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Biochemical properties of human glutaredoxins

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Cover image: Ribbon representation of reduced human Grx1 (Sun *et al*., 1998).

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To My Family

ABSTRACT

Glutaredoxins (Grxs) are highly conserved thiol-disulfide oxidoreductases that utilize electrons from the tripeptide glutathione (GSH) to catalyze thiol-disulfide exchange reactions. Bacteria, yeast and plants contain multiple dithiol Grxs, which are involved in different cellular processes like DNA synthesis, defense against oxidative stress, apoptosis, and regulation of transcription factor binding activity. Since only one Grx (Grx1) was known in mammalian cells the aim of this thesis project was to identify and characterize additional mammalian Grxs.

Two isoforms of a novel human Grx with putative mitochondrial or nuclear localization signals (Grx2a and Grx2b, respectively) were identified in the expressed sequence tag database. Analysis of the genomic sequence localized Grx2 to chromosome 1, and suggested that alternative first exon splicing generated the two isoforms. Both isoforms of human Grx2 encoded proteins of approximately 18 kDa with 34 % sequence identity to the previously characterized cytosolic Grx1. While all the regions characteristic of Grx was conserved in Grx2, the active site (CSYC) contained a serine replacing the proline in the usual consensus CPYC motif. Northern and Western blot analysis revealed ubiquitous expression of Grx2 in human tissues.

Recombinant Grx2 differed significantly in its biochemical properties from Grx1. Grx2 exhibited a ten-fold lower GSH-dependent thiol-disulfide reducing specific activity than Grx1. However, given the higher apparent affinity of Grx2 for glutathionylated substrates the efficiencies for the enzymes were almost the same. Mutant analysis showed further that the catalytic properties of Grx2 were linked to the Pro to Ser exchange in the non-conserved active site sequence. Unlike all previously characterized eukaryotic glutaredoxins, Grx2 was a substrate for both the cytosolic and mitochondrial isoforms of thioredoxin reductase. This electron transfer pathway enabled Grx2 to reduce GSH-mixed disulfides and low molecular weight disulfides, including GSSG. This activity, in conjunction with a high specificity towards glutathionylated substrates endows Grx2 with properties suitable to remove and control the levels of glutathionylated proteins and GSSG. When considering other possible cellular reductants for Grxs we also found dihydrolipoamide to catalyze Grx-dependent reduction of GSSG, which demonstrated that Grxs are able to work in a reverse electron flow.

In contrast to Grx1, Grx2 displayed features typical of glutathione S-transferases, such as conjugation of GSH to electrophilic compounds and binding to S-linked GSH-Sepharose. Unexpectedly, the latter property was dependent on Grx2 being in the oxidized state. A Grx2S38P active site mutant completely abolished the binding to the Sepharose, demonstrating a crucial role of the Pro to Ser exchange also for GSH binding. This suggested that Grx2 might form rather stable complexes with glutathionylated proteins during oxidative conditions.

Also, Grx1 and Grx2 revealed completely different patterns upon treatment with oxidants and reductants. Whereas Grx1 formed disulfide bonded dimers and oligomers and was partly inactivated upon oxidation, Grx2 retained activity and revealed no tendency to oligomerize. Instead the precence of a second structural disulfide was strongly indicated in Grx2, which stabilized the protein.

In summary, the significant differences between the two mammalian Grxs might be an adaptation to different redox environments or reactions catalyzed. The unusual properties of Grx2 implicate this enzyme in cellular redox regulation and defense against oxidative stress in mitochondria.

LIST OF PUBLICATIONS

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

I. 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

II. Porras P., Pedrajas J.R., Martínez-Galisteo E., Padilla C.A., **Johansson C.**, Holmgren A., and Bárcena J.A. (2002) Glutaredoxins catalyze the reduction of glutathione by dihydrolipoamide with high efficiency. *Biochem. Biophys. Res. Commun.* 295, 1046-1051

III. **Johansson C**., Lillig H.C., and Holmgren A. (2004) Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. *J. Biol. Chem*. 279, 7537-7543

IV. **Johansson C**., Lillig H.C., Lundberg M., and Holmgren A. (2004) Human mitochondrial glutaredoxin has glutathione S-transferase activity and a redox dependent affinity for S-linked glutathione. *Manuscript*

V. **Johansson C.**, Lillig H.C., Elgán H.T., Berndt D.K., and Holmgren A. (2004) Analysis of the redox state upon oxidation of human glutaredoxins and identification of a native structural disulfide in glutaredoxin 2. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AP1	Activator protein 1
ASK1	Apoptosis signalling kinase 1
CD	Circular dichroism
CDNB	1-Chloro-2,4-dinitrobenzene
CLIC	Chloride ion channels
CoA	Coenzyme A
DCNB	1,2-dichloro-4-nitrobenzene
DHA	Dehydroascorbate
Dsb	Disulfide bond promoting enzyme
DTT	Dithiotreitol
EST	Expressed sequence tags
FAD	Flavin adenine dinucleotide
GAPDH	Glucose-6-phosphate dehydrogenase
GdnHCl	Guanidine hydrochloride
GFP	Green fluorescent protein
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
HED	β-Hydroxyethyl disulfide
HIV	Human immunodeficiency
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NF1	Nuclear factor 1
NMR	Nuclear magnetic resonance
PDI	Protein disulfide isomerase
Prx	Peroxiredoxin
Ref1	Redox factor 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Ribonucleotide reductase
RSO_2H	Sulfinic acid
RSO_3H	Sulfonic acid
R-SOH	Sulfenic acid
R-SSG	Glutathione mixed disulfide
SH	Thiol
S-S, S_2	Disulfide bond
TGR	Thioredoxin glutathione reductase
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Wt	Wild type

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

All aerobic organisms are constitutively exposed to oxidative toxicity caused by reactive oxygen species (ROS). ROS forms endogenously as a consequence of incomplete reduction of molecular oxygen in the mitochondrial respiratory chain or exogenously from environmental stress caused by heavy metals, ionization radiation, and redox-cycling chemicals. ROS can affect the cellular redox balance and either directly or indirectly alter major functions of the cell such as signal transduction, DNA and RNA synthesis, protein synthesis and enzyme activity. To cope with the oxidative challenge cells have developed a number of defenses, such as ROS-metabolizing enzymes, disulfide reducing proteins, as well as redox active low molecular weight compounds, that work together to maintain a reducing cellular redox state. The two major redox systems that maintain the cellular thiol-disulfide redox status are the thioredoxin and the GSH/ glutaredoxin systems, that both utilize reducing equivalents from NADPH. The thioredoxin system is composed of NADPH, thioredoxin (Trx) and thioredoxin reductase (TrxR) whereas the GSH/ glutaredoxin system is composed of NADPH, GSH, glutathione reductase (GR), and glutaredoxin (Grx) (Fig1). Thioredoxins and glutaredoxins are structurally similar oxidoreductases that have been conserved throughout evolution. The introduction of this thesis is focused on glutaredoxins and related enzymes.

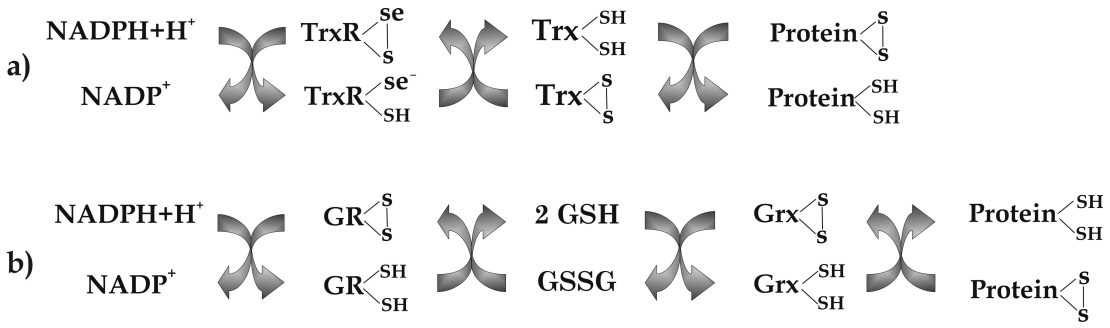

Figure 1. The thioredoxin (a) and the GSH/glutaredoxin (b) systems.

1.2 THE GLUTAREDOXIN SYSTEM

1.2.1 Glutathione

The tripeptide Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is widely distributed in animal tissues, plants and microorganisms and has been extensively studied since its discovery in the 1920s by Fredrick Gowland Hopkins. Most cells contain millimolar concentrations of GSH, which at the expense of NADPH, is kept in the reduced state by the flavoenzyme glutathione reductase (GR). GSH is in equilibrium with several oxidized disulfide species, including glutathione disulfide (GSSG) and protein-GSH mixed disulfides, which together constitute the major cellular redox buffer (Smith *et al*., 1996). The ratio of the GSH/GSSG redox couple correlates with the biological status of the cell and is therefore used to define the cellular redox state (for a review see (Schafer and Buettner, 2001)). In a resting cell, GSSG constitutes less than 1% of the cytosolic GSH, with a ratio of GSH to GSSG that exceeds 100. These levels are kept either by excretion of GSSG out of the cell or by NADPH-dependent reduction of the oxidized disulfide species back to GSH.

Normal metabolism requires constant and rapid replenishment of GSH, which is accomplished through both the reduction of oxidized glutathione and *de novo* synthesis. GSH is synthesized in the cytosol from its constitutive amino acids by two ATP dependent enzymes. The first step in the biosynthesis is the formation of γ-glutamyl-cysteine by the enzyme γ-glutamyl-cysteine synthetase, and this step is rate-limiting. The second step is the addition of glycine, which is catalyzed by the enzyme glutathione synthetase. GSH is degraded by the membrane bound enzyme γ-glutamyltranspeptidase that transfer the γ-glutamyl group in GSH to acceptors such as amino acids, dipeptides, and water. Cysteinylglycine formed in the transpeptidase reaction is then split by dipeptidases on the cell surface to cysteine and glycine (Griffith *et al*., 1978).

GSH is widely distributed in different cellular compartments. Several attempts have been made to determine the subcellular levels since the compartmentalization may result in different redox environments. GSH is shown to play a major role in maintenance of the mitochondrial redox status. The mitochondrial pool of GSH is separated from the cytosol and imported by specific ATP-dependent transport processes (Griffith and Meister, 1985; Meister and Anderson, 1983). Several studies indicate that mitochondria contain elevated levels of GSH, with a half-life that is longer

than the nuclear and cytosolic pools (Meredith and Reed, 1982; Romero and Sies, 1984; Romero *et al*., 1984; Söderdahl *et al*., 2003). Studies on the nuclear pool of GSH have given unequal results (Jevtovic-Todorovic and Guenthner, 1992; Söderdahl *et al*., 2003), and it is currently not clear whether the nucleus possesses a pool of GSH that is separated from the cytosol. The GSH pool in the endoplasmic reticulum is much more oxidized than both the cytosolic and mitochondrial pools, with an estimated ratio of GSH/GSSG to 3:1, a thiol-disulfide ratio compatible with disulfide bond formation in the secretory pathway (Hwang *et al*., 1992). GSH and GSSG are also found outside cells, but in general at 100 to 1000 times lower concentrations than intracellular GSH.

Glutathione possesses several important functions and has a central role in many redox dependent processes. Most interest has been focused on its role in protecting cells from oxidative and nitrosative damage, and by providing reducing equivalents for enzymes involved in the metabolism of reactive oxygen species (ROS), reactive nitrogen species (RNS) or in the repair of damaged proteins. GSH has also a key role in the metabolism of xenebiotics to which exposure is an everyday occurrence. GSH conjugated to electrophilic compounds are exported out of the cell or further metabolized. Recent evidence reveals in addition that the glutathione redox couple regulates protein function by reversible formation of mixed disulfides between protein cysteines and GSH (see below, protein S-glutathionylation) (Cotgreave and Gerdes, 1998).

Hence, it is not surprising that an imbalance in GSH, either by decreased GSH levels or changed ratio of GSH/GSSG, is observed in a wide range of pathologies, including cancer, neurodegenerative disorders, cystic fibrosis, HIV and ageing (for a review see (Townsend *et al*., 2003)).

1.2.2 Glutathione reductase

The flavoenzyme glutathione reductase (GR) catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to two molecules of GSH. The enzyme belongs to the pyridine nucleotide disulfide oxidoreductase family that includes lipoamide dehydrogenase (LipDH) and thioredoxin reductase (TrxR). Glutathione reductase has been isolated and characterized from many organisms, and the primary sequence of the reactive center in *E. coli*, yeast and humans are highly conserved, and

the mechanism of the reduction of GSSG is practically identical (Collinson and Dawes, 1995; Greer and Perham, 1986; Krauth-Siegel *et al*., 1985). The structure of human GR and the mechanism of GSSG reduction are known in fine detail. The human GR has a molecular weight of 102 kDa and consists of two identical subunits organized ”head to tail”. Each subunit consists of three domains, a $NADP^+$-binding domain, a flavine adenine dinucleotide (FAD)-binding domain and an interface domain. Each subunit contains further six cysteine residues, two of which form a redox-active disulfide, essential for the enzyme activity. In the catalysis two electrons are transferred from NADPH to FAD, and further to the redox active site cysteine residues in the same subunit. The dithiol in each subunit finally reduces GSSG, which is bound to the FAD domain in one subunit and the interface of the other subunit (Karplus and Schulz, 1987; Karplus and Schulz, 1989; Pai *et al*., 1988). Furthermore, GR from both eukaryotes and prokaryotes are known to be self regulated by covalent modifications controlled by the cellular redox status, the pyridine nucleotide and glutathione pools. This has further been supported by rapid loss in activity upon treatment of the pure enzymes with a reductant such as GSH and DTT (Lopez-Barea and Lee, 1979; Mata *et al*., 1985; Pinto *et al*., 1984; Pinto *et al*., 1985).

