sensitive to oxidative damage and ROS could inactivate these proteins resulting in uncontrolled cell growth (25,26).

1.1.1.2 Neurodegeneration

Alzheimer's disease is a classic example of a neurodegenerative disease linked to ROS. Copper is an important source for free radical production in the brain (27) due to its interaction with amyloid-beta (Aβ) peptide. Aβ is a redox active metallopeptide that binds copper and becomes electrochemically active which leads to the conversion of oxygen into hydrogen peroxide, setting up conditions that could promote Fenton reaction (*Figure 2*) and thus the production of hydroxyl radicals (28).

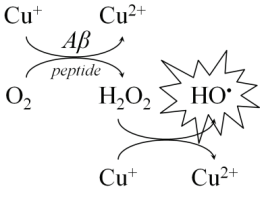

Figure 2. **Model of Aβ-mediated oxidative stress in Alzheimer's disease.** In the presence of oxygen, Cu^{+} is oxidized to Cu^{2+} by the peptide Aβ and producing H_2O_2 which reacts with another Cu^{+} producing hydroxyl ($HO^{\bullet}$) via the Fenton reaction.

1.1.1.3 Metabolic disorders

Risk factors such as an unhealthy diet, obesity and increased age may contribute to an oxidative environment that may alter insulin sensitivity either by impairing glucose tolerance or increasing insulin resistance. A metabolic disorder that is often linked to oxidative stress is diabetes mellitus (29). Diabetes mellitus is characterized by impaired glucose uptake in musculature and adipose tissue.

The majority of diabetes mellitus patients are capable of producing insulin but with time become increasingly insulin resistant (diabetes mellitus type 2) consequently increasing blood glucose levels which leads to chronic hyperglycemia. Hyperglycemia is known to generate ROS (30), which may lead to pancreatic β-cell dysfunction, impaired glucose tolerance, mitochondrial dysfunction and ultimately cause the development of diabetes mellitus and diabetes related organ complications (31).

Another interesting link between ROS and diabetes is the ROS-dependent activation of nicotinamide adenine dinucleotide phosphate oxidase (NOX) caused by hyperglycemia. Nicotinamide adenine dinucleotide phosphate (NADPH), the substrate of NOX, plays a major role in the defense against oxidative stress. When NADPH is oxidized via NOX, electrons are released. These electrons may be coupled to oxygen to generate superoxide, hydrogen peroxide and subsequently hydroxyl radical (*Figure 3*) (32).

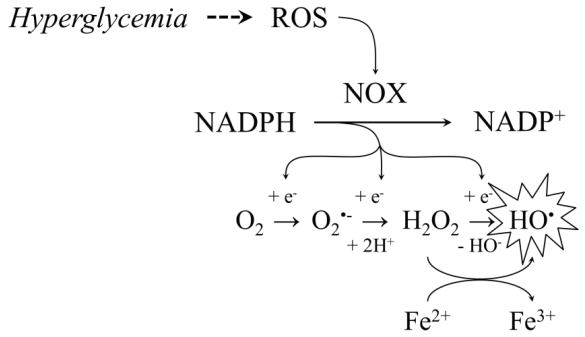

Figure 3. **Formation of oxygen radicals by hyperglycemia.** Hyperglycemia stimulates the formation of ROS. NOX is activated and electrons are continuously released from the oxidation of NADPH, enhancing the formation of more oxygen radicals.

1.1.2 Antioxidants

Antioxidants are molecules that serve as defense against excessive ROS formation and oxidative stress by preventing oxidation. Vitamin A, vitamin C, vitamin E, ubiquinol or coenzyme Q 10 (CoQ10) and glutathione are examples of non-enzymatic antioxidants while superoxide dismutase, catalase, various peroxidases, thioredoxin and glutaredoxin, among others, are enzymatic antioxidants.

1.1.2.1 Vitamins

Carotenoids (vitamin A-analogues) are lipid-soluble molecules found in dietary products such as poultry, fish meat, fruits and vegetables. Vitamin A can also be found in dietary supplements in the form of α- or β-carotene. β-Carotene is cleaved in the human intestine to retinal, which is an active antioxidant that prevents lipid peroxidation (33).

Ascorbate (vitamin C) is the most efficient antioxidant in human plasma known for its capacity to scavenge ROS. Vitamin C has been described to be an abundant antioxidant in many cell types participating in the reduction of superoxide and peroxides. Its main function is to prevent damage to lipoproteins such as low density lipoproteins (LDL), in human plasma, or to the lipids on the cell membrane (34).

α-Tocopherol (vitamin E) is a lipid-soluble antioxidant that is produced by plants and anchored to biological membranes. α-Tocopherol can turn into a radical (tocopheroxyl radical) capable of sequestering the free electron from a reactive radical. Tocopheroxyl is then be reduced to α-tocopherol by another antioxidant including vitamin C and ubiquinol among others (35).

1.1.2.2 Ubiquinone / Ubiquinol (CoQ10)

Ubiquinone (oxidized form) and Ubiquinol (reduced form) are ubiquitously expressed in aerobic organisms. Its main function is to transfer electrons between mitochondrial complex II and III during oxidative phosphorylation. CoQ10 is a lipid-soluble molecule that can be found in low concentrations in plasma, bound to cell membranes and to LDL affording protection against lipid peroxidation (36).

1.1.2.3 Glutathione

Glutathione, a tripeptide composed of the amino acids glutamic acid, cysteine and glycine, is the most abundant antioxidant in mammalian cells and crucial for maintenance of the redox balance in cells. Glutathione exists both in reduced (GSH) and oxidized (GSSG) form whose function depends on its reactive thiol (SH) in the cysteinyl moiety (*Figure 4*). The GSH form exist in a 100 times higher molar ratio than

GSSG, uses its thiol for detoxification of ROS (37). Glutathione is an antioxidant that can either neutralize ROS *per se* or be a cofactor of other ROS scavengers such as glutathione-S-transferase (GST) and glutathione peroxidase (GPx) (38,39). The antioxidant capability of GSH lies in its thiol which is able to accommodate the loss of a single electron in reduction reactions. The reaction leads to the formation of thiyl radicals ($GS^{\bullet}$) which can dimerize to form GSSG.

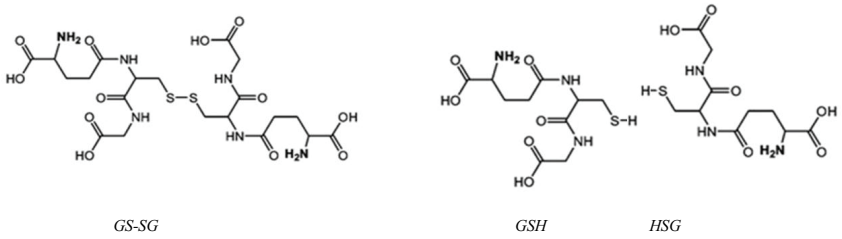

Figure 4. **Glutathione.** Glutathione disulfide (left panel) and two reduced glutathione molecules (right panel).

In addition to its ability to act as an antioxidant, glutathione is involved in posttranslational modifications of proteins via S-glutathionylation which regulates the activity of proteins that are crucial in maintaining cellular functions such as metabolism, differentiation and cell growth (40). S-glutathionylation refers to the addition of GS- to cysteine (Cys) residues in proteins thus forming mixed disulfides (*Figure 4*). S-glutathionylation is not only a mechanism for inactivation of proteins via "oxidation" of the Cys-residue, but also a way to protect proteins from other unwanted modifications or irreversible damage as result of oxidative stress. For instance, there are protein modifications caused by ROS such as irreversible oxidation to sulfinic and sulfonic acid that may lead to irreparable damage of the protein or proteosomal degradation (41). Therefore, S-glutathionylation is effective in protecting the Cys-residue of proteins from irreversible oxidation and overcomes the effects of oxidative stress conditions. Furthermore, S-glutathionylation can be reversed via reverse thiol-disulfide exchange reactions that may occur either spontaneously or catalyzed by a number of oxidoreductases such as glutathione-S-transferase, thioredoxin, glutaredoxin and sulfiredoxin (*Figure 5*) (42-45).

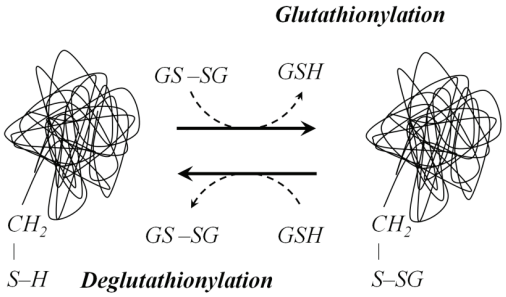

Figure 5. **Protein glutathionylation and deglutathionylation**.