In mammalian cells, GR activity is detected both in the cytosol and in the mitochondria, and the two enzymes have revealed indistinguishable biochemical properties (Taniguchi *et al*., 1986), which suggested that the two isoenzymes were encoded by a single gene. Analysis of the genomic sequence of human GR further predicted an N-terminal arginine rich mitochondrial sequence between two different in frame start codons. Consequently, each of the two start codons has been proposed to initiate synthesis of the mitochondrial and cytosolic GR proteins, respectively (Kelner and Montoya, 2000; Taniguchi *et al*., 1986).

By the NADPH-dependent reduction of GSSG, GR participates in protection of cells against oxidative stress and maintenance of the cellular redox balance. However, GR null mutants in both *E. coli* and *Saccaromyces cerevisiae* are viable and display an unperturbed ratio of GSH relative to GSSG, confirming that the enzyme is not essential in maintaining the glutathione redox status (Tuggle and Fuchs, 1985). Thus, it seems reasonable that reducing equivalents can be transferred from the Trx system for reduction of GSSG, i.e. a cross talk must exist between the Trx and the Grx systems.

1.2.3 Glutaredoxin

Glutaredoxins (Grxs) comprise a family of low molecular weight thiol-disulfide oxidoreductases that are highly conserved throughout evolution. Grx was discovered in 1976 as a GSH-dependent dithiol hydrogen donor for ribonucleotide reductase in an *E. coli* mutant lacking thioredoxin (today Trx1) (Holmgren, 1976). However, with the discovery of general GSH-disulfide oxidoreductase activity of Grxs and protein sequencing, it is now clear that Grxs were described earlier in the literature as enzyme activities in cell extracts. These include homocysteine transhydrogenase (Racker, 1955), glutathione-disulfide transhydrogenase (Thompson, 1960), thiol-disulfide transhydrogenase (Nagai, 1968) and thioltransferase (Askelöf, 1974; Ericksson, 1970).

Grxs catalyze the reduction of disulfide bonds in a variety of protein and non-protein substrates utilizing two redox active cysteines in a conserved Cys-Pro-Tyr-Cys active site motif that during catalysis cycles between a dithiol and disulfide. The intramolecular active site disulfide formed upon reduction of a disulfide substrate is selectively recycled to the reduced form by two molecules of GSH, with the formation of glutathione disulfide (GSSG) (Holmgren, 1976; Holmgren, 1979a; Holmgren, 1979b). However, most dithiol Grxs characterized have a strong preference for GSH-mixed disulfides as substrates, which are reduced by a mechanism requiring only the more N-terminal active site cysteine (Bushweller *et al*., 1992; Gravina and Mieyal, 1993). The high specificity for GSH-mixed disulfides, and the ability to use reducing equivalents from GSH, distinguishes Grx from thioredoxin (Trx), which favors intramolecular protein disulfide substrates and is regenerated by thioredoxin reductase (TrxR).

Grxs participate in numerous reactions in the cell, such as reduction of ribonucleotides to deoxyribonucleotides by reducing ribonucleotide reductase (Holmgren, 1979a; Luthman *et al*., 1979), the reduction of sulfate by virtue of Grx being an electron donor for PAPS reductase (Tsang, 1981), and reduction of dehydroascorbate to ascorbate (vitamin C) (Wells *et al*., 1990). Grx has also been described as a regulator of transcription factors (Bandyopadhyay *et al*., 1998; Hirota *et al*., 2000; Zheng *et al*., 1998) and apoptosis (Chrestensen *et al*., 2000; Daily *et al*., 2001a; Song *et al*., 2002). Grxs account for essentially all cellular protein-SG deglutathionylase activity (Chrestensen *et al*., 2000; Jung and Thomas, 1996), and this property has given the enzymes a prominent role in homeostasis of protein sulphydryl

groups, both in a protective mode under oxidative stress and in a regulatory mode, whereby reversible glutathionylation represents a mechanism of redox-mediated signal transduction (Daily *et al*., 2001a; Luikenhuis *et al*., 1998; Murata *et al*., 2003; Rodriguez-Manzaneque *et al*., 1999).

These, classical dithiol Grxs are present in various organisms such as plants, bacteria, viruses and mammals. Comparison of their three-dimensional structures, which comprises a typical "thioredoxin-fold", has revealed three highly conserved areas that are functionally important and characterize the proteins; (i) two redox active cysteines in a conserved CPYC active site motif, (ii) a glutathione binding site (Bushweller *et al*., 1994; Yang *et al*., 1998), which explains its high specificity for GSH and GSH-mixed disulfides and (iii) a hydrophobic surface area involved in substrate binding (Xia *et al*., 1992). The mammalian Grxs as well as the Grxs characterized in yeast, bacteria and viruses are described in special sections later in the thesis.

1.2.3.1 The Glutaredoxin family

In the last years the Grx family has expanded due to identification of new protein types, expressed in several isoforms with different subcellular localization, and forms that structurally are more similar to proteins of the glutathione S-transferase (GST) superfamily but exhibit activities typical for Grx. Evolutionary conserved Grx-like proteins with a monothiol active site have also been identified, as well as monothiol and dithiol Grx domains as modules in larger proteins.

The identification of these new members of the Grx family, which seem to be both structurally and functionally different from the "classical" dithiol Grxs initially identified, demands a new general nomenclature. Today, Grxs are numbered in order of the discovery in a particular species but the number has no functional meaning. When comparing Grxs across species this can be rather confusing, since e.g. Grx2 from one species not necessarily corresponds to Grx2 in another species. To simplify the nomenclature Xia et al (Xia *et al*., 2001) recently suggested a three-subfamily classification for glutaredoxins. The first group would consist of the "classical" low molecular weight Grxs with a dithiol active site and include mammalian Grx1 and Grx2 (see paper I-V), *E. coli* Grx1 and Grx3, yeast Grx1 and Grx2, virus Grxs and the phage T4 Grxs. The second group would consist of Grxs with a variable molecular mass and a

monothiol active site. Yeast Grx3-5 would belong to this group as well as other identified but so far uncharacterized Grx-like proteins with a monothiol active site. The third group would correspond to proteins having an N-terminal Grx-like domain, with either a dithiol- or monothiol active site, and a C-terminal helical domain, with a general structure reminiscent of the GST superfamily. Human glutathione S-transferase GSTO1, mouse glutathione S-transferase p28, human chloride intracellular channel protein CLIC1, as well as *E. coli* Grx2 are proposed to belong to this group (Xia *et al*., 2001).

1.3 THE THIOREDOXIN SYSTEM

1.3.1 Thioredoxin reductase

Thioredoxin reductase (TrxR) is a widely distributed flavoprotein that catalyzes the NADPH-dependent reduction of the active site disulfide in thioredoxin (Trx). TrxR belongs to the pyridine nucleotide-disulfide oxidoreductase family of dimeric flavoenzymes, which includes lipoamide dehydrogenase (LipDH) and glutathione reductase (GR). TrxR in lower species, such as bacteria and yeast, as well as the cytosol of plants, are highly conserved through evolution and consist of two identical subunits of 35 kDa, each with a non-covalently bound FAD and a redox active disulfide. These enzymes have in general narrow substrate specificity and are specific for the Trx from the same or a closely related species (Holmgren, 1985; Williams *et al*., 2000).

TrxRs in mammalian cells are entirely different to the enzymes in lower organisms, and are instead larger and more closely related to glutathione reductase. The mammalian TrxRs are composed of two identical subunits of 55 kDa, which are organized ”head to tail” in a similar fashion as glutathione reductase. Each subunit contains a NADPH-binding domain, an FAD-binding domain with a pair of redox-active cysteines, an interface domain, and a C-terminal elongation containing an essential selenocysteine (Sec) in a Gly-Cys-Sec-Gly-COOH motif (Sandalova *et al*., 2001; Tamura and Stadtman, 1996; Zhong *et al*., 2000; Zhong and Holmgren, 2000).

Mammalian TrxR exhibits broad substrate specificity and can, apart from donating electrons to Trx, also reduce PDI (Lundström and Holmgren, 1990) and various non-protein chemically unrelated low molecular compounds such as 5,5’Dithiobis-2-nitrobenzoic acid (DTNB) (Holmgren, 1977), lipoic acid (Arner *et al*., 1996), selenium containing compounds (Kumar *et al*., 1992), dehydroascorbate (DHA)

(May *et al*., 1997), lipid hydroperoxides and hydrogen peroxide (Björnstedt *et al*., 1995; Zhong and Holmgren, 2000). In the catalyzed reactions, electrons are transferred from NADPH via the non-covalently bound FAD to the redox active disulfide. The electrons are then transferred from the dithiol of one subunit to the C-terminal selenylsulfide in the other subunit. The selenolate formed, finally reduces any of the substrates mentioned above (Sandalova *et al*., 2001; Zhong *et al*., 1998; Zhong and Holmgren, 2000).

Three separate TrxR isoenzymes have been found in mammals. The isoenzymes share more than 50 % identity at the amino acid level, and have the same overall domain structure with a selenocysteine-containing active site motif. The cytosolic TrxR1 has been isolated from various mammalian tissues and is the isoenzyme that so far is best characterized. (Holmgren, 1977; Luthman and Holmgren, 1982b; Oblong *et al*., 1993). The second isoenzyme, TrxR2 is localized to the mitochondrial matrix and has been reported to display similar kinetic properties as TrxR1 (Miranda-Vizuete *et al*., 1999; Miranda-Vizuete *et al*., 2000; Rigobello *et al*., 1998; Watabe *et al*., 1999). The third TrxR isoenzyme contains an N-terminal extension in form of a monothiol Grx domain, and has in contrast to TrxR1 and TrxR2 been shown to reduce glutathione disulfide (GSSG). Since this enzyme possesses activities both as a thioredoxin reductase and glutathione reductase, it has been termed TGR (Thioredoxin and Glutathione Reductase). TGR has in addition been shown to possess low Grx activities, and the Grx domain was suggested to receive electrons from the C-terminal selenocysteine-containing center, enabling a GSH-independent pathway (Agorio *et al*., 2003; Alger and Williams, 2002; Sun *et al*., 2001a). In contrast to TrxR1 and TrxR2 that are ubiquitously expressed, TGR is mainly found in testis (Sun *et al*., 2001a). Furthermore, recent reports have indicated that the three TrxR isoenzymes are expressed in a large number of isoforms by differential splicing (Osborne and Tonissen, 2001; Rundlöf *et al*., 2000; Sun *et al*., 2001b). In humans no more than 21 different transcripts of TrxR1 has been found, predicted to encode five isoforms with alternative N-terminal domains. Interestingly, one isoform encoded an N-terminal dithiol Grx domain with an atypical CTRC active site sequence (Rundlöf *et al*., 2004).

Mammalian TrxRs, whose activity is not sensitive for either changes in the glutathione redox buffer or decreases in the cellular pH, have been demonstrated to have a central role in the cellular protection against oxidative damage and in the cellular redox regulation. For recent extensive reviews about TrxR function and

regulation see (Arner and Holmgren, 2000; Nordberg and Arner, 2001; Rundlöf and Arner, 2004).

1.3.2 Thioredoxin

Thioredoxins (Trxs) are small thiol-disulfide oxidoreductases that are found in all living organisms studied. Trxs contain two redox active cysteines in a conserved CGPC active site motif that during the catalysis cycle between a dithiol and an intramolecular disulfide. The active site disulfide formed in the catalysis is, in contrast to Grxs, reduced by TrxR and not by GSH. Trxs from different species show a high degree of conservation with the same predicted overall fold and few insertions in the loop regions (Arner and Holmgren, 2000; Eklund *et al*., 1984). Mammalian cells contain two classical Trx isoenzymes, termed Trx1 and Trx2, which are localized to the cytosol and to the mitochondria, respectively. Trx1 and Trx2 show 35 % sequence identity and reveal similar catalytic properties *in vitro* (Spyrou *et al*., 1997). Both isoenzymes are expressed in all tissues and play a central role in the cellular defense against oxidative stress (Damdimopoulos *et al*., 2002; Tanaka *et al*., 2002; Tanaka *et al*., 2001). As demonstrated by early embryonic lethality in knockout mice, both Trx1 and Trx2 are essential for normal development (Matsui *et al*., 1996; Nonn *et al*., 2003).

Trx1, which is yet the best studied, is a general disulfide reductant that interacts with a variety of enzymes, receptors, and transcription factors (Holmgren, 1985). Trx1 is involved in many different cellular processes including DNA synthesis [Engstrom, 1974 #414], regulation of transcription factors (Hirota *et al*., 1997; Hirota *et al*., 1999; Nakamura *et al*., 1997; Schenk *et al*., 1994), and apoptosis (Saitoh *et al*., 1998). Trx1 can also be secreted (Rubartelli *et al*., 1995), by a so far unknown mechanism, and act exogenously as a cytokine (Rosen *et al*., 1995), chemokine, or growth factor (Bertini *et al*., 1999; Pekkari *et al*., 2000). Several studies have also demonstrated that cells can take up Trx1, by a mechanism that does not require its redox active cysteines (Gasdaska *et al*., 1995; Powis *et al*., 1994). Unlike mitochondrial Trx2 and Trxs from lower species, mammalian Trx1 contains three additional conserved cysteine residues (at position 62, 69 and 73), with potential regulatory roles. Cys62 was recently shown to form an intramolecular disulfide bond with Cys69 upon oxidative conditions. Formation of the second intramolecular disulfide was suggested to have a profound effect on the three-dimensional structure, that transiently would inhibit Trx1 activity

(Watson *et al*., 2003). An intermolecular disulfide bond formed between Cys73 in a Trx1 homodimer has also been observed, making the active site disulfide inaccessible to TrxR (Weichsel *et al*., 1996). Cys73 has in addition been shown to form a GSH-mixed disulfide upon oxidative conditions (Casagrande *et al*., 2002) and Cys69 to be nitrosylated (Haendeler *et al*., 2002).

Furthermore, mammalian cells contain apart from these two classical Trxs also a large number of proteins composed of a Trx domain in fusion with other protein domains, including Txl-1 and Txl-2 (Miranda-Vizuete *et al*., 1998; Sadek *et al*., 2003), the spermatid specific SpTrx1 and SpTrx2 (Sadek *et al*., 2001), TXM (Matsuo *et al*., 2001), ERDj5 (Cunnea *et al*., 2003) and the PDI-family of proteins (Ferrari and Soling, 1999), which today encompasses 24 members (Holmgren A, personal communication).

1.4 THE THIOREDOXIN /GLUTAREDOXIN FOLD

Glutaredoxins are part of the thioredoxin fold superfamily of proteins, which includes thioredoxins (Trxs), protein disulfide isomerases (PDIs), glutathione S-transferases, glutathione peroxidases, peroxiredoxins and the bacterial Dsb family (Choi *et al*., 1998; Kemmink *et al*., 1996; Martin, 1995). The individual subfamilies have in general low sequence identity but all share a similar three-dimensional architecture, comprised of a central core of four-stranded mixed β-sheet surrounded by three α-helices. This folding motif was first identified in a crystal structure of *E. coli* Trx1 and has therefore been called the ”thioredoxin fold”. However, Trx itself contains an additional β-strand at its N- terminus compared to the other members of this superfamily (Holmgren *et al*., 1975).

The three-dimensional structure of oxidized, reduced and GSH-mixed forms of Grxs from numerous species have been determined, including bacteriophage T4 Grx1 (Eklund *et al*., 1992; Ingelman *et al*., 1995), Vaccinia virus Grx1 (Kelley and Bushweller, 1998), *E. coli* Grx1 (Bushweller *et al*., 1994; Sodano *et al*., 1991; Xia *et al*., 1992), Grx2 (Xia *et al*., 2001), Grx3 (Nordstrand *et al*., 2000; Nordstrand *et al*., 1999a), pig Grx1 (Katti *et al*., 1995) and human Grx1 (Sun *et al*., 1998; Yang *et al*., 1998), demonstrating a well preserved three-dimensional architecture despite low sequence similarities.

Mammalian Grx1 has short α-helical extensions at the N- and C-terminus compared to the prokaryotic glutaredoxins. Thus, the four-stranded mixed β-sheet is surrounded by a total of five α-helices (Katti *et al*., 1995; Sun *et al*., 1998; Yang *et al*., 1998). The strands β2, β3, and β4 are antiparallel, whereas β2 and β1 are parallel. The β-sheet is flanked on one side by helices 2, 4 and 5 and on the other side by helices 1 and 3 (Fig. 2). The active site is located on helix 2 in mammalian Grx1, compared to helix 1 in the prokaryotic Grxs. The presence of the additional helices in mammalian Grx1 is suggested to increase the "stiffness" of the fold and to be important for its function (Berardi and Bushweller, 1999; Yang *et al*., 1998).

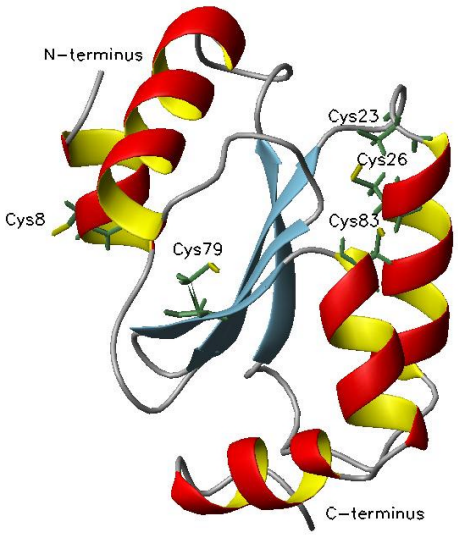

Figure 2. Ribbon representation of fully reduced human Grx1 demonstrating a typical thioredoxin fold The figure was generated with the program MOLMOL (Sun *et al*., 1998).

Comparison of the oxidized and reduced forms of the proteins reveals only minor structural alterations, especially around the active site area (Nordstrand *et al*., 2000; Xia *et al*., 1992). However, the three-dimensional structures of *E. coli* Grx1 revealed an increased solvent exposed hydrophobic area in the reduced protein, which was suggested to favor interactions with protein substrates (Xia *et al*., 1992). In contrast to the oxidized and reduced form of Grx, significant changes of the helices are associated with GSH or peptide binding (Berardi and Bushweller, 1999; Bushweller *et al*., 1994; Nordstrand *et al*., 1999a). The binding site for the GSH moiety in the GSH-Grx mixed disulfide intermediate is located in a cleft on the protein surface encompassing residues

from helices 2 and 3, the active site loop and the loop connecting helix 3 and β-strand 3. Both the location and the predicted non-covalent interactions contributing to stabilization of the complex, appear very similar between different Grxs (Sun *et al*., 1998; Yang *et al*., 1998). In human Grx1 electrostatic interactions are predicted between the carboxylate of the Gly residue in GSH and Lys19 and Arg67, and between the carboxylate group of γ-Glu and Arg71. Electrostatic interactions are further expected between the carboxylate of γ-Glu and the positive charge of the dipole of helix 4. Hydrogen bonds are also predicted to form between the carboxylate of the Gly residue and Gln57, between the carbonyl groups of both γ-Glu and Cys and Val69, and between the carboxylate of γ-Glu and Cys82 and Ser83. In addition, numerous van der Waals interactions are predicted between Tyr24, Cys25, Thr68, Val69 and Pro70 residues in Grx1 and the GSH moiety (see Fig. 3) (Sun *et al*., 1998; Yang *et al*., 1998).

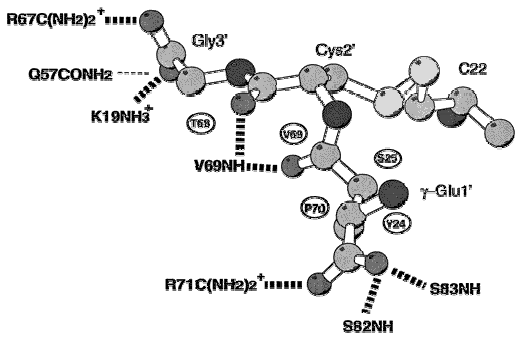

Figure 3. Predicted hydrogen bonds and salt interactions between the GSH moiety and the human Grx1C7SC25SC78SC82S mutant. Residues expected to be involved in van der Waals interactions with glutathione are shown by circles (Yang *et al*., 1998).

1.4.1 The active site

Thiol-disulfide oxidoreductases of the thioredoxin fold superfamily of proteins exert their catalysis utilizing two redox active cysteines in a CXXC motif, located at the N-terminus of an α-helix. Despite many similarities between the proteins in this superfamily, some members like Grx and Trx, are mainly reductases, whereas others are responsible for introduction and isomerization of disulfide bonds. The source of these differences in reducing /oxidizing capacity is not well understood but the pK_a of the active site thiols is one of the factors that has been found to be a key determinant. The pK_a value of the more N-terminal active site cysteine thiol in the thioredoxin superfamily of proteins is significantly lower than that of a free cysteine, which is about

8.5, whereas the more C-terminal active site cysteine thiol has a pK_a that is usually more basic (Dillet *et al*., 1998; Gan and Wells, 1987; Grauschopf *et al*., 1995; Kallis and Holmgren, 1980; Kortemme *et al*., 1996; Nelson and Creighton, 1994). As indicated by Table I both the pK_a value of the N-terminal active site thiol and the redox potential ($E^{o'}$ value) of various proteins in the thioredoxin superfamily vary over a wide range. The redox potential, which reflects the relative stabilities of the reduced and oxidized forms of the protein, are directly linked to the pK_a of the N-terminal active site thiol. In general, a lower pK_a is associated with a more positive redox potential and a more oxidizing protein (Mossner *et al*., 2000).

TABLE I

pK_a-value of the more N-terminal active site thiol and the redox potential of various thiol-disulfide oxidoreductases

Thiol-disulfide oxidoreductase	**Active site sequence**	**pK_a-value of the N-terminal active site thiol**	**Redox potential mV**
E. coli Trx1	CGPC	7.1	-270
E. coli NrdH	CVQC	-	-248
E. coli Grx1	CPYC	~ 5	-233
T4 Grx1	CVYC	-	-230
E. coli Grx3	CPYC	~ 5	-198
human Grx1	CPYC	3.5	-
S.cerevisiae Grx5	CGFS	5	-
human PDI	CGHC	6.7	-175
E. coli DsbA	CPHC	3.2	-124

The intervening $-X_1-X_2-$ dipeptide sequence in the active site motif, that differs between members of the thioredoxin superfamily but is conserved within each protein family, has been shown to be the main determinant of the redox properties of these enzymes (Grauschopf *et al*., 1995; Joelson *et al*., 1990; Mossner *et al*., 1998). This correlation was first demonstrated by a 35 mV increase in redox potential of a Trx mutant with the active site dipeptide mimicking that of PDI (Krause *et al*., 1991). Later, the same observation was made with other dipeptide mutant variants, demonstrating a shift in redox potential towards that of the natural enzymes (Grauschopf *et al*., 1995; Kortemme *et al*., 1996). Today, these observations are largely understood in terms of the number of hydrogen bonds that the intervening residues provide to stabilize the

thiolate charge (Foloppe *et al*., 2001; Nordstrand *et al*., 1999b). However, as demonstrated by the active site mutant variants, the dipeptide sequence is not alone sufficient to convert one thiol-disulfide oxidoreductase into another, also other relatively unknown factors modulates the redox potential for a given enzyme. The *E. coli* Grx1 and Grx3 is an additional example demonstrating that also other factors influence the redox properties of the proteins. Despite sharing similar folds with identical CPYC active site motif located at the same position, the redox potential of these proteins differ by 35 mV (Åslund *et al*., 1997). Recently, molecular dynamics simulations suggested that the structure and dynamics of the catalytic cysteines differed between the two proteins, with Grx3 being more rigid than Grx1. This suggested further that the hydrogen bonds that stabilized the N-terminal active site thiolate formed less frequently in the reduced form of Grx1 compared to Grx3 (Foloppe and Nilsson, 2004; Foloppe *et al*., 2001).

1.4.2 The consensus CPYC motif

Grxs characterized from most species contain a highly conserved Cys-Pro-Tyr-Cys motif in their active site, located at the N-terminus of helix2 in the mammalian Grx and helix1 in the prokaryotic Grxs. The more N-terminal active site thiol is solvent exposed and has a significantly depressed pK_a, determined to 3.5 in mammalian Grx1 (Mieyal *et al*., 1991b; Yang and Wells, 1991), below 4 in yeast Grx1 (Gan *et al*., 1990) and approximately 5 in *E. coli* Grx1 and Grx3 (Foloppe and Nilsson, 2004; Foloppe *et al*., 2001). In *E. coli* Grx3, the N-terminal active site thiolate is stabilized primarily by formation of three hydrogen bonds to the active site Tyr13 and Cys14 (see Fig. 4), and similar interactions are predicted in other Grxs containing the same active site motif (Foloppe and Nilsson, 2004; Foloppe *et al*., 2001; Nordstrand *et al*., 1999b).

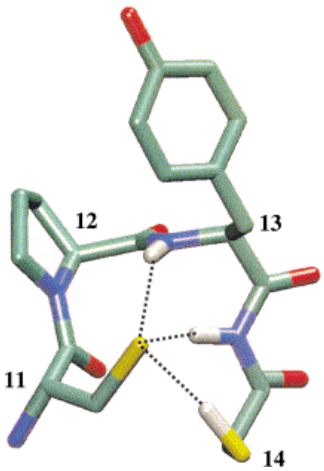

Figure 4. Stabilization of the N-terminal active site thiolate in *E. coli* Grx3 by direct hydrogen bonding with the amide protons of Tyr13 and Cys14 as well as the thiol proton of Cys14 (Foloppe *et al*., 2001).

The importance of the hydrogen bonds for lowering the pK_a of the N-terminal thiol is reinforced by mutational studies demonstrating that replacement of the C-terminal active site Cys by an Ala increase the pK_a value with 1.9 units, whereas replacement with a more polar Ser decrease the pK_a further (Foloppe *et al*., 2001). This is also consistent with GSH-mixed disulfide reductase activity measurements of the *E. coli* Grxs, demonstrating loss of more than 90 % of the specific activity in a Cys to Ala mutant, whereas Cys to Ser mutants retained approximately 30 % activity (Bushweller *et al*., 1992; Nordstrand *et al*., 1999b). In the mammalian Grx1, on the contrary, a Cys to Ser mutant results in increased activity (Yang *et al*., 1998; Yang and Wells, 1991).