1.1.2.4 Superoxide dismutases and catalases

Within a cell, superoxide dismutases (SOD) constitute the first line of defense against ROS. They catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (*reaction 1*). SODs are metal-containing enzymes that exist in different compartments of the cell and in different forms. Cytosolic SOD contain copper and zinc (Cu/Zn-SOD) while mitochondrial SOD has manganese bound (Mn-SOD) (46).

$$2O_2^{\bullet -} \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{(1)}$$

Much like SOD, catalase is a metalloprotein that catalyzes dismutation of hydrogen peroxide into water and oxygen (*reaction 2*). Thus, catalases reduce the risk of formation of hydroxyl radicals (47).

$$2H_2O_2 \xrightarrow{Catalase} H_2O + O_2 \quad \textbf{(2)}$$

1.1.2.5 Peroxidase

Peroxidases are enzymes that utilizes H_2O_2 as substrate (48). Glutathione peroxidases are the most studied peroxidases. These are tetrameric proteins where each monomer contains a selenium atom at the catalytic site in the form of selenocysteine. In its selenol form (protein-Se), GPx reacts with peroxide (H_2O_2) (*reaction 3*) or lipid peroxide (LOOH) (*reaction 4*) forming selenenic acid (protein-SeOH). The selenenic acid group is then reduced back to a selenol by two GSH which in turn are oxidized to GSSG while LOOH is reduced to its corresponding alcohol (LOH) (39).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \qquad \textbf{(3)}$$
$$LOOH + 2GSH \rightarrow GSSG + H_2O + LOH \quad \textbf{(4)}$$

1.2 THE THIOREDOXIN FAMILY

Thioredoxins (Trxs), Trx-like proteins, glutaredoxins (Grxs), PDI, GST and GPx are members of the thioredoxin family (49-53). These proteins participate in post-translational modification of proteins, redox-state regulations and catalysis of protein reduction (54-57). Members of the thioredoxin family are characterized by a common structural motif known as the thioredoxin fold comprised of a central core of four-stranded β-sheet surrounded by three α-helices (*Figure 6*) (58-60). All members of the Trx family share a well conserved active site (-Cys-X-X-Cys-) which catalyzes the reversible reduction of disulfides (49,61,62). The active site Cys residues are located proximal to the N-terminal and exposed towards the surface while the C-terminal Cys-residues are buried in the structure (63)

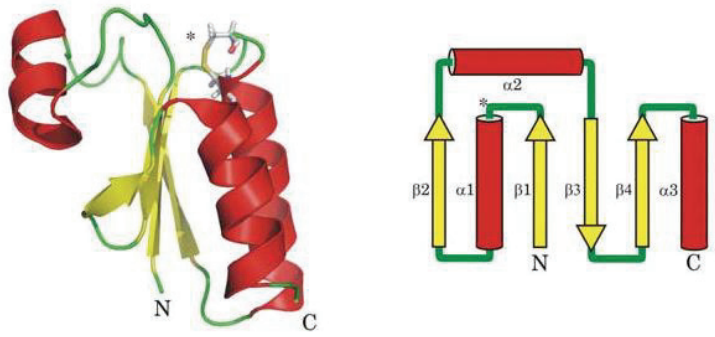

***Figure 6*. The thioredoxin fold.** Bacterial Grx1 displays the typical Trx fold composed of four-stranded β-sheet surrounded by three α-helices (left panel). The right panel presents the arrangement of the β-sheet (arrows), the α-helices (barrels). The position of the active site is shown by the asterisk.

Trx and Grx are part of the thioredoxin and glutaredoxin systems, respectively. Together with GSH, these systems are important for the maintenance of a reduced intracellular milieu via redox regulation through reduction of disulfides to sulfhydryl groups (44). These two systems have both specific and shared functions. The Trx system is often described as an antioxidant (64), but has also been demonstrated to be crucial for the regulation of transcription factors, modulation of intra- and extracellular signaling pathways and to be involved in immune response (65-69). The Grx system has multiple functions in maintaining intracellular GSH-disulfide-dithiol exchange, apoptosis, cellular differentiation and, like Trx, defense against oxidative stress (70-74).

1.2.1 The thioredoxin system

The thioredoxin system is composed of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH (*Figure 7*). It was discovered in 1964 as the hydrogen donor of ribonucleotide reductase (RNR), the enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides, in *Escherichia coli* (E. coli) (75). In the Trx system, electrons are transferred from the coenzyme NADPH to TrxR and on to Trx which in turn is capable of reducing a wide range of proteins (76). Additionally, the Trx system is ubiquitous hence it exists in basically all life forms and is one of the most important systems that regulate cellular redox signaling and maintain the delicate redox homeostasis (77-83). Moreover, it is actively involved in the regulation of cell proliferation and growth, apoptosis, activation of transcription factors and defense against oxidative stress (64,84-89).

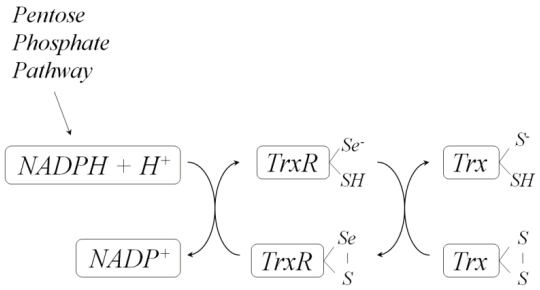

Figure 7. **The mammalian thioredoxin system.** Thioredoxin *(Trx)* is reduced by thioredoxin reductase *(TrxR)* using electrons from nicotinamide adenine dinucleotide phosphate *(NADPH)*.

1.2.1.1 Thioredoxin reductase

Thioredoxin reductase is a flavoenzyme that belongs to the pyridine nucleotide-disulfide reductase family, originally purified from E. coli. (90-92). The mammalian TrxR is a 112 kDa homodimer, arranged in a head-to-tail conformation, with a redox active dithiol/disulfide in each unit that contains a NADPH, a flavin adenine dinucleotide (FAD) and an interface domain (93). In addition, mammalian TrxRs share a conserve N-terminal active site composed of -Cys-Val-Asn-Val-Gly-Cys- whereas the C-terminal comprises a selenium containing active site that is not present in lower organisms. TrxRs has been characterized in various species including rat, calf and human displaying a broad substrate specificity including thioredoxin, vitamin K, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (94-96). There are so far three known mammalian TrxRs: cytosolic TrxR1, mitochondrial TrxR2 and the testis-specific thioredoxin-glutathione reductase (TGR) (95,97,98). TGR differs markedly from

TrxR1 and TrxR2 because it has an extended N-terminal site that is composed of a monothiol glutaredoxin domain thus TGR possess the unique ability to not only reduce Trx but also GSSG and Grx.

1.2.1.2 Thioredoxin

Thioredoxin is a small protein with a molecular mass of 12 kDa first isolated in pure form as the hydrogen donor for ribonucleotide reductase from *Escherichia coli* (85,99). Trx is a ubiquitous protein that exist in different species and tissues with a conserved redox-active dithiol/disulfide in the active site Cys-Gly-Pro-Cys (56,100). Trx is well known for participating in regulation of the enzyme activity of many proteins via thiol redox control involving reversible thiol-disulfide exchange (101,102). The structure of Trx is that of a globular protein, composed of five strands of β-sheets that are surrounded by four α-helices with an active site located at the N-terminal (*Figure 8*) (58). In contrast to lower organisms, mammalian Trx has three additional cysteine residues (Cys-62, Cys-69 and Cys-73), apart from Cys-32 and Cys-35 that forms the active site of the protein (103).

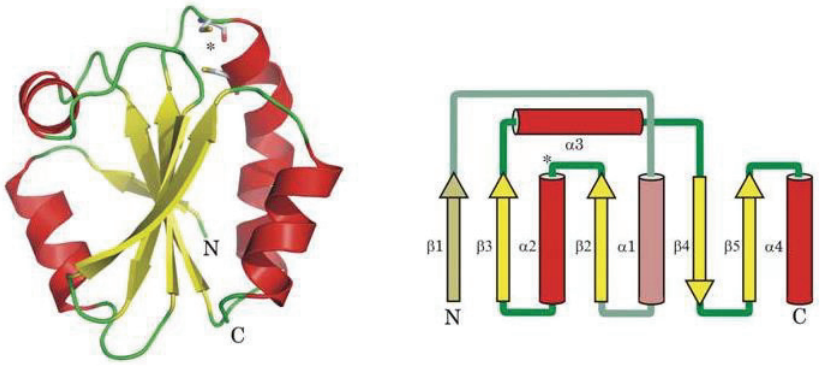

Figure 8. **Human thioredoxin.** Human thioredoxin is a globular protein with the typical thioredoxin family Cys–X–X–Cys active site motif (left panel). The protein is composed of five β-sheets (arrows), the four α-helices (barrels) (right panel). The active site located at the N-terminal is indicated by the asterisk.

Trxs are important oxidoreductases involved in redox regulations via thiol-disulfide exchange reactions to protect against oxidative stress and uphold the intracellular redox homeostasis (104). The active disulfide in the oxidized form of Trx is reduced by electrons transferred from NADPH through TrxR (*reaction 5*) and consequently Trx may reduce the Cys-residues of other proteins (*reaction 6*) (105).

$$Trx - S_2 + NADPH + H^+ \xrightarrow{TrxR} Trx - (SH)_2 + NADP^+ \quad \textbf{(5)}$$

$$Trx - (SH)_2 + protein - S_2 \longrightarrow Trx - S_2 + protein - (SH)_2 \quad \textbf{(6)}$$

There are three known Trxs in human: cytosolic Trx1, mitochondrial Trx2 and spermatozoon specific spTrx (106-108).

Trx1 is the most studied of the Trxs. Even though Trx1 is mainly cytosolic, it may translocate to the nucleus during oxidative stress (109). In addition, Trx1 is also known to exists in the extracellular milieu acting either as a co-cytokine displaying chemokine-like activity or electron donor for plasma glutathione peroxidase (110,111).

The function of Trx1 goes beyond being an oxidoreductase. Besides regulating the activity of other proteins via thiol-disulfide reduction, Trx may also regulate other proteins via protein-protein complex formation. Even though the mechanism for binding is not completely known, it is believed to occur via formation of mixed disulfides through cysteine-cysteine interaction. One example is the binding of Trx1 to apoptosis signaling kinase 1 (ASK1) (67) where reduced Trx1 forms a complex with the N-terminal portion of ASK1 resulting in suppressed kinase activity of ASK1 while oxidation of Trx1 leads to dissociation and activation of ASK1.

The activity of Trx1 is regulated by Thioredoxin interacting protein (Txnip), a 46 kDa protein, whose main function is to act as negative regulator of Trx1 (112). Txnip is ubiquitously present and its expression regulated by oxidative stress (113). Like ASK1, Txnip binds to reduced Trx1 via disulfide bonds (114).