Similarly, the functional importance of the two intervening residues, separating the redox active cysteines, has been analyzed by mutagenesis studies. The Tyr residue is highly conserved in most Grxs but is replaced by Phe in pig and vaccinia virus Grx1, which suggests that the aromatic ring is essential for catalysis. Replacement of this residue by a Pro or Ala in T4 Grx1, resulted in less than 10 % of the wild type GSH-mixed disulfide reductase activity. Interestingly, in vaccinia virus and phage Grx2, the Tyr residue is replaced by a Ile and a Gly, respectively. The functional importance of this exchange is not clear but indicates that the redox properties of these enzymes are significantly different from other Grxs.

Furthermore, the conserved Pro residue is suggested to decrease the pK_a of the more N-terminal active site thiol by favoring stabilization of the thiolate, both by formation of hydrogen bonds (Foloppe *et al*., 2001) and by electrostatic interactions with the helix dipole (Kortemme and Creighton, 1995). The results from mutation studies are however somewhat contradictory. In T4 Grx1, which contains the CVYC active site motif, a Val to Pro exchange resulted in 10 mV decreased redox potential, and thus in a more reducing protein (Joelson *et al*., 1990).

1.5 THE CATALYTIC MECHANISM OF GLUTAREDOXINS

Glutaredoxins are oxidoreductases that catalyze GSH-dependent thiol-disulfide exchange reactions with high specificity for GSH-mixed disulfides. Grxs exert the catalysis utilizing either one or two cysteines in the CXXC active site motif, which is referred to as the monothiol and dithiol mechanism, respectively.

In the dithiol mechanism, which is utilized to reduce protein disulfides, the solvent exposed N-terminal active site thiolate does a nucleophilic attack on the protein disulfide. This results in a relatively solvent inaccessible Grx-protein mixed disulfide intermediate, which subsequently is attacked by the C-terminal active site thiol, releasing the reduced substrate. Since this reaction requires a nucleophilic attack of the C-terminal active site thiol, which is protonated and largely buried in the reduced protein, it has been suggested that the Grx-protein mixed disulfide changes the local environment so that the pK_a of this thiol decreases, and thus increases its reactivity (Berardi and Bushweller, 1999). In this reaction, an intramolecular disulfide bond is formed in the active site of the Grx, which subsequently is reduced by two molecules of GSH, via a GSH-mixed disulfide intermediate (see Fig. 5). The rate of this reaction is dependent on a number of factors such as the pK_a of the N-terminal active site thiol that initiates the reaction but also on the substrate specificity.

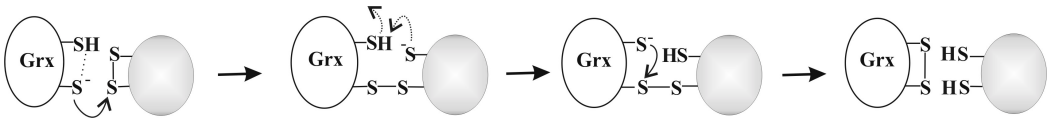

Figure 5. The dithiol mechanism, utilized in the Grx catalyzed reduction of protein disulfides.

In the monothiol mechanism, which is used in the specific reduction of GSH-mixed disulfides, only the more N-terminal active site thiol is required (Bushweller *et al*., 1992; Yang *et al*., 1998). This reaction is initiated by a nucleophilic attack of the N-terminal active site thiolate on the GSH sulfur, liberating the reduced non-glutathione moiety. In this reaction a solvent accessible Grx-GSH mixed disulfide intermediate is formed, which subsequently is reduced by a second GSH molecule, generating GSSG (see Fig. 6). The overall reaction displays a ping-pong pattern of two-substrate kinetics, with the rate-limiting step being reduction of the Grx-GSH mixed disulfide intermediate (Srinivasan *et al*., 1997).

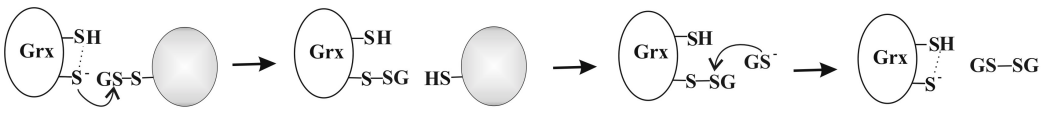

Figure 6. The monothiol mechanism, utilized in the Grx catalyzed reduction of GSH-mixed disulfides.

In addition Grxs have been demonstrated to possess GSH-dependent dehydroascorbate reductase (DHA) activity *in vitro*. The DHA reductase activity of Grx presumably proceeds via a monothiol mechanism, since retained activity is demonstrated for monothiol active site mutants. The reaction is proposed to involve a thiohemiketal intermediate between the Grx and DHA, which are attacked by GSH, releasing ascorbic acid. Similar to the Grx catalyzed reduction of GSH-mixed disulfides, a Grx-GSH mixed disulfide intermediate is formed, that subsequently is reduced by a second GSH molecule, forming GSSG (Wells *et al*., 1990).

The GSH-dependent thiol-disulfide exchange reactions catalyzed by Grx are *in vitro* frequently determined by coupling the formation of GSSG to NADPH oxidation by GR. The reaction rate can in this way easily be followed spectrophotometrically by the decrease in absorbance at 340 nm. The Grx catalyzed reduction of DHA can in addition be followed by the formation of ascorbic acid at 265 nm.

1.6 OXIDATIVE STRESS AND MODIFICATION OF PROTEIN THIOLS

The term oxidative stress is commonly used to describe a situation in which the cellular redox homeostasis is altered because of excessive production of reactive oxygen species (ROS), and /or impairment of cellular antioxidant mechanisms (Klatt and Lamas, 2000). During such conditions the levels of GSSG increases markedly, and the ratio of GSH to GSSG can decrease to values between 10 and 1 instead of the normal ratio of around 100 to 1 (Gilbert, 1995), which changes the cellular redox state to a more positive potential (Cotgreave and Gerdes, 1998; Klatt and Lamas, 2000). ROS, which includes a number of reactive molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$), is associated with the cellular turnover of oxygen by aerobic organisms (Gille and Sigler, 1995). ROS are primarily generated in the mitochondria by incomplete reduction of oxygen in the respiratory chain, but are also formed in other parts of the cell both from enzymatic and non-enzymatic reactions (for a review see (Dröge, 2002; Nordberg and Arner, 2001)). ROS can at low levels function as important redox signals, but excessive production can have hazardous effects and damage membrane lipids, DNA, and proteins (Kirlin *et al*., 1999; Lee *et al*., 1999; Suh *et al*., 1999; Yeldandi *et al*., 2000).

An important hallmark in situations associated with oxidative stress, are oxidatively modified proteins that may alter protein function, lead to formation of protein-protein cross linkages, fragmentation of polypeptide chains and finally to proteolytic degradation (Berlett and Stadtman, 1997). Cysteine residues are particularly sensitive to oxidation and may even under mild oxidative conditions form intra- or intermolecular disulfide bonds, that may serve to stabilize the protein structure, protect critical thiols against irreversible oxidation, or regulate protein activity. Reversible disulfide bond formation is a well-established mechanism by which cells modulate enzyme activity and transduce oxidative stress into inducible expression of a wide variety of genes involved in the cellular defense. Proteins with activities regulated through formation of intramolecular disulfide bonds have been identified from prokaryotes to mammals, and both Grx and Trx are involved in the mechanism. One of the best studied transcription factor that function as a ”redox -sensor” by forming an intramolecular disulfide bond when the environment becomes oxidizing is the *E.coli* transcription factor OxyR. Oxidized OxyR activate the expression of antioxidant genes involved in the response to H_2O_2 and is subsequently inactivated by enzymatic reduction of the disulfide by *E.coli* Grx1 (Zheng *et al*., 1998). Likewise, Yap1, a functional homologue to the bacterial OxyR regulates the hydrogen peroxide homeostasis in yeast (Delaunay *et al*., 2000; Delaunay *et al*., 2002; Toone *et al*., 2001). No similar system has yet been disclosed in mammalian systems, but the evolutionary conservation in *E. coli* and yeast of a disulfide activated transcription system as a key response system to hydrogen peroxide makes the presence of such system in higher eukaryotes highly plausible. Examples of mammalian transcription factors whose DNA binding activity is reliant on the redox status of critical cysteine thiols include AP1, NFκ-B, NF-1, Sp-1 and p53 (Nakamura *et al*., 1997; Pineda-Molina *et al*., 2001; Sun and Oberley, 1996). The tumor suppressor PTEN undergoes in addition, reversible intramolecular disulfide bond formation and inactivation by H_2O_2 (Lee *et al*., 2002).

Apart from forming intra- and intermolecular disulfides upon changes in the redox potential or exposure to ROS, cysteine residues may oxidize to sulfenic (RSOH), sulfinic (RSO_2H) or sulfonic (RSO_3H) acid. Whereas sulfenates are generally unstable, sulfinates and sulfonates are relatively stable oxidation states that under biological conditions not easily are reduced back to cysteines. Sulfenates easily become irreversibly oxidized to sulfinates and sulfonates, alternatively they can react with GSH to form either intramolecular disulfides or GSH-mixed disulfides (see below).

Sulfenates can be reduced either non-enzymatically by GSH or enzymatically by Trx or Prx, but cannot directly be reduced by Grx (Biteau *et al*., 2003; Claiborne *et al*., 1999). Sulfenates have been shown to modulate the activity of a number of proteins such as protein tyrosine phosphatase-1B (PTP-1B) (Denu and Tanner, 1998; Lee *et al*., 1998) and the insulin receptor kinase activity (Schmid *et al*., 1999), and have been suggested to play a significant role in cellular redox regulation. Sulfenates have furthermore been observed as part of catalytic redox cycles in enzymes (Claiborne *et al*., 2001; Schröder *et al*., 2000). Additionally, cysteine thiols have in the past decade been shown to react with reactive nitrogen species (RNS), which are important members of the ROS family (Stamler, 1994; Stamler and Hausladen, 1998). The attachment of NO to protein thiols, known as S-*nitrosylation*, is similar to sulfenates a rather unstable modification that readily can be removed non-enzymatic (Liu *et al*., 1998) or enzymatically (Zai *et al*., 1999). Moreover, nitrosylation of cysteine thiols may induce formation of intra- and intermolecular disulfides, mixed disulfides with low molecular weight thiols, or formation of sulfenic, sulfinic and sulfonic acid (Stamler and Hausladen, 1998). Today, nitrosylation is established as an important mechanism in NO signalling, and regulates the function of a growing list of proteins including caspases (Mannick *et al*., 1999) and ryanodine receptors (Eu *et al*., 1999). For a more extensive literature review about nitrosylation see (Demple, 2002; Stamler and Hausladen, 1998).

1.6.1 Protein S-glutathionylation

Changes in the cellular redox conditions as well as generation of ROS and RNS, may induce formation of mixed disulfides between protein thiols and cysteine, homocysteine, γ–glutamyl-cysteine and glutathione (Cotgreave and Gerdes, 1998; Thomas *et al*., 1995). These reactions, which are referred to as S-*thiolation*, are reversible processes that occur at different rates depending on the nature of the thiol in the protein. Among the mixed disulfides formed, glutathione is the predominant ligand, and this cysteine modification is generally referred to as S-*glutathionylation* (Schuppe-Koistinen *et al*., 1994a). Glutathionylation has been implicated in the buffering of oxidative stress, protecting proteins from irreversible oxidation and regulation of protein activity (Cotgreave and Gerdes, 1998).

GSH-mixed disulfides can be formed in response to changes in the GSH/ GSSG ratio, trough thiol-disulfide exchange reactions between a protein thiol and GSSG (see Fig. 7, reaction 1), a reaction that both removes GSSG and generates GSH, and thus restores the redox conditions. However, glutathionylated proteins can also be formed without any detectable changes in the cellular redox state by activation of the protein thiol or GSH. This can occur either by a radical reaction, by one electron oxidation of the protein thiol or GSH, and then formation of a mixed disulfide (Fig. 7, reaction 2a and 2b), or from two electron oxidation of the protein thiol to sulfenic acid, followed by reaction with GSH (Fig. 7, reaction 3). Alternatively, GSH-mixed proteins may arise from reactions between GSH and nitrosylated proteins (Fig. 7, reaction 4), or from reactions between nitrosoglutathione (GSNO) and protein thiols (Fig. 7, reaction 5) (Klatt and Lamas, 2000; Stamler, 1994; Thomas *et al*., 1995). In order to restore the cellular redox conditions a host of defensive mechanisms are triggered including the reduction of the mixed disulfides in reactions dependent on GSH (Fig. 7, reaction 6). These reactions, referred to as *deglutathionylation*, are efficiently catalyzed by Grxs (Gravina and Mieyal, 1993). Interestingly, some reports indicate that Grxs also catalyses the reverse reaction, i.e. glutathionylation (Lind *et al*., 1998; Lundström-Ljung *et al*., 1999; Starke *et al*., 2003). This requests some degree of caution in overexpression studies aimed at evaluating the role of Grxs under conditions of oxidative stress.

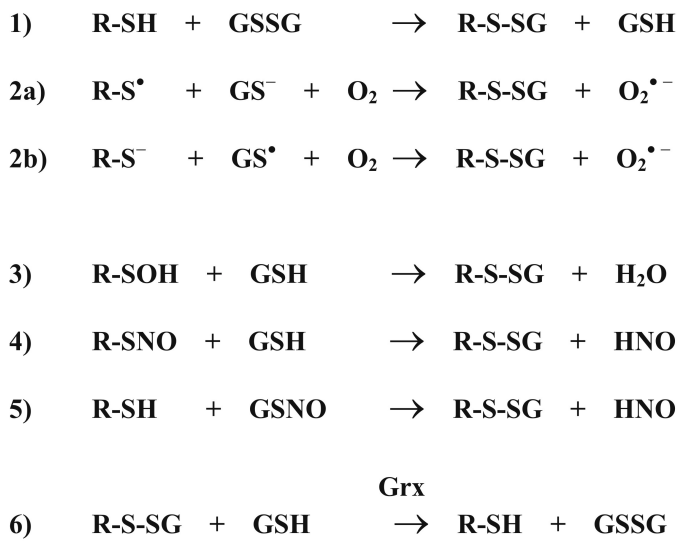

Figure 7. Proposed reactions by which glutathionylated proteins may form.

1.6.1.1 Proteins detected to undergo glutathionylation

Glutathionylation has been shown to alter the function of a growing list of proteins, and may serve to transduce redox changes and oxidative or nitrosative stimuli into a functional response at various levels of cellular signalling. This has rapidly increased the interest for glutathionylated proteins in the last years and the list of proteins demonstrated to be glutathionylated during constitutive metabolism or during oxidative stress increases continuously (Lind *et al*., 2002). One of the first proteins demonstrated to be glutathionylated and inactivated upon oxidative stress was the central metabolic enzyme glucose-6-phosphate dehydrogenase (GAPDH)(Lind *et al*., 1998; Schuppe-Koistinen *et al*., 1994b). Other proteins recently detected to undergo glutathionylation that are suggested to be targets for redox dependent regulation includes phosphofructokinase, aldose reductase, fatty acid synthase, carbonic anhydrase, hydroxy methylglutaryl-CoA reductase, glutathione transferase, a HIV protease (reviewed by (Cotgreave and Gerdes, 1998; Thomas *et al*., 1995)), β-actin (Wang *et al*., 2001; Wang *et al*., 2003), α-ketoglutarate dehydrogenase (Nulton-Persson *et al*., 2003), tyrosine hydroxylase (Borges *et al*., 2002), protein phosphates 1B and 2A (Barrett *et al*., 1999; Rao and Clayton, 2002), creatine kinase (Reddy *et al*., 2000), protein kinase C (Ward *et al*., 2002; Ward *et al*., 2000), cAMP-dependent protein kinase (Humphries *et al*., 2002), human Trx1 (Casagrande *et al*., 2002) and peroxiredoxin 1 and 4 (Lind *et al*., 2002; Sullivan *et al*., 2000).

A clustered group of proteins also detected to undergo glutathionylation during constitutive as well as during induced oxidative stress includes ubiquitin-conjugating enzymes (Figueiredo-Pereira *et al*., 1998; Jahngen-Hodge *et al*., 1997) and a number of molecular chaperones (Lind *et al*., 2002). Many chaperones were previously suggested to utilize protein-GSH mixed disulfides as part of their catalytic mechanism but the functional significance of this modification is still obscure (Freeman *et al*., 1999). Glutathionylation has furthermore been demonstrated to regulate the DNA binding activity of the transcription factors NF1 (Bandyopadhyay *et al*., 1998), the AP-1 subunit c-Jun (Klatt and Lamas, 2002; Klatt *et al*., 1999) and NF-κB (Pineda-Molina *et al*., 2001). It has therefore been speculated that glutathionylation may represent a general mechanism for transducing oxidative stress into induction of gene expression (Klatt and Lamas, 2000)

Interestingly, the 75- and 51- kDa subunits of mitochondrial complex I were recently shown to undergo glutathionylation following oxidation of the mitochondrial

glutathione pool. Since glutathionylation of these subunits correlated with an elevated production of superoxide formation and increased release of H_2O_2 from mitochondria to the cytoplasm, it was suggested as a mechanism by which mitochondria signal its glutathione redox state to the rest of the cell (Taylor *et al*., 2003).

The functional significance of many of the proteins described above that are demonstrated to undergo glutathionylation remains obscure, but the fact that the identified proteins are involved in numerous physiological processes such as metabolism, growth, differentiation, cell cycle progression and cytoskeletal functions, suggests that glutathionylation is a general mechanism of redox regulation. For a more extensive literature review about proteins being glutathionylated, see (Klatt and Lamas, 2000; Lind *et al*., 2002).

1.7 GLUTAREDOXINS IN VARIOUS ORGANISMS

1.7.1 Mammalian glutaredoxins

Two classical dithiol glutaredoxin isoenzymes with different subcellular localization have been identified in mammalian cells. The cytosolic Grx1 is today well characterized, whereas identification and characterization of the second mammalian glutaredoxin, Grx2 is part of this thesis (paper I-V, see also ref (Gladyshev *et al*., 2001)).

The 12 kDa Grx1 have been purified from a number of species, including rat (Axelsson *et al*., 1978; Mannervik and Axelsson, 1975), bovine (Luthman *et al*., 1979), rabbit (Hopper *et al*., 1989), pig (Gan and Wells, 1986) and human (Mieyal *et al*., 1991a; Padilla *et al*., 1996b), demonstrating a sequence identity of more than 80 % between the different species. Grx1 comprises approximately 105 amino acids and contains a consensus CPYC active site motif – the only exception being the pig enzyme in which a Phe replaces the conserved Tyr. Grx1 from most animals contain two additional cysteines C-terminal of the active site (Cys78 and Cys82 in human Grx1), located on β-strand 4 and α-helix 4, respectively (Katti *et al*., 1995; Sun *et al*., 1998). Human Grx1 further contains a third cysteine, located in the N-terminal helix (Cys7) (Padilla *et al*., 1996a; Padilla *et al*., 1995). The cysteine corresponding to Cys78 in human Grx1 is replaced by a conserved histidine in most Grx, whereas the second

cysteine (Cys82 in human Grx1), also is found in *E. coli* Grx3, rice Grx1 and Grx1 from the parasites *Plasmodium falciparum* and *Plasmodium bergei*. Hence, the second non-active site cysteine seems at least to be partly conserved. These additional cysteines are all solvent exposed and presumed to be important for regulation of the Grx function (Gan and Wells, 1986; Klintrot *et al*., 1984; Luthman and Holmgren, 1982a)(See also paper V). Furthermore, Grx1 purified from animal tissue is found to be acetylated in the N-terminus but this modification does not seem to affect the activity, since the recombinant enzyme has revealed identical kinetic properties to those of the native enzyme (Padilla *et al*., 1996b; Yang and Wells, 1990).

Grx1 is predominantly localized to the cytosol, but immunological studies reveal that the protein also, by an unknown mechanism, can be translocated to the nucleus (Padilla *et al*., 1992; Rozell *et al*., 1993; Stavreus-Evers *et al*., 2002). The nuclear localization is relevant since Grx1 has been shown to regulate the activity of several transcription factors *in vitro*, including NF1, NFκB and AP1 (Bandyopadhyay *et al*., 1998; Hirota *et al*., 2000). Grx1 has further been found in the human plasma, indicating that the protein can be secreted from cells (Nakamura *et al*., 1998). A possible role for Grx1 extra cellularly is not known, but human Grx1 has been shown to catalyze the reduction of plasma glutathione peroxidase (GPx3) *in vitro* (Björnstedt *et al*., 1994). Additionally, Grx1 has been shown to penetrate neuronal cells, indicating that the protein, by an unknown mechanism, can be translocated through membranes (Daily *et al*., 2001a).

The gene for human Grx1 is localized to chromosome 5q14 and the gene organization and a putative promotor has been identified. An AP1 binding site was further identified and suggested to regulate the transcription of Grx1 under oxidative stress (Krysan and Lou, 2002). Although Grx1 has been found in the nucleus, the gene does not code for any known signals for nuclear import (Padilla *et al*., 1996a; Park and Levine, 1997).

1.7.1.1.1 The biological role of Grx1

Grx1 has been ascribed many different functions in the cell, both by *in vivo* and *in vitro* experiments. The bovine and human Grx1 can function as GSH-dependent hydrogen donors for ribonucleotide reductase (Luthman and Holmgren, 1982a; Padilla *et al*., 1995). The apparent K_m value of bovine Grx1 for ribonucleotide reductase was

determined to be 0.6 µM, which is lower than the 1.8 µM determined for the bovine Trx1 (Luthman and Holmgren, 1982a). However, neither Grx1 from rabbit or pig appeared to supply a partly purified ribonucleotide reductase preparation with reducing equivalents (Hopper *et al*., 1989). Similarly, mouse fibroblasts depleted of glutathione were not affected in growth rate, DNA synthesis or the size of the deoxyribonucleotide pool (Spyrou and Holmgren, 1996). Moreover, as revealed by the immunological studies (see below) the expression of Grx1 does not correlate with the distribution of ribonucleotide reductase. Obviously, Grx1 has many important functions in addition to being a hydrogen donor for ribonucleotide reductase. It should be noted that no other protein disulfide, other than the one in ribonucleotide reductase, has been reported to be substrates for Grx1. This demonstrates that Grx1 is not a general disulfide reductase like the mammalian Trx. On the other hand, Trx does not reduce GSH-mixed disulfides showing the functional distinction between thioredoxins and glutaredoxins.

Grx1 has been shown to reduce dehydroascorbate (DHA) to ascorbate (vitamin C) *in vitro*. This reaction has been analyzed by mutagenesis studies and shown to proceed by a GSH-dependent monothiol mechanism (Washburn and Wells, 1999; Wells *et al*., 1990; Yang and Wells, 1991). Ascorbic acid is an essential cofactor for enzymes that participate in the biosynthesis of collagen, norepinephrine and carnitine (Englard and Seifter, 1986; Frei *et al*., 1989; Rebouche, 1991), and its deficiency may result in scurvy. Ascorbate is furthermore an important antioxidant, and has been implicated in the protection against ROS (Meister, 1992). Thus, the regeneration of ascorbate by Grx1 may be important *in vivo*. Other enzymes that have been reported to reduce DHA *in vitro* includes 3α-hydroxysteroid dehydrogenase (Del Bello *et al*., 1994), TrxR (May *et al*., 1997) and PDI (Wells *et al*., 1990).

The mammalian Grx1 has, both by *in vitro* and *in vivo* studies, been demonstrated to reduce glutathionylated substrates with high efficiency and specificity (Chrestensen *et al*., 2000; Gravina and Mieyal, 1993). This reaction has been extensively studied with various glutathionylated substrates and shown to proceed by a monothiol mechanism (Gravina and Mieyal, 1993; Mieyal *et al*., 1991b; Srinivasan *et al*., 1997; Yang *et al*., 1998). Human Grx1 has been shown to deglutathionylate a number of proteins detected to undergo glutathionylation either under constitutive metabolism or oxidative stress, and has by these reactions been implicated in a number of cellular processes (see also proteins detected to undergo glutathionylation).

Grx1 appears to play important roles in the cellular protection against oxidative stress and apoptosis by apparent different mechanisms. Overexpression of mouse Grx1 protects H9c2-vector cells from hydrogen peroxide-induced apoptosis by regulating the redox state of the serine/threonine kinase Akt. This finding was supported by *in vitro* experiments, demonstrating that Grx1 by a GSH-dependent mechanism prevented formation of an intramolecular disulfide in Akt, and thus its association with protein phosphate 2A, which inactivates Akt by dephosphorylation (Murata *et al*., 2003). Interestingly, the activity of protein phosphatase 2A is also regulated by glutathionylation, presumably via Grx1 (Rao and Clayton, 2002).

Furthermore, acute exposure of cells to cadmium, known to inhibit the enzymes in the glutaredoxin and the thioredoxin system, results in induced levels of glutathionylated proteins and subsequently apoptosis. The finding that cells with high expression of Grx1 were more resistant to cadmium-induced apoptosis, suggested that loss of Grx1 activity was an event that initiated apoptosis, but the underlying mechanism was never elucidated (Chrestensen *et al*., 2000).

Moreover, human Grx1 has been shown to be a negative regulator of the apoptosis signal-regulating kinase 1 (ASK1) by interacting with its C-terminal portion. The Grx1-ASK1 complex was demonstrated to dissociate upon formation of either an intramolecular disulfide or a GSH-mixed disulfide in the active site of Grx1, which subsequently led to activation of the ASK1-SEK1-JNK1 signal transduction pathway and cell death (Song and Lee, 2003; Song *et al*., 2002).

Furthermore, human Grx1 as well as *E. coli* Grx2, have been demonstrated to protect cerebellar neurons from dopamine-induced apoptosis in an experimental model of Parkinson's disease. For both proteins the effect was mediated by activation of NFκB via the intranuclear redox factor1 (Ref1) (Daily *et al*., 2001a). Despite significantly different activities in the GSH-dependent reduction of β-hydroxyethyl disulfide (Vlamis-Gardikas *et al*., 1997), the protective profiles were almost identical. The mechanism by which the proteins penetrated the neurons is not clear, but in the case of *E. coli* Grx2, it was demonstrated to be independent of the redox active cysteines, that on the contrary were needed for the protein to exert its protective effect. The underlying protecting mechanism for *E. coli* Grx2 was further analyzed, and revealed dual parallel activation of the Ras-Phosphoinositide 3-kinase/akt and the Jun N-terminal kinase (JNK)/AP1 cascade, mediated by Ref1 (Daily *et al*., 2001a; Daily *et al*., 2001b).