Human Trx2 has a molecular size of 18 kDa with extension at the N-terminal where the mitochondrial translocational signal is located. Trx2 shares the catalytic active site of Trx1, but lacks the structural cysteines. It has an important role in preventing mitochondrial dysfunction and regulating mitochondrial-mediated mediated apoptosis (115,116).

SpTrx is a testis specific Trx. SpTrx is homologous to cytosolic Trx1 with a conserved CGPC active site and the three additional structural cysteines, but lacks oxidoreductase activity (117).

1.2.2 The glutaredoxin system

The glutaredoxin system (Grx system) comprises glutathione reductase (GR), glutathione (GSSG/GSH), glutaredoxin (Grx) and NADPH (*Figure 9*). In the Grx system, electrons are transferred from NADPH to GR, on to GSH and finally to Grx which reduces proteins (118). The reduction of protein disulfides via the Grx system may occur via dithiol or monothiol mechanisms. In the dithiol mechanism, two electrons are transferred from the active site of Grx to the disulfide of another protein. The monothiol mechanism utilizes only one active cysteine to catalyze either the formation or reduction of glutathione mixed disulfides; hence the monothiol mechanism implies the catalysis of both glutathionylation and deglutathionylation of proteins (119).

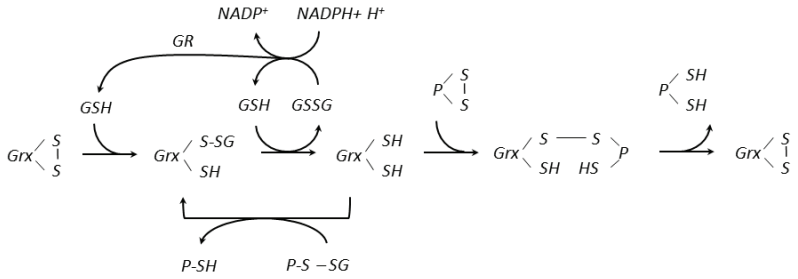

Figure 9. **The glutaredoxin system.** Glutathione reductase *(GR)* reduces GSSG to two GSH molecules by transferring electrons from NADPH. The disulfides in the active site of Grx are reduced by the two GSH molecules to dithiol in a two-step reaction. Subsequently, dithiol glutaredoxin reduces a disulfide *(P-S_2)* to its dithiol form *(P-$(SH)_2$)*. In a monothiol mechanism, Grx uses a single active site Cys residue to reduce a glutathionylated protein *(PS-SG)* to its monothiol form *(P-SH)*.

1.2.2.1 Glutathione reductase

Glutathione reductase is the enzyme responsible for maintaining the GSH supply, since it reduces GSSG using electrons transferred from NADPH. Like thioredoxin reductase, glutathione reductase is a homodimeric, flavin-containing protein that belongs to the pyridine nucleotide-disulfide oxidoreductase family. There are two isoforms of glutathione reductase: one cytosolic and one mitochondrial (120).

1.2.2.2 Glutaredoxin

Glutaredoxin (Grx) is a small oxidoreductase with a molecular size of 12 kDa, discovered as a glutathione (GSH)-dependent hydrogen donor for RNR in an E. coli mutant lacking thioredoxin (Trx) (70,121,122). Different glutaredoxins are involved in monothiol or dithiol mechanisms, which are distinct but functionally connected since both rely on the N-terminal Cys-residue in the CXXC/S active site and on the affinity for GSH (123,124). Grx1 and Grx2 are well characterized glutaredoxins in mammalian cells. These Grxs differ in cellular localization and substrate specificity. Grx1 acts through a dithiol oxido-reductase mechanism and is predominately localized in the cytosol, but may also be localized in the nucleus (125). Grx2 with the active site CSYC, was identified as the first iron-sulfur [Fe–S] thioredoxin family protein and acts as a dithiol Grx. Grx2 is mainly localized in the mitochondria (Grx2a) but other isoforms have been localized in the nucleus and the cytosol (Grx2b and Grx2c) (126,127). There are a total of four glutaredoxins described in humans: two dithiol (Grx1 and Grx2), a multidomain monothiol (Grx3) (128) and a mitochondrial single-domain monothiol (Grx5) (129).

A unique characteristic of glutaredoxins is their ability to catalyze glutathione dependent redox regulations via glutathionylation and reverse-S-glutathionylation (130). While the mechanism for catalyzed protein glutathionylation is not well understood, reverse-S-glutathionylation is well known to be catalyzed by many members of the Grx family (131). As mentioned above, reverse-S-glutathionylation involves a thiol-disulfide exchange reaction between glutathione and a protein. During the catalysis, the thiolate moiety of Grx attacks the protein-mixed (protein-SSG), resulting in glutathionylated Grx (Grx-SSG) and the reduced protein (protein-SH) (*Figure 9*). Subsequently, the glutaredoxin-glutathione mixed-disulfide intermediate can either be reduced by another glutathione molecule, forming reduced Grx and GSSG, or alternatively, a dithiol Grx may form an intra-molecular disulfide and release GSH.

Even though glutaredoxins contain two cysteines in the active site, catalysis of reverse-S-glutathionylation occurs via monothiol mechanism. For instance, Grx1 has Cys22-Cys25 in the active site, but only Cys22 participates in the reverse-S-glutathionylation mechanism due to its low pKa, hence acting as a thiolate at physiologic pH, which is prone to attack a disulfide bond (132).

1.2.2.3 The role of thioredoxin and glutaredoxin in diseases

As already adumbrated, oxygen radicals may have a strong impact on redox-sensitive transcription factors which modulate diverse cellular processes and could trigger the conditions required for the development of various diseases. In addition, the redox-regulation of these transcription factors may be controlled by the thioredoxin and glutaredoxin systems (133).

For instance, there is evidence that activation of ASK1 may play an important role in Aβ peptide toxicity and the development of Alzheimer's disease (134). As mentioned earlier, Trx1, as well as Grx1, may regulate the activity of ASK1 via protein-protein interaction inhibiting the proapoptotic MAPK cascade (135). Additionally, it has been shown that levels of Trx1 decrease, while levels of Grx1 and TrxR increase in brain tissue from Alzheimer's patients (136,137). Hence, it is believed that there is a loss of function or direct inhibition of Trx1, which might be compensated by the elevated levels of Grx1 as previously demonstrated in *Escherichia coli* (70). However, the loss of Trx1 results in ASK1 dysregulated activity and consequently enhanced apoptosis. Therefore suggesting that the compensatory increase of Grx1 and TrxR levels is not sufficient to cope with the decrease of Trx1 levels.

Transcription factors are important gate keepers preventing the radical transformation of a healthy tissue into a malignant tissue. Various antioxidant systems, including the thioredoxin and glutaredoxin systems, help maintaining the function of some of these transcription factors. As mentioned earlier, the tumor suppressor protein p53 is an important transcription factor because it prevents severely damaged cells from dividing until repaired or triggers apoptosis if the cellular damage is beyond repair. The binding of p53 to DNA occurs via Cys-residues located at it DNA binding domain. The thiol-redox status of these Cys-residues may be regulated by Trx. Thus Trx, via supporting the binding of p53 to DNA, promotes gene activation to either trigger DNA repair or apoptosis (138).

As previously mentioned, the activity of Trx is regulated by Txnip. In fact, the interaction between ASK1 and Trx1 is consequently regulated by Txnip, via binding to the catalytic cysteines of Trx1, which inhibits Trx1 activity and its ability to bind to ASK1 (139,140). In addition, glucose has been shown to trigger the expression of Txnip in a variety of cells and tissues, thus pointing Txnip as glucose- and insulin-sensitive homeostatic regulator of the glucose uptake (141,142). Hence, insulin deficiency or hyperglycemia may increase Txnip levels in cells, resulting in impaired peripheral glucose uptake (143). In the pancreas, β-cells compensate the impaired glucose uptake by secreting more insulin. However, if this compensation fails, the resulting hyperglycemia may lead to increased cellular expression of Txnip and consequent inhibition of Trx1-ASK1 binding and triggering β-cell apoptosis (144). This vicious cycle would eventually trigger a cascade of events that may result in the development of diabetes mellitus type 2.

2 PRESENT INVESTIGATION

2.1 AIMS OF THE THESIS

Our primary goal was to develop sensitive assays to assess the activity of thioredoxin, thioredoxin reductase and glutaredoxin, respectively, to enable analysis of clinical patient samples. We modified known substrates for the Trx and Grx systems by labeling these molecules with fluorescent probes. After further method development and optimization, these methods were applied to analyze Trx, TrxR and Grx in various types of patient samples. Listed below are the individual aims of this thesis.

- To develop highly sensitive fluorescent assays for characterization of Trx in Trx-dependent disulfide reduction and activity determinations of Trx in samples used in biochemical research.

- To characterize Grx catalyzed protein glutathionylation and reverse glutathionylation and develop a sensitive activity assay for Grx activity determination by using a novel fluorescent disulfide substrate.

- To describe the biochemical profile of two subjects with mutated SBP2 gene in respect to selenoproteins including TrxR.

- To establish the physical properties of Grx5 by crystallization and characterize its catalytic properties as thiol-disulfide reductase and electron donor properties for ribonucleotide reductase.

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I - *Sensitive activity assays of mammalian thioredoxin and thioredoxin reductase: Fluorescent disulfide substrates, mechanisms and use with tissue samples*

The Trx system is central for regulation of cell redox signaling and redox environment and is closely linked to various human diseases (133). In fact, Trx and TrxR are suggested biomarkers for disease monitoring. Thus, new techniques are needed to determine and monitor Trx and TrxR activity in human patient samples. Traditionally, the methods that have been used to determine Trx activity are spectrophotometric either using consumption of NADPH at 340 nm or determination of SH-groups with DTNB at 412 nm (145). However, these methods display several limitations related to background, specificity and sensitivity.