Moreover, the activity of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine, is significantly decreased upon glutathionylation (at Cys177, 249, 263, 329, 330 and 380) . Human Grx1 has been reported to fully restore the activity by reducing the GSH-mixed disulfides formed upon diamide-induced oxidative stress. This suggests a potential role for Grx1 in the dopamine biosynthesis (Borges *et al*., 2002). Additionally, Grx1 was recently suggested to regulate the activity of the mitochondrial complex I by reversible glutathionylation of redox active thiols on the 75 and 51 kDa subunits (Taylor *et al*., 2003). The ready formation of GSH-mixed disulfides on complex I might be of pathological significance since complex I dysfunction is implicated in several neurodegenerative disorders such as Parkinson's disease and Huntington's disease (Cooper *et al*., 1992; Schapira *et al*., 1990; Tabrizi *et al*., 2000). The mechanism by which complex I dysfunction occurs in these diseases and how this effect causes neurodegeneration is not entirely clear, nor is it clear to what extent Grx1 can enter mitochondria. Administration of 1-Methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), a model neurotoxin that inhibits complex I and causes Parkinson's disease-like symptoms in mice, increases both transcription of Grx1 mRNA and Grx activity. The correlation between Grx1 and complex I dysfunction has further been validated by selective down-regulation of Grx1 using antisense oligonucleotides, that prevented recovery of complex I after MPTP treatment (Kenchappa and Ravindranath, 2003). Collectively, these results suggest a potential protective role for Grx1 in the pathogenesis of Parkinson's disease, one the most common chronic neurodegenerative disorders affecting older adults, characterized by a loss in dopaminergic striatal neurons in substantia nigra.

Human Grx1 have also been shown to regulate the activity of a HIV-1 aspartyl protease, required for viral maturation, by reversible glutathionylation of two highly conserved cysteines (Cys67 and Cys95) and is further shown to be assembled into HIV viral particles. Compelling evidence indicate that infection by human immunodeficiency virus (HIV) leads to oxidative stress with significant changes in the levels of oxidized and reduced glutathione (for a review see (Townsend *et al*., 2003)), it is therefore likely that cysteine residues of both cellular and viral proteins are glutathionylated under HIV infection (Davis *et al*., 1996; Davis *et al*., 1997). Hence, Grx1 might be important for HIV maturation.

Furthermore, both *in vivo* and *in vitro* studies have revealed that human Grx1 in part regulate growth factor-induced actin polymerization. The ubiquitous protein G

actin, has been shown to be glutathionylated at Cys374, and deglutathionylation catalyzed by Grx1, has been shown to increase the rate of polymerization 6-fold (Wang *et al*., 2001). This has been verified by Grx1 knockout cells (by RNA interference), which showed no actin polymerization or reorganization (Wang *et al*., 2003).

Finally, *in vitro* studies have shown that the DNA-binding of nuclear factor I (NFI) is decreased by glutathionylation of a conserved cysteine residue (Cys3) in the DNA-binding domain. Human Grx1 was shown to reduce the mixed disulfide and thus to restore the DNA-binding activity of NFI (Bandyopadhyay *et al*., 1998).

1.7.1.1.2 Tissue distribution of mammalian Grx1

Immunohistochemistry and western blot analysis have shown that Grx1 is expressed in all tissues, with highest expression in tissues with high metabolic turnover e.g. skeletal muscle, liver, kidney and brain. The expression pattern in the ovary, in epithelial tissue of the skin and tongue also reflected induced expression during the course of cell differentiation (Gan and Wells, 1988; Rozell *et al*., 1993). Grx1 is further induced, both at mRNA and protein level, in differentiated macrophages (Takashima *et al*., 1999).

Immunohistochemistry studies have shown that human Grx1 is present in placenta (Padilla *et al*., 1995; Sahlin *et al*., 2000a), oocytes (Rozell *et al*., 1993), corpus luteum (Garcia-Pardo *et al*., 1999) and cervix (Sahlin *et al*., 1997; Sahlin *et al*., 2000b). The levels of human Grx1 in cervix have been shown to increase in term pregnancy and immediately after delivery, suggesting that Grx1 may be involved in cervical ripening, which is known as an inflammatory process (Sahlin *et al*., 1997; Sahlin *et al*., 2000b). Furthermore, a strong nuclear and cytoplasmic immunostaining of human Grx1 has been shown during the time the endometrium is receptive for blastocyst implantation. The immunostaining was especially strong within the pinopodes, which appear on the endometrial surface during this period, and coincides with the decrease of serum progesterone. The function of Grx1 in these cells is not known, but it was suggested that Grx1 might play an important role in protecting cells from oxidative stress associated with the implantation of the blastocyst. It was further suggested that progesterone might be one factor involved in the regulation of Grx1 expression in the human endometrium. These studies suggest that Grx1 might have an important function in reproduction (Stavreus-Evers *et al*., 2002).

1.7.1.2 PICOT

Mammalian cells contain a highly conserved protein of 38.5 kDa that is termed PICOT (PKC-interacting cousin of thioredoxin), since it was identified in a yeast two-hybrid screen as a protein interacting with protein kinase Cθ (PKC). PICOT contains an N-terminal Trx module and two highly conserved domains (called PICOT-homology domains) with predicted structures that resemble that of a Grx but with a CGFS monothiol active site motif. PICOT is expressed in various tissues, and when overexpressed in T-cells it inhibits the activation of c-Jun N-terminal kinase and the transcription factors AP1 and NFκB (Witte *et al*., 2000). The wide distribution of this protein in evolutionary distant organisms is indicative of a function, which is indispensable (Fomenko and Gladyshev, 2002; Isakov *et al*., 2000).

1.7.2 Yeast glutaredoxins

Two dithiol Grxs are presently characterized in the yeast *Saccaromyces cerevisiae* that share a high degree of similarity with other dithiol Grxs from different species. The isoenzymes, designated Grx1 and Grx2 share 64 % sequence identity and contain the consensus CPYC active site motif (Luikenhuis *et al*., 1998). The 12 kDa Grx1 is localized to the cytosol whereas Grx2 is expressed in two isoforms of 11.9 and 15.9 kDa, that are directed to the cytosol and mitochondria, respectively (Pedrajas *et al*., 2002). In addition to the GSH thiol-disulfide activity typical for Grxs, both Grx1 and Grx2 possess functionalities typical for glutathione S-transferases (GSTs) and glutathione peroxidases (GPx). Therefore, they are ideally suited to detoxify the wide range of xenebiotics and oxidants that might be generated during diverse stress conditions. The purified recombinant Grx1 and Grx2 have been shown to efficiently catalyze the GSH-dependent reduction of the mixed disulfide between GSH and β-mercaptoethanol, with Grx1 being almost twice as active as Grx2 (Collinson and Grant, 2003). Nonetheless, null mutant analyses have demonstrated that Grx2 accounts for the majority of the oxidoreductase activity *in vivo*. Furthermore, recombinant Grx1 and Grx2 catalyze the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB), by a mechanism requiring only the more N-terminal active site cysteine, an activity typical for GSTs (see below). The yeast Grxs have also, most surprisingly, been shown to catalyze a GSH-dependent reduction of peroxides,

with highest rates for hydrogen peroxide, followed by cumene hydroperoxide and tert-butyl peroxide. Both the GSH-conjugating activities and the GSH-dependent reduction of peroxides are demonstrated to be efficient and comparable to the rates determined for yeast phospholipid glutathione peroxidase (PHGPx2) and various eukaryotic GPxs. The results were further supported by deletion analysis, that showed overlapping functions with the yeast GSTs and GPxs *in vivo* (Collinson and Grant, 2003; Collinson *et al*., 2002). However, partly contradictory results have been obtained from another group that were unable to detect glutathione peroxidase activity of recombinant Grx2, thus proposing functional differences between the two Grxs (Pedrajas *et al*., 2002).

It remains to be established whether the two dithiol yeast Grxs can function as hydrogen donors for ribonucleotide reductase. This seems likely given that the level of deoxynucleotides is unaltered in a mutant lacking both yeast Trxs (Muller, 1994). Similarly, a quadruple mutant lacking Trx1 and Trx2, as well as Grx1 and Grx2 is not viable, but a single Trx or Grx is necessary and sufficient for growth (Draculic *et al*., 2000). This result is reminiscent of the situation in *E. coli* where one of the potent disulfide reducing proteins Grx1, Trx1, Trx2 is necessary and sufficient to sustain growth under aerobic conditions (Stewart *et al*., 1998).

Furthermore, both Grx1 and Grx2 are regulated via stress-responsive elements and both genes are induced in response to various stress conditions. Grx1 is induced to significantly higher levels than Grx2 after heat and osmotic shock, whereas Grx2 is rapidly induced in response to ROS and upon entry into stationary phase growth. These data support the idea that the two enzymes play distinct roles during normal cellular growth and in response to stress conditions (Collinson *et al*., 2002; Grant *et al*., 2000; Luikenhuis *et al*., 1998; Rodriguez-Manzaneque *et al*., 1999).

1.7.2.1 Monothiol glutaredoxins

Recently a second family of Grx-like proteins containing a CGFS active site motif was identified in *Saccaromyces cerevisiae* (Rodriguez-Manzaneque *et al*., 1999). The proteins, termed Grx3-5, are included in a large protein superfamily that contains the conserved PICOT-homology domain, defined after the human PICOT protein. Whereas the PICOT-homology domain corresponds to the major part of Grx5, Grx3 and Grx4 have N-terminal extensions that, based on sequence homologies, appear to constitute Trx-like domains (Isakov *et al*., 2000). None of the monothiol Grxs is able to substitute for the classical dithiol Grxs or Trxs in yeast, as demonstrated by the lethal

phenotype of a quadruple mutant lacking Trx1, Trx2, Grx1 and Grx2 (Draculic *et al*., 2000). Conversely, a triple mutant of the three monothiol Grxs is not viable, indicating indispensable functions that cannot be covered by the dithiol Grxs (Rodriguez-Manzaneque *et al*., 1999).

Grx3 and Grx4 have not been biochemically characterized and no clear phenotypes have been described in yeast cells lacking the proteins (Rodriguez-Manzaneque *et al*., 1999). While no functional data for Grx3 and Grx4 are available, Grx4 has recently been shown to interact with Bud32, a putative yeast kinase distantly related to the eukaryotic protein serine/threonine kinase superfamily. The functional significance of this interaction is not clear, but the finding that Bud32 phosphorylates Grx4 at Ser134 *in vitro*, is suggestive of Grx4 regulation by phosphorylation (Lopreiato *et al*., 2004). This possibility is noteworthy from the point of view that also the human PICOT protein, which is structurally similar to Grx4, also associates with a protein kinase (Witte *et al*., 2000).

In contrast to Grx3 and Grx4, absence of Grx5 induces severe growth defects (Rodriguez-Manzaneque *et al*., 1999). Grx5 has been shown to be located to the mitochondrial matrix and to be involved in the synthesis and assembly of iron/sulfur centers, which is consistent with the accumulation of iron in the mitochondria and decreased activities of iron-sulfhur containing enzymes in cells lacking Grx5. Interestingly, a mutant lacking both the dithiol Grx2 and Grx5 is inviable, indicating that there might be some overlapping functions between these two proteins (Rodriguez-Manzaneque *et al*., 2002). Grx5, which corresponds to a protein of 13.5 kDa is the only yeast monothiol Grx that so far has been biochemically characterized (Rodriguez-Manzaneque *et al*., 2002). A three-dimensional model of Grx5 have revealed a classic Trx/ Grx fold, with the putative catalytic cysteine (Cys60) lying opposite to another conserved motif, suggested to be involved in GSH binding. The pK_a of the catalytic cysteine thiol has been determined to 5.0, which is similar to the N-terminal active site thiol in the dithiol Grxs. Furthermore, incubation with GSSG has been shown to promote formation of an intramolecular disulfide bond within the protein, formed between Cys60 and a non-conserved cysteine residue (Cys117). The redox potential for the disulfide was determined to –175 mV, but could only slowly be reduced by GSH. This suggested that GSH might not be the physiologic reductant. Enzymatic characterization has shown that Grx5 is inactive in the classical assay for dithiol Grxs, measuring the GSH-dependent reduction of hydroxyethyl disulfide (HED). However, it

has been demonstrated that Grx5 has the potential to form a GSH-mixed disulfide with Cys60, and further to reduce glutathionylated carbonic anhydrase. The deglutathionylase activity detected was thus much less efficient than that determined for the dithiol Grxs, indicating that Grx5 is not specific for GSH-mixed disulfides (Tamarit *et al*., 2003).

Recently two additional monothiol Grxs, containing a CPYS and CSYS active site motif were discovered in *Saccaromyces cerevisae* but these proteins have so far neither functionally nor genetically been characterized (Fomenko and Gladyshev, 2002).

1.7.3 Bacterial glutaredoxins

The best-characterized bacterial Grxs are the three dithiol Grxs (Grx1-3) in *Escherichia coli*, each containing the consensus CPYC active site motif. The three Grxs have been extensively studied both *in vitro* and *in vivo*.