We optimized the synthesis of two fluorescent substrates, dieosin-diglutathione (Di-E-GSSG) and fluorescein labeled insulin (FITC-insulin) to develop novel assays for Trx and TrxR activity. Di-E-GSSG and FITC-insulin substrates have been described as disulfide substrates for protein disulfide isomerase (PDI) in the presence of dithiothreitol (DTT) (146,147). Our findings suggest that the native characteristics of these substrates of the thioredoxin system are changed after chemical modification. Hence, the fluorescent emission of these substrates is quenched due to the proximity of the two fluorochromes attached to the N-terminal of the molecule. However, upon reduction, the fluorochromes are separated and the fluorescence emission is increased.

Di-E-GSSG was initially a promising fluorescent substrate for Trx because it displayed a major fluorescence increase upon reduction and displayed high affinity to Trx compared to GSSG. However, Di-E-GSSG proved to be a weak substrate of TrxR consequently yielding a background reaction when measuring more complex biological samples, thus it was disqualified for further use.

Conversely, FiTC-insulin showed high substrate specificity for Trx with higher affinity than insulin. This substrate was the key component to develop novel highly sensitive assays in 96 well microtiter plates for activity determination of both Trx and TrxR. We were thus able to follow the oxidation state of Trx and quantify Trx and TrxR activity in the picomole range. The assay in turn facilitated measurements of Trx from various types of blood cells such as peripheral blood mononuclear cells (PBMC), lymphocytes or monocytes which until today has only been possible with ELISA.

2.2.2 Paper II - *Determination of glutaredoxin enzyme activity and S-glutathionylation using fluorescent glutathione*

While Di-E-GSSG seemed to bind or react non-specifically with thiols, it was highly probable that mixed disulfides were being formed. Despite its very low substrate activity with glutathione reductase, we discovered that Di-E-GSSG was instead an excellent substrate for Grx activity measurements and developed two alternative assays. One of the assays is based on the classical HED assay (148), but replacing HED with Di-E-GSSG. This alternative assay consist in reduction of Grx driven by lipoamide dehydrogenase and lipoamide (149) using Di-E-GSSG as substrate. This new method allows the quantification of Grx1 and Grx2 activities in nanomolar concentrations.

The distinctive feature of Di-E-GSSG to react with thiols suggested that this molecule in addition to behave as a disulfide substrate could potentially be also used to study protein glutathionylation. One protein that is commonly used for glutathionylation studies is albumin, thus we tested glutathionylation with Di-E-GSSG using albumin from bovine serum (BSA). BSA consists of 577 amino acid residues, of which 35 are cysteines, forming 17 disulfide bridges between 34 of them, rendering only one free cysteine residue, Cys34, which is located in the crevice on the protein surface of domain I 33. Upon reaction with BSA, Di-E-GSSG formed a mixed disulfide with the free thiol of residue Cys34, forming a covalent bond and yielding albumin glutathionylated with eosin-glutathione (E-GS-BSA). E-GS-BSA showed a quantum yield fluorescence that increased 20-fold upon reduction with DTT.

Given that glutathionylated proteins are good substrates for glutaredoxins, we tested de-glutathionylation of E-GS-BSA through the electron transport chain of NADPH, GR and GSH in the presence of Grx. The results obtained suggested that E-GS-BSA was an excellent molecule for Grx catalyzed de-glutathionylation reactions, which facilitated the determination of the catalytic activity of Grx. We developed a new assay using E-GS-BSA as key component where we were capable of detecting as low as 30 fmol Grx1 and 70 fmol of Grx2. Furthermore, we were able to quantify Grx1 in plasma which so far only been performed with ELISA, but with the additional advantage of quantifying the amount of active protein which was not possible with former immunoassay techniques.

Di-E-GSSG was also used for S-glutathionylation studies. Di-E-GSSG was utilized to glutathionylate Grx and we were able to follow how a fluorescently labeled monothiol Grx containing E-GS- was reversibly transferred from BSA.

2.2.3 Paper III - *Mutations in the selenocysteine insertion sequence–binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans*

Two subjects with heterozygous defects in the selenocysteine insertion sequence–binding protein 2 presented a clinical profile with elevated circulating levels of thyroxine (T4) in addition to low concentrations of selenium and selenoproteins; selenoprotein P (SEPP) and glutathione peroxidase 3 (GPx3). The biochemical profile of the subjects suggested deficiency of T4 deiodinase enzymes as well as other selenoproteins, and similar or equivalent to the phenotype of childhood cases with defects in SBP2 (150). The subjects also demonstrated clinical symptoms including azoospermia, axial muscular dystrophy, skin photosensitivity, abnormal immune cell function, and marked insulin sensitivity in addition to cellular features such as increased ROS production, membrane lipid peroxidation and oxidative DNA damage, and accelerated telomere shortening, which were directly attributed to loss of selenoprotein function.

Azoospermia has been linked to selenium deficiency (151). Our findings suggested that deficient spermatogenetic development in the subjects was closely associated with insufficient activity of thioredoxin-glutathione reductase, glutathione peroxidase 4 and selenoprotein V, which resulted from failure to efficiently incorporate selenium into selenoproteins.

Both subjects presented axial muscular dystrophy. Selenoprotein N1 mutation was reported to cause rigid spine muscular dystrophy (152) and tissue analysis from the subjects showed features that were similar to selenoprotein N related myopathies. Additionally, dermal fibroblast protein analysis from both subjects demonstrated decreased proteins levels selenoprotein N. Hence, the findings suggest that the muscular dystrophy was likely caused by selenoprotein N deficiency.

TrxR is a scavenger of free radicals generated from ultraviolet radiation (UVR) in the human skin (153). Since these subjects presented skin photosensitivity, we tested TrxR activity in skin biopsies from one of the patients. The TrxR activity in the skin biopsy showed significant decreased activity as well as increased levels of ROS when compared to healthy tissue.

It has been reported that mice whose T-cells are devoid of selenoproteins produce an excess of ROS upon activation resulting in inhibited T-cell proliferation (154). One might assume that the SBP2 defect would affect TrxR activity in the T-cells of the subject. To test this, blood samples were obtained and PBMCs were isolated from the patients in order to determine TrxR activity in these cells. These results showed low levels of TrxR activity compared to healthy controls thus suggesting loss of antioxidant defense in the immune cells.

2.2.4 Paper IV - *The crystal structure of human GLRX5: iron−sulfur cluster co-ordination, tetrameric assembly and monomer activity*

Grx5 plays an important role in mitochondrial iron-sulfur cluster biosynthesis and iron metabolism (155). However, little was known about the structure of human [2Fe-2S]-bound Grx5 and its catalytic properties to reduce disulfides or mixed disulfides with GSH.

We successfully managed to purify and characterize the physical and the catalytic properties of human Grx5. The molecular mass analysis revealed that Grx5 had a molecular size of 12851 Da with intra-molecular disulfides while holo-Grx5 was determined to be 12946 Da.

Crystallized Grx5 showed that [2Fe-2S] clusters were coordinated by two GSH molecules and two protein chains in a tetrameric organization. Each monomer displayed the classical Trx fold composed of five α-helices and four β-sheets. The active site Cys^{67} was located to the vicinity of helix α2 at the N-terminus.

The catalytic properties of human Grx5 were determined by measuring the reduction of mixed disulfides in a system coupled with GSH while compared with human Grx2. Both Grx5 and Grx2 are localized in the mitochondria and their stability is dependent on the assembly of [Fe-S]-cluster. When GSH was used as an electron donor, micromolar concentrations of Grx5 catalyzed the reduction of E-GS-BSA while Grx2 reduced E-GS-BSA at nanomolar concentrations under the same conditions. These results indicated that the relative catalytic activity of Grx5 was approximately 500-fold lower than Grx2. We also studied the reduction of the Grx−GSH intermediate of Grx5 and Grx2. The results revealed that GSH−Grx5 mixed disulfide is reduced at least 100-fold lower rate than GSH−Grx2. Furthermore, Grx5 did not only catalyze the reduction of mixed disulfides, it could also catalyze transfer electrons from GSH to ribonucleotide reductase (RNR). In this regard our data showed that Grx5 transferred electrons to R1-R2 subunits but only in the presence of high amounts of GSH (>10 mM).

3 CONCLUSIONS

Thioredoxin, thioredoxin reductase and glutaredoxin are essential enzymes for regulation of the redox homeostasis. They have all been linked to diseases thus emphasizing the importance of the study of these proteins in order to improve understanding for its enzymatic mechanism and their associated signaling pathways.

Even though there are currently well established methods to analyze the activity of these systems, they present major drawbacks such as high background and lack of sensitivity which, may occur particularly in samples with limited amount of protein or samples that are highly oxidized. Sometimes when working with clinical sample, there is only certain amount of material available, which is a very important limiting factor to carry out certain experimental approaches. Based on this fact, there is an evident need for novel tools that require very small amount of sample and generate highly reproducible results when studying Trx, TrxR and Grx in a clinical context.

Our pursue to obtain novel tools to study thioredoxin, thioredoxin reductase and glutaredoxin lead us to modify insulin and glutathione disulfide, which are two natural substrates of thioredoxin and glutaredoxin, respectively, by attaching fluorescent molecules. Therefore, we were able to improve the biochemical characteristics of these substrates to develop new measurement techniques for this family of redox enzymes.

As a result of the chemical modification of the free amino groups of insulin or glutathione disulfide, with either FITC or EITC to yield FiTC-insulin and Di-E-GSSG, respectively, we managed to obtain two excellent substrates of the thioredoxin system with improved substrate characteristics compared with the native form. These observations set the platform for developing novel assays for the thioredoxin system thus facilitating further characterization of thioredoxin and thioredoxin reductase. Of the two substrates, Di-E-GSSG displayed valuable substrate characteristics, but it was not suitable for enzymatic assays of the thioredoxin system due to its lack of specificity. On the other hand, FiTC-insulin, was utilized to develop fluorescent 96 well plate assays to measure the enzymatic activity of thioredoxin and thioredoxin reductase, respectively. The assays enabled quantitative determination of very low concentrations of human thioredoxin ($\geq$ 0.4 picomoles) or thioredoxin reductase ($\geq$ 40 femtomole). We were able to quantify thioredoxin in samples that are commonly used in biomedical research from cell, tissue (biopsies) and blood plasma.