The first glutaredoxin, Grx1, was purified to homogeneity in 1979 as a GSH-dependent hydrogen donor for ribonucleotide reductase1a in a mutant lacking Trx1 (Holmgren, 1979a). Later, the two additional glutaredoxins were isolated from extracts of mutants lacking Trx1 and Grx1 (Åslund *et al*., 1994). Grx1 and Grx3 are approximately 10 kDa proteins, that share 33 % sequence identity and very similar three-dimensional structures (see Trx/Grx fold). In spite of their overall structural similarity, these Grxs have very different redox potentials. The redox potential has been determined to -233 and -198 mV, respectively, indicating that Grx1 is a stronger reductant that Grx3. This provides a biophysical basis for the biochemical data which have revealed that only Grx1 is a potent disulfide bond reductant, whereas Grx2 and Grx3 may be limited to the reduction of GSH-mixed disulfides (Åslund *et al*., 1994). Apart from reducing the disulfide in ribonucleotide reductase, (with a K_m value reported to be 10-fold lower than Trx1) (Holmgren, 1979a), Grx1 has been shown to reduce arsenate reductase and 3´-phosphoadenylyl sulfate (PAPS) reductase (Gladysheva *et al*., 1994; Shi *et al*., 1999; Tsang, 1981; Tsang and Schiff, 1978). The latter activity is also possessed by *E. coli* Trx1, demonstrating that Grx1 has overlapping functions with Trx1 *in vivo*. This is further supported by genetic evidence,

since mutants lacking Trx1 and Grx1 are unable to grow on minimal medium (Russel and Holmgren, 1988). Albeit Trx2 is able to support sulfate reduction both *in vivo* when overexpressed and *in vitro* (Lillig *et al*., 1999; Stewart *et al*., 1998), it appears that the endogenously levels of Trx2 are too low to support sulfate reduction. However, the levels of Trx2 are sufficient to support reduction of ribonucleotide reductase, since Trx2 has to be knocked out in conjunction with Trx1 and Grx1 in order to create a lethal phenotype. The overlap in function between Trx1, Trx2 and Grx1 is also shown by the fact that expression of any of the three proteins is sufficient to support growth under aerobic conditions in the Trx1, Trx2, Grx1 triple mutant (Stewart *et al*., 1998).

Grx1 has also been shown to exhibit general GSH-mixed disulfide reducing activities, and reduce *in vitro* various GSH-mixed disulfides, such as the mixed disulfide formed between GSH and β-mercaptoethanol, glutathionylated ribonuclease (RNase-SG), glutathionylated PAPS reductase, and glutathionylated arsenate reductase (Holmgren, 1979a; Lillig *et al*., 2003; Lundström-Ljung *et al*., 1999; Shi *et al*., 1999). The mechanism has been extensively studied and shown to proceed by a reaction requiring only the N-terminal active site cysteine (Bushweller *et al*., 1992).

Grx1 is regulated by the redox sensitive transcription factor OxyR that becomes active upon oxidative stress by formation of a disulfide bond, and induces the expression of a number of antioxidant defensive genes. Once the level of Grx1 increases, Grx1 is able to reduce the disulfide bond in OxyR, thus rendering the regulatory protein inactive (Zheng *et al*., 1998).

The role of Grx3 *in vivo* is not as well elucidated as for Grx1. The level of Grx3 has been demonstrated to remain the same during all stages of growth (Potamitou *et al*., 2002a). Grx3 exhibits *in vitro* only 5 % of the catalytic activity of Grx1 with ribonucleotide reductase (Åslund *et al*., 1994). Apparently this activity is not sufficient to support growth since a mutant lacking Grx1, Trx1 and Trx2 is lethal, indicating that Grx3 may not be an important reductant of ribonucleotide reductase *in vivo* (Stewart *et al*., 1998). Grx3 reduces the mixed disulfide between β-mercaptoethanol and GSH with a two-fold higher turnover rate than Grx1, but other GSH-mixed disulfides are catalyzed with similar rates as Grx1 (Lundström-Ljung *et al*., 1999; Shi *et al*., 1999). The different activities demonstrated for Grx1 and Grx3 have been proposed to be due to a lower plasticity of Grx3 compared to Grx1, explained by an additional C-terminal helix as well as a shorter loop connecting helix1 and β2 (Berardi and Bushweller,

1999). Grx3 contains, an additional Cys in the C-terminus of the third helix that corresponds to Cys82 in human Grx1. Similar to the additional Cys present in mammalian Grxs, this residue has been observed to be involved in dimerization of the protein and has been suggested as a potential site for regulation (Åslund *et al*., 1994).

Grx2 is atypical when compared to all presently known members of the Grx family. Grx2 has a molecular mass of 24.3 kDa and shows low sequence similarity to other Grxs. Grx2 cannot reduce the disulfide in ribonucleotide reductase or PAPS reductase, but catalyzes the reduction of the mixed disulfide formed between GSH and β-mercaptoethanol and RNase-SG with higher efficiency than both Grx1 and Grx3 (Lundström-Ljung *et al*., 1999; Vlamis-Gardikas *et al*., 1997; Åslund *et al*., 1994). Grx2 has also been reported to reduce glutathionylated PAPS reductase and glutathionylated arsenate reductase (Lillig *et al*., 2003; Shi *et al*., 1999).

Grx2 has been shown to be highly abundant in the cell, and to account for as much as 80 % of the GSH-disulfide oxidoreductase activity in a wild type strain (Potamitou *et al*., 2002a; Vlamis-Gardikas *et al*., 2002). The levels of Grx2 increases three-fold in stationary phase of growth, and the expression has been shown to be regulated by guanosine-3´,5´-tetraphosphate (ppGpp) and σ^S (Potamitou *et al*., 2002b), which also regulate the expression of other stationary phase proteins.

Grx2 is structurally similar to glutathione *S*-transferases (GSTs) and could be considered as a link between the Grxs and the GSTs. Grx2 consists of a N-terminal domain that structurally is very similar to Grx1, Grx3 and human Grx1, and a C-terminal domain consisting of 6 α-helices. Nonetheless, the CPYC active site is positioned further into helix 1 and is in close contact to the C-terminal domain. The location of the active site is suggested to mask the C-terminal active site cysteine and explain why Grx2 is unable to utilize a dithiol mechanism in its catalysis (Xia *et al*., 2001). Grx2 possess no activity as a GST, in spite of the structural similarities (unpublished observations).

Apart from the three dithiol Grxs described above, a monothiol Grx-like protein with a CGFS active site motif has been identified in *E. coli*, homologues to the monothiol Grxs identified in other species (Fomenko and Gladyshev, 2002; Rodriguez-Manzaneque *et al*., 1999). This protein (Grx4) is currently being functionally and structurally characterized (Potamitou, 2003).

1.7.3.1 NrdH

A Grx-like protein termed NrdH has been identified in *E. coli* and several other organisms, in particular organisms lacking GSH (Jordan *et al*., 1996). NrdH has a molecular weight of approximately 9 kDa and shows high sequence homology to *E. coli* Grx3, but the residues involved in GSH binding are not conserved in the protein. NrdH shows no sequence homology to the Trxs, but "behaves" functionally as a Trx. NrdH is, similar to Trx, an efficient substrate for *E. coli* TrxR and is unable to use reducing equivalents from GSH. NrdH contains a CVQC active site motif, which differs from both the Trxs and Grxs, and the redox potential has been determined to -248 mV. NrdH has been shown to reduce various disulfide substrates *in vitro*, which shows that the protein, similar to Trx, is functional as a general disulfide reductase (Jordan, 1997). The *in vivo* function of NrdH is not completely clear, but it can act as a hydrogen donor for class 1b ribonucleotide reductase (NrdEF) and it is part of an nrdHIEF operon (Jordan *et al*., 1996; Jordan and Reichard, 1998). The crystal structure of recombinant oxidized NrdH has revealed a typical Grx fold, with highest similarity to *E. coli* Grx3 and T4 Grx1. NrdH lacked the GSH binding cleft present dithiol Grxs, and contained instead a wide hydrophobic pocket at the surface, suggested to be important for the interaction with TrxR (Stehr *et al*., 2001).

1.7.4 Phage and virus glutaredoxins

1.7.4.1 Bacteriophage glutaredoxins

When *E. coli* is infected by bacteriophage T4, two phage encoded Grxs with 21 % sequence homologue, and a ribonucleotide reductase are produced. The first bacteriophage T4 Grx was identified 1969 and first termed T4 Trx, since it was reduced by *E. coli* TrxR with a similar efficiency as *E. coli* Trx1. Later, this protein was renamed T4 Grx1 since it also was reduced by GSH and contained several characteristics of a Grx (Berglund, 1969; Holmgren, 1978; Nikkola *et al*., 1991). T4 Grx1, which is a 10 kDa protein is today well characterized both functionally and structurally (Eklund *et al*., 1992; Ingelman *et al*., 1995; Sjöberg and Söderberg, 1976; Söderberg *et al*., 1978). It shows 32 % sequence identity to *E. coli* Grx1 and the CVYC active site has a similar redox redox potential of –230 mV (Holmgren and Sjöberg, 1972; Joelson *et al*., 1990; Nikkola *et al*., 1993a; Åslund *et al*., 1997). T4 Grx1 is a

hydrogen donor for T4 ribonucleotide reductase, but not for *E. coli* ribonucleotide reductase. This, together with its ability to use reducing equivalents from either GSH or *E. coli* TrxR enables an efficient DNA synthesis in the infected cells. T4 Grx1 reduces in addition, several low molecular weight substrates *in vitro* (Holmgren, 1978). Mutational studies of the active site sequence have demonstrated to have minor effects on the interactions with TrxR and ribonucleotide reductase, indicating that the interacting area extends beyond the active site motif (Joelson *et al*., 1990). Mutant studies have also identified charged residues on the surface of the protein which are important for interaction with the reductase as well as several residues involved in GSH binding (Nikkola *et al*., 1993b; Nikkola *et al*., 1991).

The second T4 Grx, a 12 kDa protein with the CPGC active site, was described in 1996. T4 Grx2 is expressed in all stages of the T4 infection cycle and can *in vitro* serve as a GSH-dependent hydrogen donor for the phage ribonucleotide reductase. T4 Grx2 has also been shown to catalyze the GSH-dependent reduction of dehydroascorbate and HED (Gvakharia *et al*., 1996a).

1.7.4.2 Virus glutaredoxins

Glutaredoxins have been identified in several mammalian viruses. Best characterized are the two Grxs encoded in the vaccinia virus, a member of the poxvirus family that replicates in the cytoplasm of infected eukaryotic cells and encodes numerous enzymes involved in DNA replication and transcription (Moss, 1990). The first Grx is a 12 kDa protein with a CPFC active site motif, called O2L since it is a product of the o2l gene. This Grx shows more than 40 % sequence identity to the mammalian Grx1, but is absent in other poxviruses (Johnson *et al*., 1991; Kelley and Bushweller, 1998). The second Grx in the vaccinia virus is a product of the g4l gene and is called G4L. G4L is conserved in all other poxviruses examined thus far, and encodes a 14 kDa protein with the active site CSIC (Senkevich *et al*., 2000; White *et al*., 2000). The O2L and G4L proteins do not show any sequence similarity to one another, and the similarity between the O2L and the mammalian Grx1 suggests that this Grx has been acquired by the poxviruses at a much later stage of evolution (Gvakharia *et al*., 1996a).

The viral glutaredoxins have similar activities *in vitro*, and reduce efficiently both dehydroascorbate and hydroxyethyl disulfide (HED) in a GSH-dependent manner (Ahn and Moss, 1992; Gvakharia *et al*., 1996b). However, the two proteins seem to

have different functions *in vivo*. The O2L Grx appears to be involved in the nucleotide biosynthesis, but is nonessential for the vaccinia virus replication (Rajagopal *et al*., 1995). G4L is expressed late in the infection and is a component of a disulfide bond formation pathway that is essential for virus reproduction. In this redox pathway G4L directly oxidizes thiols in two substrate viron proteins L1R and F9L. GL4 then passes the electrons further to the redox protein A2.5L that forms a disulfide-linked heterodimer with E10R, an FAD-dependent sulphydryl oxidase that uses oxygen as electron acceptor. (Senkevich *et al*., 2000; White *et al*., 2000).

1.8 OTHER ENZYMES AND ANTIOXIDANTS RELATED TO GLUTAREDOXINS

1.8.1 Glutathione S-transferases

Glutathione S-transferases (GST) constitutes a multi-gene family of enzymes involved in the detoxification of a wide variety of xenebiotics and physiological compounds. GSTs are found in virtually every living species examined, including plants, bacteria and animals (Hayes and Pulford, 1995). Most GSTs exist as dimeric proteins with subunit molecular weights of approximately 25 kDa. Each subunit contains a hydrophilic GSH-binding site, which is conserved between all GSTs, and a hydrophobic site for the binding of diverse electrophilic compounds, which diverge between different GSTs.

GSTs catalyze the nucleophilic attack of GSH to electrophilic compounds by activating the thiol group of GSH, by hydrogen bonding to a Tyr, Ser or Cys in its active site. The interactions between GSH and GSTs are well studied and reveal numerous similarities with the interactions stabilizing the Grx-GSH mixed disulfide (Armstrong, 1997). A large number of chemicals serve as substrates for GSTs, both environmental toxicants and drugs. Chlorinated nitrobenzenes (CDNB and DCNB) have long served as standard substrates for nearly all GSTs but the specific activity vary greatly between different isoforms. Another model substrate is the diureticum ethacrynic acid ((2,3- dichloro-4(2-methylenebutyryl)phenoxy) acetic acid, ECA), which is a good substrate for certain GSTs and an inhibitor of others (Awasthi *et al*., 1993; Ploemen *et al*., 1993). The GSH conjugates formed are generally less reactive towards cellular macromolecules and more water-soluble, and can therefore be more easily eliminated from the cell (Armstrong, 1997).