Nevertheless, even though Di-E-GSSG was unspecific for thioredoxin measurements, it was an excellent disulfide substrate of glutaredoxin and, like glutathione disulfide, Di-E-GSSG could glutathionylate cysteine residues resulting in glutathionylated proteins. Therefore it was possible to perform protein-glutathionylation studies by measuring the fluorescence or to detect this process when fluorescent glutathionylated proteins are subjected to SDS-PAGE and exposed to ultraviolet light. Additionally, glutathionylated proteins are good substrates for glutaredoxins hence fluorescent glutathionylated albumin showed to be an outstanding substrate. We were positively surprised when we were able to detect glutaredoxin activity in plasma samples which had not been achieved or reported before as far as we are concerned.

Thus, we can state that:

- Di-E-GSSG can be utilized as a tool to study other molecules that hypothetically could be glutathionylated.

- If a protein is glutathionylated it can also be deglutathionylated hence enabling experiments on proteins that are hypothetically capable of catalyzing reverse glutathionylation reactions.

- Di-E-GSSG may set the foundation for tools that could be used for further analysis around the mechanisms of catalyzed protein glutathionylation.

Our enrollment in the clinical analysis of the unique case of two patients with a mutation provided a great opportunity to test our new assay. The mutation of these patients featured many physiological alterations that resulted from impaired cellular antioxidant defense. It was therefore expected that the catalytic activity of thioredoxin reductase would be affected. However the true challenge from our study was not only to assess the activity of thioredoxin reductase but the limited amount of sample from the subjects and healthy controls. Hence, these experiments required the most accurate measurement technique and sensitive tools in order to obtain highly reproducible results. Our novel FiTC-insulin assay for thioredoxin reductase turned to be a valuable tool for this type of studies, providing results that were accurate and convincing due to its sensitivity. Therefore providing evidence that our novel methodology is suitable for these difficult analyses, which are highly relevant in biomedical research.

The usefulness of E-GS-BSA extended to a sensitive substrate for reverse-S-glutathionylation catalysis studies. When characterizing the catalytic properties of human Grx5, one might not have been able to decipher its catalytic properties if was not for the sensitivity of the substrate. Our results revealed that E-GS-BSA presented evident substrate features that were optimal for studying enzymes that catalyze reverse glutathionylation.

In conclusion, since Trx, TrxR and Grx have been suggested as potential biomarkers for several diseases such as: cancer, diabetes, neurodegenerative and cardiovascular diseases; the actual relevance of the novel methodologies presented here, goes beyond the enzymatic measurements performed, as we are describing methods which might assist the detection or/and in the analysis of disease progression with clear biomedical applications.

4 ACKNOWLEDGEMENTS

The work in this thesis was performed at the division of biochemistry, department of medical biochemistry and biophysics, Karolinska institutet.

I would like to express my deepest gratitude to my supervisor **professor Arne Holmgren** for all the trust, support and confidence you gave me and my work. You spotted my strengths and weaknesses and made me work on my weaknesses while boosting my strengths, always keeping me on the right track. You taught me what is today one of most cherished mottos "*it is ok to make mistakes as long as we learn from them and never repeat them again*". You were not only a supervisor or a boss you were also my mentor who taught me a lot of things about science and life. I will always remember your stories while having coffee and the fun we had that time when we were working in the lab shoulder to shoulder. Thank you.

I want to thank my co-supervisor **Dr. Johanna Ungerstedt** with whom I share a bond of trust and respect after working together in common projects. One of my wisest decisions during my thesis was to suggest you as my co-supervisor since you complemented me on many things that I lacked. Your criticism and great advice made do better and we always kept honesty and openness which made me confident about myself and my work even in my faults. We do not make wonders like the A-team but we do make great things as a team. Much cherish and respect.

To my other co-supervisor **Dr Jun Lu** or whom I came to call my shifu (師傅). I could always count on you for help and advice. You were the first person at work who stretched the hand for me whenever I needed it. I still remember those words of confidence you recently gave me when you said that I have become independent and self-critical being a sign of great maturity. 谢谢我师傅.

To the head of our division **professor Elias Arnér** for all the great criticism and advice when I presented on the Wednesday seminars. I was always prepared with pen and paper every time I had a presentation mainly because of all your constructive criticism, good advice and the ideas you gave me. This encouraged me to do better in my work and my presentations. I could always count on you for spotting those things that I missed.

To **Lena Ringdén** for always cheering me up when I needed it the most. You always saw the better of me and it always felt great to talk to someone at work about anything but science. When I was most stressed, you made me disconnect for a while every time we stopped for a chit-chat and all the countless times you assisted me in matters that were out of my hands. I always felt appreciated by you, the feeling is mutual.

To my sidekick in the lab **Mr. M.D. Ph.D. Tomas N. Gustafsson** who I share a special bond with even though we have different personalities and we are generally very different. Nevertheless, we do share important ground rules such as rationality, the enjoyment of great culinary experiences, a healthy common sense and the importance of not being socially challenged. Keep up the great work my friend.

To **Dr Alexios Vlamis-Gardikas** for all the help you gave me while you were in the lab and whom I shared great conversations about science and life. To **Jacek Andrzejewski** for always being helpful and kind.

To my dear colleague **Xu Zhang**, who always kept such contagious good mood and enthusiasm in the office. To **Deepika Nair** for sharing your joy and happiness in and outside the lab. It is great to work with such kind person like you. To **Dr. Jianqian Xu** for the friendship and great laughs we shared in the lab. To **Dr. Hanna-Stina Martinsson Ahlzén** for all the conversations and thoughts we shared while having coffee. To **Dr. Lucia Coppo** for the great work we did together. To **William Stafford** for all the good times we had outside the lab including the outdoors swimming, parties, cigars and Scotch. To **Dr. Qing Chen** for all the assistance and help throughout these years. To Lena **Haffo, Emelie Barreby and Harer Osman**, the students who brought a lot of laughter to the office.

To present members of biochemistry **Marcus Cebula, Irina Pader, Xiaoxiao peng, Dr. Katarina Johansson, Dr. Helena Wållberg, Dr. Rajib Sengupta, Dr. Yatao Du, Dr. Huihui Zhang, Xiaoyuan Ren** for the great team spirit and the nice environment you´ve created in biochemistry.

To past members of biochemistry **Dr. Lars Bräutigam, Dr. Victor Croitoru, Dr. Maria Lönn, Dr. Isaac Hashemy, Dr. Farnaz Zahedi, Dr. Sofi Eriksson, Dr. Steffanie Prast-Nielsen, Dr. Christoff Huddeman** for the great time we had while you were at biochemistry.

Dr. Essam Refai, **Johanna Sandelius**, **Chad Tunell** and **Linus Östberg** for the great times we shared while organizing pubs, BBQs, afterwork and dinners. We had a lot of fun in- and outside work and made my staying at MBB much more fun.

Herr Dr. Pascal Dammeyer, one of my true a most loyal friends, whom I will forever cherish. You were and always there in times when I need a friend. All our countless chats about life, work, food, drinks and friendship still bring us closer together.

To my dear friend **Dr Javier Avila Cariño**, for all the great advice and the good times we spent "a lo mero macho".

To my friends since childhood **Rikard Lindström, Pablo (mi carnal) Bonomi, Mauricio (Marre / gudfarn) Bistnér** and let's not forget **Martin Edvardsson**. For all the crazy things we did, all the laughs we shared, all the tears we dropped in joy and sadness and all the things we have ahead of us, thank you. I can always count on you for anything (even though Dolores still does not understand our sense of humor).

A **Don Jacobo Fernández** y a la **Dra. Berta Rivas**, grandes compañeros de experiencias culinarias. Por todos los viajes hechos y por hacer… y en especial, por su amistad incondicional. See you soon in Spain!

To my dear **mother**, my life mentor, for all the advice and unconditional love. You literally held my hand throughout my life journey and you are still doing it. Whatever I write will not be enough… To my **father** for being there whenever I needed, supporting me in difficult times and helping me persuade mom that some things are meant to be.

To my sister **Ingrid** for all the unconditional help, support, cherish and love. I can always count on you for anything, it´s mutual. Love you sis, always. To my brother from another mother and father **Johan** for bringing joy to me and my family. You are the closest I´ve got to a true brother. To "my brother's parents" **Ulla** and **Lennart**, for always welcoming me and Dolores with open arms.

A mi familia en España, mis suegros **Dolores** y **Jose Manuel**, mis "cuñaos" **David** y **Josito** por todo el cariño y el afecto que a lo largo de estos años me han brindado y en especial por la intensiva introducción a la gatronomía gallega (y al pulpo). A la familia **Amarelle-Guillín** por darme el cariño de un yerno, aunque no lo sea.

To **Dr. Salvado**, my beloved wife, the other half of my heart, my life companion. For the enormous support, the care, the love you have given me. I realized while writing this thesis that you are truly my pillar since I wouldn't have made it if it wasn't for your support. We have just commenced our life journey together and I'm looking forward to what is to come... I LOVE YOU… FOREVER.

5 REFERENCES

1. Halliwell, B., and Cross, C. E. (1994) Oxygen-derived species: their relation to human disease and environmental stress. *Environmental health perspectives* **102 Suppl 10**, 5-12
2. Betteridge, D. J. (2000) What is oxidative stress? *Metabolism* **49**, 3-8
3. Gorlach, A., and Kietzmann, T. (2007) Superoxide and derived reactive oxygen species in the regulation of hypoxia-inducible factors. *Methods Enzymol* **435**, 421-446
4. Cejudo-Martin, P., Morales-Ruiz, M., Ros, J., Navasa, M., Fernandez-Varo, G., Fuster, J., Rivera, F., Arroyo, V., Rodes, J., and Jimenez, W. (2002) Hypoxia is an inducer of vasodilator agents in peritoneal macrophages of cirrhotic patients. *Hepatology* **36**, 1172-1179
5. Handy, D. E., and Loscalzo, J. (2012) Redox regulation of mitochondrial function. *Antioxid Redox Signal* **16**, 1323-1367
6. Gong, P., and Cederbaum, A. I. (2006) Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protects against oxidative stress caused by CYP2E1. *Hepatology* **43**, 144-153
7. Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005) Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* **120**, 649-661
8. Forman, H. J., Maiorino, M., and Ursini, F. (2010) Signaling functions of reactive oxygen species. *Biochemistry* **49**, 835-842
9. Beckman, K. B., and Ames, B. N. (1997) Oxidative decay of DNA. *J Biol Chem* **272**, 19633-19636
10. Stadtman, E. R., and Levine, R. L. (2000) Protein oxidation. *Ann N Y Acad Sci* **899**, 191-208
11. Shatrov, V. A., Sumbayev, V. V., Zhou, J., and Brune, B. (2003) Oxidized low-density lipoprotein (oxLDL) triggers hypoxia-inducible factor-1alpha (HIF-1alpha) accumulation via redox-dependent mechanisms. *Blood* **101**, 4847-4849
12. Yla-Herttuala, S. (1998) Is oxidized low-density lipoprotein present in vivo? *Current opinion in lipidology* **9**, 337-344
13. Bokoch, G. M., and Knaus, U. G. (2003) NADPH oxidases: not just for leukocytes anymore! *Trends in biochemical sciences* **28**, 502-508
14. Wiseman, H., and Halliwell, B. (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* **313 (Pt 1)**, 17-29
15. Lee, Y. J., Galoforo, S. S., Berns, C. M., Chen, J. C., Davis, B. H., Sim, J. E., Corry, P. M., and Spitz, D. R. (1998) Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. *J Biol Chem* **273**, 5294-5299
16. Yan, S. D., Chen, X., Schmidt, A. M., Brett, J., Godman, G., Zou, Y. S., Scott, C. W., Caputo, C., Frappier, T., Smith, M. A., and et al. (1994) Glycated tau protein in Alzheimer disease: a mechanism for induction of oxidant stress. *Proc Natl Acad Sci U S A* **91**, 7787-7791
17. Brownlee, M. (1995) Advanced protein glycosylation in diabetes and aging. *Annual review of medicine* **46**, 223-234
18. Vincent, H. K., Powers, S. K., Stewart, D. J., Shanely, R. A., Demirel, H., and Naito, H. (1999) Obesity is associated with increased myocardial oxidative stress. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **23**, 67-74
19. Schmidt, A. M., Weidman, E., Lalla, E., Yan, S. D., Hori, O., Cao, R., Brett, J. G., and Lamster, I. B. (1996) Advanced glycation endproducts (AGEs) induce oxidant stress in the gingiva: a potential mechanism underlying accelerated periodontal disease associated with diabetes. *Journal of periodontal research* **31**, 508-515

20. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2003) Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* **52**, 1-8
21. Halliwell, B. (1993) Free radicals and vascular disease: how much do we know? *Bmj* **307**, 885-886
22. Marnett, L. J. (2000) Oxyradicals and DNA damage. *Carcinogenesis* **21**, 361-370
23. Tamura, M., Gu, J., Tran, H., and Yamada, K. M. (1999) PTEN gene and integrin signaling in cancer. *Journal of the National Cancer Institute* **91**, 1820-1828
24. Gottlieb, E., and Vousden, K. H. (2010) p53 regulation of metabolic pathways. *Cold Spring Harbor perspectives in biology* **2**, a001040
25. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) p53 mutations in human cancers. *Science* **253**, 49-53
26. Downes, C. P., Walker, S., McConnachie, G., Lindsay, Y., Batty, I. H., and Leslie, N. R. (2004) Acute regulation of the tumour suppressor phosphatase, PTEN, by anionic lipids and reactive oxygen species. *Biochemical Society transactions* **32**, 338-342
27. Bush, A. I. (2000) Metals and neuroscience. *Current opinion in chemical biology* **4**, 184-191
28. Lynch, T., Cherny, R. A., and Bush, A. I. (2000) Oxidative processes in Alzheimer's disease: the role of abeta-metal interactions. *Experimental gerontology* **35**, 445-451
29. Cheng, H. M., and Gonzalez, R. G. (1986) The effect of high glucose and oxidative stress on lens metabolism, aldose reductase, and senile cataractogenesis. *Metabolism* **35**, 10-14
30. Wolff, S. P., Jiang, Z. Y., and Hunt, J. V. (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic Biol Med* **10**, 339-352
31. Rains, J. L., and Jain, S. K. (2011) Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med* **50**, 567-575
32. Asaba, K., Tojo, A., Onozato, M. L., Goto, A., Quinn, M. T., Fujita, T., and Wilcox, C. S. (2005) Effects of NADPH oxidase inhibitor in diabetic nephropathy. *Kidney international* **67**, 1890-1898
33. Britton, G. (1995) Structure and properties of carotenoids in relation to function. *Faseb J* **9**, 1551-1558
34. Padayatty, S. J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J. H., Chen, S., Corpe, C., Dutta, A., Dutta, S. K., and Levine, M. (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *Journal of the American College of Nutrition* **22**, 18-35
35. Herrera, E., and Barbas, C. (2001) Vitamin E: action, metabolism and perspectives. *Journal of physiology and biochemistry* **57**, 43-56
36. Bentinger, M., Brismar, K., and Dallner, G. (2007) The antioxidant role of coenzyme Q. *Mitochondrion* **7 Suppl**, S41-50
37. Muller, E. G. (1996) A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth. *Molecular biology of the cell* **7**, 1805-1813
38. Dawson, J. R., Vahakangas, K., Jernstrom, B., and Moldeus, P. (1984) Glutathione conjugation by isolated lung cells and the isolated, perfused lung. Effect of extracellular glutathione. *Eur J Biochem* **138**, 439-443
39. Brigelius-Flohe, R. (1999) Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* **27**, 951-965
40. Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxid Redox Signal* **7**, 348-366
41. Barrett, W. C., DeGnore, J. P., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B. (1999) Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. *J Biol Chem* **274**, 34543-34546

42. Gallogly, M. M., and Mieyal, J. J. (2007) Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Current opinion in pharmacology* **7**, 381-391
43. Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) Glutathione transferases. *Annual review of pharmacology and toxicology* **45**, 51-88
44. Holmgren, A., Johansson, C., Berndt, C., Lonn, M. E., Hudemann, C., and Lillig, C. H. (2005) Thiol redox control via thioredoxin and glutaredoxin systems. *Biochemical Society transactions* **33**, 1375-1377
45. Findlay, V. J., Townsend, D. M., Morris, T. E., Fraser, J. P., He, L., and Tew, K. D. (2006) A novel role for human sulfiredoxin in the reversal of glutathionylation. *Cancer Res* **66**, 6800-6806
46. Bannister, J. V., Bannister, W. H., and Rotilio, G. (1987) Aspects of the structure, function, and applications of superoxide dismutase. *CRC critical reviews in biochemistry* **22**, 111-180
47. Zamocky, M., and Koller, F. (1999) Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. *Progress in biophysics and molecular biology* **72**, 19-66
48. Zachara, B. A. (1992) Mammalian selenoproteins. *Journal of trace elements and electrolytes in health and disease* **6**, 137-151
49. Hoog, J. O., Jornvall, H., Holmgren, A., Carlquist, M., and Persson, M. (1983) The primary structure of Escherichia coli glutaredoxin. Distant homology with thioredoxins in a superfamily of small proteins with a redox-active cystine disulfide/cysteine dithiol. *Eur J Biochem* **136**, 223-232
50. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. *Nature* **317**, 267-270
51. Darby, N. J., Kemmink, J., and Creighton, T. E. (1996) Identifying and characterizing a structural domain of protein disulfide isomerase. *Biochemistry* **35**, 10517-10528
52. Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O., and Huber, R. (1991) The three-dimensional structure of class pi glutathione S-transferase in complex with glutathione sulfonate at 2.3 A resolution. *EMBO J* **10**, 1997-2005
53. Epp, O., Ladenstein, R., and Wendel, A. (1983) The refined structure of the selenoenzyme glutathione peroxidase at 0.2-nm resolution. *Eur J Biochem* **133**, 51-69
54. Holmgren, A. (2000) Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid Redox Signal* **2**, 811-820
55. Chae, H. Z., Kang, S. W., and Rhee, S. G. (1999) Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. *Methods Enzymol* **300**, 219-226
56. Holmgren, A. (1985) Thioredoxin. *Annu Rev Biochem* **54**, 237-271
57. Holmgren, A. (1984) Enzymatic reduction-oxidation of protein disulfides by thioredoxin. *Methods Enzymol* **107**, 295-300
58. Holmgren, A., Soderberg, B. O., Eklund, H., and Branden, C. I. (1975) Three-dimensional structure of Escherichia coli thioredoxin-S2 to 2.8 A resolution. *Proc Natl Acad Sci U S A* **72**, 2305-2309
59. Schultz, L. W., Chivers, P. T., and Raines, R. T. (1999) The CXXC motif: crystal structure of an active-site variant of Escherichia coli thioredoxin. *Acta crystallographica. Section D, Biological crystallography* **55**, 1533-1538
60. Capitani, G., Markovic-Housley, Z., DelVal, G., Morris, M., Jansonius, J. N., and Schurmann, P. (2000) Crystal structures of two functionally different thioredoxins in spinach chloroplasts. *J Mol Biol* **302**, 135-154
61. Sjoberg, B. M., and Holmgren, A. (1972) Studies on the structure of T4 thioredoxin. II. Amino acid sequence of the protein and comparison with thioredoxin from Escherichia coli. *J Biol Chem* **247**, 8063-8068
62. Saarinen, M., Gleason, F. K., and Eklund, H. (1995) Crystal structure of thioredoxin-2 from Anabaena. *Structure* **3**, 1097-1108

63. Kallis, G. B., and Holmgren, A. (1980) Differential reactivity of the functional sulfhydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from Escherichia coli. *J Biol Chem* **255**, 10261-10265
64. Nordberg, J., and Arner, E. S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* **31**, 1287-1312
65. Nakamura, H., Vaage, J., Valen, G., Padilla, C. A., Bjornstedt, M., and Holmgren, A. (1998) Measurements of plasma glutaredoxin and thioredoxin in healthy volunteers and during open-heart surgery. *Free Radic Biol Med* **24**, 1176-1186
66. Akamatsu, Y., Ohno, T., Hirota, K., Kagoshima, H., Yodoi, J., and Shigesada, K. (1997) Redox regulation of the DNA binding activity in transcription factor PEBP2. The roles of two conserved cysteine residues. *J Biol Chem* **272**, 14497-14500
67. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* **17**, 2596-2606
68. Yoshioka, J., Schulze, P. C., Cupesi, M., Sylvan, J. D., MacGillivray, C., Gannon, J., Huang, H., and Lee, R. T. (2004) Thioredoxin-interacting protein controls cardiac hypertrophy through regulation of thioredoxin activity. *Circulation* **109**, 2581-2586
69. Pekkari, K., and Holmgren, A. (2004) Truncated thioredoxin: physiological functions and mechanism. *Antioxid Redox Signal* **6**, 53-61
70. Holmgren, A. (1976) Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. *Proc Natl Acad Sci U S A* **73**, 2275-2279
71. Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by dual activation of the ras-phosphoinositide 3-kinase and jun n-terminal kinase pathways. *J Biol Chem* **276**, 21618-21626
72. Takashima, Y., Hirota, K., Nakamura, H., Nakamura, T., Akiyama, K., Cheng, F. S., Maeda, M., and Yodoi, J. (1999) Differential expression of glutaredoxin and thioredoxin during monocytic differentiation. *Immunol Lett* **68**, 397-401
73. Fernandes, A. P., and Holmgren, A. (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid Redox Signal* **6**, 63-74
74. Holmgren, A., and Aslund, F. (1995) Glutaredoxin. *Methods Enzymol* **252**, 283-292
75. Moore, E. C., and Reichard, P. (1964) Enzymatic Synthesis of Deoxyribonucleotides. Vi. The Cytidine Diphosphate Reductase System from Novikoff Hepatoma. *J Biol Chem* **239**, 3453-3456
76. Arner, E. S., and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* **267**, 6102-6109
77. Johnson, T. C., Crawford, N. A., and Buchanan, B. B. (1984) Thioredoxin system of the photosynthetic anaerobe Chromatium vinosum. *J Bacteriol* **158**, 1061-1069
78. Gonzalez Porque, P., Baldesten, A., and Reichard, P. (1970) Purification of a thioredoxin system from yeast. *J Biol Chem* **245**, 2363-2370
79. Florencio, F. J., Yee, B. C., Johnson, T. C., and Buchanan, B. B. (1988) An NADP/thioredoxin system in leaves: purification and characterization of NADP-thioredoxin reductase and thioredoxin h from spinach. *Archives of biochemistry and biophysics* **266**, 496-507
80. Kim, Y. C., Yamaguchi, Y., Kondo, N., Masutani, H., and Yodoi, J. (2003) Thioredoxin-dependent redox regulation of the antioxidant responsive element (ARE) in electrophile response. *Oncogene* **22**, 1860-1865
81. Holmgren, A., Johansson, C., Berndt, C., Lonn, M. E., Hudemann, C., and Lillig, C. H. (2005) Thiol redox control via thioredoxin and glutaredoxin systems. *Biochem Soc Trans* **33**, 1375-1377

82. Myers, C. R. (2012) The effects of chromium(VI) on the thioredoxin system: implications for redox regulation. *Free Radic Biol Med* **52**, 2091-2107
83. Gustafsson, T. N., Sahlin, M., Lu, J., Sjoberg, B. M., and Holmgren, A. (2012) Bacillus anthracis thioredoxin systems, characterization and role as electron donors for ribonucleotide reductase. *J Biol Chem* **287**, 39686-39697
84. Schulze-Osthoff, K., Schenk, H., and Droge, W. (1995) Effects of thioredoxin on activation of transcription factor NF-kappa B. *Methods Enzymol* **252**, 253-264
85. Laurent, T. C., Moore, E. C., and Reichard, P. (1964) Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B. *J Biol Chem* **239**, 3436-3444
86. Yoshida, T., Nakamura, H., Masutani, H., and Yodoi, J. (2005) The involvement of thioredoxin and thioredoxin binding protein-2 on cellular proliferation and aging process. *Ann N Y Acad Sci* **1055**, 1-12
87. Cassidy, P. B., Edes, K., Nelson, C. C., Parsawar, K., Fitzpatrick, F. A., and Moos, P. J. (2006) Thioredoxin reductase is required for the inactivation of tumor suppressor p53 and for apoptosis induced by endogenous electrophiles. *Carcinogenesis* **27**, 2538-2549
88. Holmgren, A. (1979) Reduction of disulfides by thioredoxin. Exceptional reactivity of insulin and suggested functions of thioredoxin in mechanism of hormone action. *J Biol Chem* **254**, 9113-9119
89. Holmgren, A. (1979) Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem* **254**, 9627-9632
90. Thelander, L. (1967) Thioredoxin reductase. Characterization of a homogenous preparation from Escherichia coli B. *J Biol Chem* **242**, 852-859
91. Zanetti, G., and Williams, C. H., Jr. (1967) Characterization of the active center of thioredoxin reductase. *J Biol Chem* **242**, 5232-5236
92. Prongay, A. J., and Williams, C. H., Jr. (1992) Oxidation-reduction properties of Escherichia coli thioredoxin reductase altered at each active site cysteine residue. *J Biol Chem* **267**, 25181-25188
93. Zhong, L., Arner, E. S., and Holmgren, A. (2000) Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proc Natl Acad Sci U S A* **97**, 5854-5859
94. Luthman, M., and Holmgren, A. (1982) Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* **21**, 6628-6633
95. Holmgren, A. (1977) Bovine thioredoxin system. Purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction. *J Biol Chem* **252**, 4600-4606
96. Oblong, J. E., Gasdaska, P. Y., Sherrill, K., and Powis, G. (1993) Purification of human thioredoxin reductase: properties and characterization by absorption and circular dichroism spectroscopy. *Biochemistry* **32**, 7271-7277
97. Lee, S. R., Kim, J. R., Kwon, K. S., Yoon, H. W., Levine, R. L., Ginsburg, A., and Rhee, S. G. (1999) Molecular cloning and characterization of a mitochondrial selenocysteine-containing thioredoxin reductase from rat liver. *J Biol Chem* **274**, 4722-4734
98. Sun, Q. A., Kirnarsky, L., Sherman, S., and Gladyshev, V. N. (2001) Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. *Proc Natl Acad Sci U S A* **98**, 3673-3678
99. Moore, E. C., Reichard, P., and Thelander, L. (1964) Enzymatic Synthesis of Deoxyribonucleotides.V. Purification and Properties of Thioredoxin Reductase from Escherichia Coli B. *J Biol Chem* **239**, 3445-3452
100. Holmgren, A. (1968) Thioredoxin. 6. The amino acid sequence of the protein from escherichia coli B. *Eur J Biochem* **6**, 475-484
101. Holmgren, A. (1989) Thioredoxin and glutaredoxin systems. *J Biol Chem* **264**, 13963-13966
102. Holmgren, A., and Bjornstedt, M. (1995) Thioredoxin and thioredoxin reductase. *Methods Enzymol* **252**, 199-208

103. Engstrom, N. E., Holmgren, A., Larsson, A., and Soderhall, S. (1974) Isolation and characterization of calf liver thioredoxin. *J Biol Chem* **249**, 205-210
104. Holmgren, A. (1984) Enzymatic reduction-oxidation of protein disulfides by thioredoxin. *Methods Enzymol* **107**, 295-300
105. Zhong, L., Arner, E. S., Ljung, J., Aslund, F., and Holmgren, A. (1998) Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J Biol Chem* **273**, 8581-8591
106. Taniguchi, Y., Taniguchi-Ueda, Y., Mori, K., and Yodoi, J. (1996) A novel promoter sequence is involved in the oxidative stress-induced expression of the adult T-cell leukemia-derived factor (ADF)/human thioredoxin (Trx) gene. *Nucleic acids research* **24**, 2746-2752
107. Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J. (1997) Cloning and expression of a novel mammalian thioredoxin. *J Biol Chem* **272**, 2936-2941
108. Miranda-Vizuete, A., Ljung, J., Damdimopoulos, A. E., Gustafsson, J. A., Oko, R., Pelto-Huikko, M., and Spyrou, G. (2001) Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *J Biol Chem* **276**, 31567-31574
109. Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999) Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* **274**, 27891-27897
110. Bertini, R., Howard, O. M., Dong, H. F., Oppenheim, J. J., Bizzarri, C., Sergi, R., Caselli, G., Pagliei, S., Romines, B., Wilshire, J. A., Mengozzi, M., Nakamura, H., Yodoi, J., Pekkari, K., Gurunath, R., Holmgren, A., Herzenberg, L. A., Herzenberg, L. A., and Ghezzi, P. (1999) Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells. *The Journal of experimental medicine* **189**, 1783-1789
111. Bjornstedt, M., Xue, J., Huang, W., Akesson, B., and Holmgren, A. (1994) The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *J Biol Chem* **269**, 29382-29384
112. Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y., and Yodoi, J. (1999) Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* **274**, 21645-21650
113. Wang, Y., De Keulenaer, G. W., and Lee, R. T. (2002) Vitamin D(3)-up-regulated protein-1 is a stress-responsive gene that regulates cardiomyocyte viability through interaction with thioredoxin. *J Biol Chem* **277**, 26496-26500
114. Patwari, P., Higgins, L. J., Chutkow, W. A., Yoshioka, J., and Lee, R. T. (2006) The interaction of thioredoxin with Txnip. Evidence for formation of a mixed disulfide by disulfide exchange. *J Biol Chem* **281**, 21884-21891
115. Lowes, D. A., and Galley, H. F. (2011) Mitochondrial protection by the thioredoxin-2 and glutathione systems in an in vitro endothelial model of sepsis. *Biochem J* **436**, 123-132
116. Zhang, R., Al-Lamki, R., Bai, L., Streb, J. W., Miano, J. M., Bradley, J., and Min, W. (2004) Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. *Circulation research* **94**, 1483-1491
117. Miranda-Vizuete, A., Sadek, C. M., Jimenez, A., Krause, W. J., Sutovsky, P., and Oko, R. (2004) The mammalian testis-specific thioredoxin system. *Antioxid Redox Signal* **6**, 25-40
118. Lillig, C. H., Berndt, C., and Holmgren, A. (2008) Glutaredoxin systems. *Biochim Biophys Acta* **1780**, 1304-1317
119. Bushweller, J. H., Aslund, F., Wuthrich, K., and Holmgren, A. (1992) Structural and functional characterization of the mutant Escherichia coli glutaredoxin (C14----S) and its mixed disulfide with glutathione. *Biochemistry* **31**, 9288-9293

120. Kelner, M. J., and Montoya, M. A. (2000) Structural organization of the human glutathione reductase gene: determination of correct cDNA sequence and identification of a mitochondrial leader sequence. *Biochem Biophys Res Commun* **269**, 366-368
121. Holmgren, A. (1979) Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. *J Biol Chem* **254**, 3664-3671
122. Holmgren, A. (1979) Glutathione-dependent synthesis of deoxyribonucleotides. Characterization of the enzymatic mechanism of Escherichia coli glutaredoxin. *J Biol Chem* **254**, 3672-3678
123. Gonzalez-Fernandez, R., Gaytan, F., Martinez-Galisteo, E., Porras, P., Padilla, C. A., Sanchez Criado, J. E., and Barcena, J. A. (2005) Expression of glutaredoxin (thioltransferase) in the rat ovary during the oestrous cycle and postnatal development. *Journal of molecular endocrinology* **34**, 625-635
124. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001) Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *J Biol Chem* **276**, 26269-26275
125. Sahlin, L., Wang, H., Stjernholm, Y., Lundberg, M., Ekman, G., Holmgren, A., and Eriksson, H. (2000) The expression of glutaredoxin is increased in the human cervix in term pregnancy and immediately post-partum, particularly after prostaglandin-induced delivery. *Mol Hum Reprod* **6**, 1147-1153
126. Lonn, M. E., Hudemann, C., Berndt, C., Cherkasov, V., Capani, F., Holmgren, A., and Lillig, C. H. (2008) Expression pattern of human glutaredoxin 2 isoforms: identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid Redox Signal* **10**, 547-557
127. Lillig, C. H., Berndt, C., Vergnolle, O., Lonn, M. E., Hudemann, C., Bill, E., and Holmgren, A. (2005) Characterization of human glutaredoxin 2 as iron-sulfur protein: a possible role as redox sensor. *Proc Natl Acad Sci U S A* **102**, 8168-8173
128. Witte, S., Villalba, M., Bi, K., Liu, Y., Isakov, N., and Altman, A. (2000) Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. *J Biol Chem* **275**, 1902-1909
129. Johansson, C., Roos, A. K., Montano, S. J., Sengupta, R., Filippakopoulos, P., Guo, K., von Delft, F., Holmgren, A., Oppermann, U., and Kavanagh, K. L. (2010) The crystal structure of human GLRX5: iron-sulfur cluster co-ordination, tetrameric assembly and monomer activity. *Biochem J* **433**, 303-311
130. Gravina, S. A., and Mieyal, J. J. (1993) Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. *Biochemistry* **32**, 3368-3376
131. Gallogly, M. M., Starke, D. W., and Mieyal, J. J. (2009) Mechanistic and kinetic details of catalysis of thiol-disulfide exchange by glutaredoxins and potential mechanisms of regulation. *Antioxid Redox Signal* **11**, 1059-1081
132. Jao, S. C., English Ospina, S. M., Berdis, A. J., Starke, D. W., Post, C. B., and Mieyal, J. J. (2006) Computational and mutational analysis of human glutaredoxin (thioltransferase): probing the molecular basis of the low pKa of cysteine 22 and its role in catalysis. *Biochemistry* **45**, 4785-4796
133. Lillig, C. H., and Holmgren, A. (2007) Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal* **9**, 25-47
134. Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagano, T., and Ichijo, H. (2005) Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ* **12**, 19-24
135. Song, J. J., and Lee, Y. J. (2003) Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1. *Biochem J* **373**, 845-853
136. Lovell, M. A., Xie, C., Gabbita, S. P., and Markesbery, W. R. (2000) Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain. *Free Radic Biol Med* **28**, 418-427

137. Akterin, S., Cowburn, R. F., Miranda-Vizuete, A., Jimenez, A., Bogdanovic, N., Winblad, B., and Cedazo-Minguez, A. (2006) Involvement of glutaredoxin-1 and thioredoxin-1 in beta-amyloid toxicity and Alzheimer's disease. *Cell Death Differ* **13**, 1454-1465
138. Ueno, M., Masutani, H., Arai, R. J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999) Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J Biol Chem* **274**, 35809-35815
139. Junn, E., Han, S. H., Im, J. Y., Yang, Y., Cho, E. W., Um, H. D., Kim, D. K., Lee, K. W., Han, P. L., Rhee, S. G., and Choi, I. (2000) Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* **164**, 6287-6295
140. Lu, J., and Holmgren, A. (2012) Thioredoxin system in cell death progression. *Antioxid Redox Signal* **17**, 1738-1747
141. Schulze, P. C., Yoshioka, J., Takahashi, T., He, Z., King, G. L., and Lee, R. T. (2004) Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* **279**, 30369-30374
142. Parikh, H., Carlsson, E., Chutkow, W. A., Johansson, L. E., Storgaard, H., Poulsen, P., Saxena, R., Ladd, C., Schulze, P. C., Mazzini, M. J., Jensen, C. B., Krook, A., Bjornholm, M., Tornqvist, H., Zierath, J. R., Ridderstrale, M., Altshuler, D., Lee, R. T., Vaag, A., Groop, L. C., and Mootha, V. K. (2007) TXNIP regulates peripheral glucose metabolism in humans. *PLoS medicine* **4**, e158
143. Yoshihara, E., Chen, Z., Matsuo, Y., Masutani, H., and Yodoi, J. (2010) Thiol redox transitions by thioredoxin and thioredoxin-binding protein-2 in cell signaling. *Methods Enzymol* **474**, 67-82
144. Minn, A. H., Hafele, C., and Shalev, A. (2005) Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology* **146**, 2397-2405
145. Arner, E. S., and Holmgren, A. (2001) Measurement of thioredoxin and thioredoxin reductase. *Current protocols in toxicology / editorial board, Mahin D. Maines* **Chapter 7**, Unit 7 4
146. Raturi, A., and Mutus, B. (2007) Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. *Free Radic Biol Med* **43**, 62-70
147. Heuck, A. P., and Wolosiuk, R. A. (1997) Di-fluoresceinthiocarbamyl-insulin: a fluorescent substrate for the assay of protein disulfide oxidoreductase activity. *Anal Biochem* **248**, 94-101
148. Luthman, M., and Holmgren, A. (1982) Glutaredoxin from calf thymus. Purification to homogeneity. *J Biol Chem* **257**, 6686-6690
149. Porras, P., Pedrajas, J. R., Martinez-Galisteo, E., Padilla, C. A., Johansson, C., Holmgren, A., and Barcena, J. A. (2002) Glutaredoxins catalyze the reduction of glutathione by dihydrolipoamide with high efficiency. *Biochemical and biophysical research communications* **295**, 1046-1051
150. Dumitrescu, A. M., Liao, X. H., Abdullah, M. S., Lado-Abeal, J., Majed, F. A., Moeller, L. C., Boran, G., Schomburg, L., Weiss, R. E., and Refetoff, S. (2005) Mutations in SECISBP2 result in abnormal thyroid hormone metabolism. *Nat Genet* **37**, 1247-1252
151. Flohe, L. (2007) Selenium in mammalian spermiogenesis. *Biological chemistry* **388**, 987-995
152. Mercuri, E., Talim, B., Moghadaszadeh, B., Petit, N., Brockington, M., Counsell, S., Guicheney, P., Muntoni, F., and Merlini, L. (2002) Clinical and imaging findings in six cases of congenital muscular dystrophy with rigid spine syndrome linked to chromosome 1p (RSMD1). *Neuromuscular disorders : NMD* **12**, 631-638
153. Schallreuter, K. U., and Wood, J. M. (2001) Thioredoxin reductase - its role in epidermal redox status. *J Photochem Photobiol B* **64**, 179-184

154. Shrimali, R. K., Irons, R. D., Carlson, B. A., Sano, Y., Gladyshev, V. N., Park, J. M., and Hatfield, D. L. (2008) Selenoproteins mediate T cell immunity through an antioxidant mechanism. *J Biol Chem* **283**, 20181-20185
155. Rodriguez-Manzaneque, M. T., Tamarit, J., Belli, G., Ros, J., and Herrero, E. (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Molecular biology of the cell* **13**, 1109-1121