The mammalian GSTs have been extensively investigated and based on their primary structure, catalytic activity, immunogenicity, sensitivity to inhibitors, they have been grouped in seven distinct classes; alpha (A), mu (M), pi (P), theta (T), zeta (Z), sigma (S), kappa (k) and omega (O) (Andersson *et al*., 1994; Jakobsson *et al*., 1996; Pemble *et al*., 1996). The omega-class of GSTs contain in contrast with other mammalian GSTs, an active site cysteine, that is able to form a disulfide bond with GSH. The proteins exhibit low activity towards the classical GST substrates but show significant GSH-dependent dehydroascorbate and GSH-mixed disulfide reducing activities, reactions typical for glutaredoxins. These proteins show in addition, structural similarities to *E. coli* Grx2 (Board *et al*., 2000).

The crystal structure has been determined for all the cytosolic GST classes and despite low overall level of sequence identity across the classes all structures follow a similar fold, with each subunit consisting of two distinct domains. The first domain adopts a topology similar to that of the thioredoxin fold, with a four stranded β-sheet and three flanking α-helices. This domain is highly conserved in the GSTs and provides most of the GSH binding site. The orientation of the GSH molecule in the active site is approximately the same for all isoenzymes but there are substantial differences in the electrostatic interactions. The second domain consists of a number of α-helices that varies widely between the different classes, and differences in this domain may be responsible for differences in substrate specificity (Dirr *et al*., 1994; Sheehan *et al*., 2001).

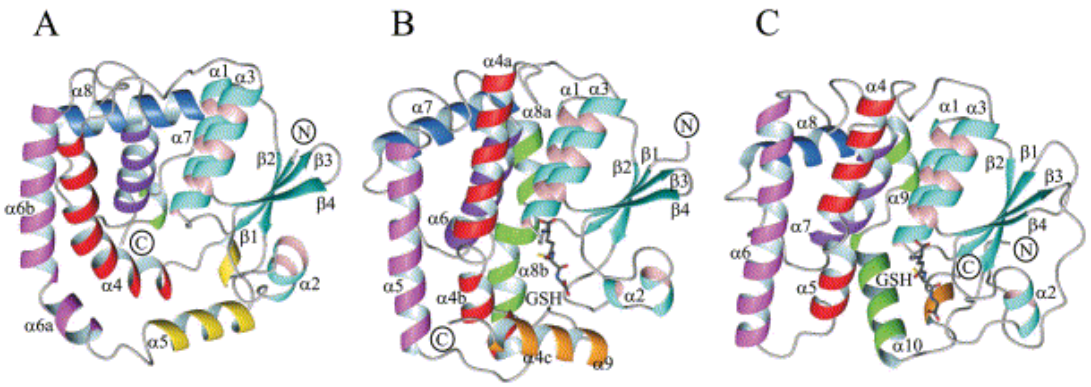

Figure 8. Comparison of the three dimensional structure of a) reduced *E coli* Grx2 and b) human θ-class GST (glutathione complex) an c) human ω–class GST (glutathione complex) (Xia *et al*., 2001).

1.8.2 Chloride ion channel proteins

Recently, a family of intracellular chloride ion channel proteins (CLICs) was identified in mammalian cells. The family consists of seven members that exist in both soluble and integral membrane forms. They have a broad tissue distribution and are found both in plasma membranes and in organelle membranes such as mitochondria, nuclear membranes, trans-golgi vesicles, secretory vesicles and the endoplasmic reticular membrane. CLICs have furthermore, been linked to several functions including apoptosis, pH and cell cycle regulation (Fernandez-Salas *et al*., 2002) (Cromer *et al*., 2002; Schlesinger *et al*., 1997; Valenzuela *et al*., 2000).

A major step forward in understanding the function of these proteins has been the recent crystal structure of one of the family members, CLIC1 (Harrop *et al*., 2001). The structure of the soluble form of CLIC1 was shown to consist of two domains, an N-terminal domain possessing a Trx fold closely resembling Grx, and a α-helical C-domain, which is typical or the GST superfamily. The Trx/ Grx domain was shown to contain a Grx-like active site and an intact GSH-binding site that covalently bound GSH via a conserved cysteine residue. This suggested that the function of CLIC1 is redox regulated, possibly via reactive oxygen species or nitrogen species. Furthermore, CLIC1 has been shown to form an intramolecular disulfide bond upon oxidation, which resulted in a structural alteration and formation of non-covalent dimers. This suggested that formation of the disulfide was essential for transition of the soluble CLIC1 into an integral membrane channel form (Littler *et al*., 2003).

1.8.3 Glutathione peroxidases

Glutathione peroxidases (GPxs) constitute a GSH-dependent protein family involved in the cellular defense against oxidative stress. GPxs catalyze the reduction of H_2O_2 and lipid hydroperoxides to water or the corresponding alcohol, using reducing equivalent from GSH (Brigelius-Flohe, 1999). These enzymes are classified into two groups; one of which contains a selenocysteine at its active center, while the other does not. Mammalian cells contain at least four isoenzymes belonging to the former group (GPx1-4). GPx1 and GPx4 are both localized to the cytosol and the mitochondria, and the enzymes are ubiquitously expressed in different tissues. GPx4 is also called PHGPx since it in contrast to the other enzymes preferentially reduces lipophilic substrates such

as phospholipid hydrogen peroxides. GPx2 and GPx3 are cytosolic enzymes, mainly localized to the gastrointestinal tract and kidney, respectively (Mates *et al*., 1999). GPx3 is also found in the human plasma, and has been demonstrated to utilize reducing equivalents from TrxR1, Trx1 and Grx1 (Björnstedt *et al*., 1994).

1.8.4 Peroxiredoxins

Peroxiredoxins (Prxs) comprise a recently defined family of peroxidases that catalyzes the reduction of H_2O_2 and organic hydroperoxides. Prxs, which have a molecular size of 20-30 kDa, are present in all living organisms. Members of the Prx family are divided into two subgroups; 2-Cys Prx proteins, which contain both an N-terminal cysteine and a C-terminal cysteine, and 1-Cys Prx proteins, which contain only the N-terminal cysteine (Rhee *et al*., 2001). The crystal structures of 1-Cys and 2-Cys Prx enzymes have revealed that both enzymes exist either as monomers, or in a head-to tail-dimeric structure. The crystal structures revealed further that the proteins consist of two domains, with the N-terminal domain adopting a Trx-fold (Chae *et al*., 1994; Choi *et al*., 1998; Hirotsu *et al*., 1999). The N-terminal Cys acts as the primary catalytic site for the reduction of peroxides and forms a sulfenic acid intermediate during catalysis. In the dithiol Prxs, the sulfenic acid is reduced either by the C-terminal cysteine in the same protein, forming an intramolecular disulfide bond, or by the C-terminal cysteine in the other subunit in the dimeric protein, forming an intermolecular disulfide bond. In both cases the disulfides are reduced by Trx, but not by Grx or GSH. In the monothiol Prxs on the contrary, no intermolecular disulfide is formed, and the regeneration of the cysteine thiol is not entirely clear. Of the six mammalian Prxs, five are dimeric dithiol enzymes (Prx I-V) and one contains a single redox active cysteine (PrxVI). The ubiquitous and abundant precence of Prx enzymes indicate that they play an important role in the detoxification of ROS, but they may also be involved in signalling cascades by regulating the intracellular levels of H_2O_2 (Rhee *et al*., 2001).

Recently, both monothiol and dithiol Prxs fused with a dithiol Grx domain were found in several pathogenic bacteria. The Prx and Grx domains of these hybrid proteins showed sequence homology with human PrxV and *E. coli* Grx3, respectively. The enzymes showed GSH-dependent peroxidase activity, and the Grx domain was suggested to act as an electron donor to the Prx domain (Cha *et al*., 2004; Kim *et al*.,

2003; Pauwels *et al*., 2003).

2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

Most organisms have been found to contain multiple dithiol Grxs that utilize electrons from GSH to catalyze thiol-disulfide exchange reactions. However, before this thesis project only one Grx (Grx1), mainly localized to the cytosol, was identified in mammalian cells. Based on its *in vitro* specificity towards GSH-mixed disulfides, Grx1 has been ascribed a plethora of functions in the cell - even in cellular compartments where there are no indications that the enzyme exists. Our aims were therefore to identify novel mammalian Grxs. Early in the studies we identified a second Grx, termed Grx2. The specific aims with this investigation were then to:

- Determine the subcellular localization of Grx2.
- Recombinantly express Grx2 and determine its substrate specificities.
- Elucidate the catalytic mechanism and the role of a Pro to Ser exchange in the active site of Grx2.
- Identify alternative reductants for the mammalian Grxs and for Grxs from other species.
- Elucidate the function of the additional structural cysteines present in the mammalian Grxs.

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms.

Since most species contain a family of glutaredoxins and only one Grx was identified in mammalian cells (Grx1) we searched for similar proteins in the expressed sequence tag (EST) Database. Two human clones that differed in their 5´- sequences by the presence of either a mitochondrial or a nuclear translocation signal (termed Grx2a and Grx2b, respectively) were identified in testis and colon cDNA libraries. Analysis of the genomic sequence demonstrated that the gene for Grx2 was localized on chromosome 1q31.2-31.3 and suggested that alternative first exon splicing generated the two isoforms. The predicted amino acid sequences corresponded to two variants of a Grx-like protein of approximately 18 kDa with 34 % sequence identity to the cytosolic human Grx1. While all the regions characteristic of a Grx were conserved, the active site (CSYC) contained a serine replacing the conserved proline of the consensus CPYC motif. Grx2 further contained two additional Cys residues, at positions that did not correspond to the three structural cysteines present in human Grx1. Furthermore, homologues (i.e. orthologues) to the human Grx2a isoform were identified in mouse and rat (with >70 % sequence identity), whereas no sequences corresponding to the Grx2b isoform were identified.

To analyze the expression pattern of human Grx2 we utilized a commercial multiple tissue blot with poly(A) RNA extracted from different human tissues and a probe common for both Grx2 isoforms. The Grx2 probe hybridized to a mRNA with the expected size of 0.9 kb in all tissues investigated, indicative of ubiquitous transcription of Grx2. Interestingly, to a minor extent, the Grx2 probe also hybridized to a larger mRNA of ~ 8 kb, indicating the presence of additional Grx2 isoforms. The GAPDH-control showed an expression pattern similar to Grx2. Thus, it would appear that Grx2 is ubiquitously expressed in a similar vein as the housekeeping gene GAPDH. The expression of Grx2 was further analyzed at the protein level, in different human cancer cell lines. Western blot analysis using specific antibodies raised against the recombinant protein confirmed that the protein, with the size of 16-18 kDa, was expressed in all cell lines tested.

In order to explore the subcellular localization of Grx2 we analyzed mitochondrial, nuclear and cytosolic fractions from Jurkat cells by Western blot analysis. Grx2 was predominantly detected in nuclear extracts, whereas only a weak band was detected in mitochondrial fractions. To investigate whether the potential translocation signals in Grx2a and Grx2b were functional, both isoforms were fused to the green fluorescent protein (GFP). Cells expressing a Grx2a-GFP fusion protein showed a fluorescent pattern that overlapped with the Mito-Tracker Red mitochondrial dye, thus indicating that the mitochondrial translocation signal was functional. In contrast, in cells expressing a Grx2b-GFP fusion protein, the fluorescence was predominantly observed in the perinuclear area, and only a weak fluorescence overlapped with the propidium iodide nuclear dye. Thus, the functionality of the potential nuclear targeting signal of Grx2b could not be assessed by the fusion protein approach. Truncated variants of Grx2 (i.e. corresponding to exon II-IV) lacking targeting signals were also expressed in fusion with GFP (either in the N- or C-terminus) and showed a cytosolic pattern of fluorescence.

To explore whether Grx2 exhibited GSH-dependent oxidoreductase activity, we expressed both isoforms of Grx2, as well as a truncated form containing only the region conserved for the two isoforms (exon II-IV), in *E. coli* BL21 (DE3) with N-terminal histidine-tags. The recombinant proteins were active in standard assays for glutaredoxin activity, monitoring GSH-coupled reduction of hydroxyethyl disulfide (HED) or dehydroascorbate (DHA). However, the specific activity was ten-fold lower than that of Grx1. The low activity was not due to the presence of the histidine tag, since a truncated form lacking the histidine-tag, revealed the same activity. Similar to human Grx1, none of the Grx2 variants were able to reduce insulin disulfides, indicating a restriction towards GSH-mixed disulfides, as is the case for most Grxs with the exception of *E. coli* Grx1. Moreover, whereas the activity of reduced Grx1 decreased to approximately 50 % upon preincubation with GSSG, the activity of Grx2 remained unchanged. This finding implied that Grx2 might retain its activity upon oxidative conditions when the level of GSSG increases.

Together these results demonstrated the presence of a second dithiol Grx in mammalian cells that in human is directed to the mitochondria, and possibly also to the nuclei.

2.2.2 Paper II

Glutaredoxins catalyze the reduction of glutathione by dihydrolipoamide with high efficiency

Glutaredoxins are known to catalyze thiol-disulfide oxidoreductions using electrons from NADPH via GSH. Under normal conditions the reduction of Grxs by GSH is fast, however, the rate of reduction falls substantially as the [GSH]/[GSSG] ratio decreases. In this study we demonstrated that Grxs from different species could utilize electrons from dihydrolipoamide, an important coenzyme for several enzymes and multienzyme complexes such as the pyruvate- and α-ketoglutarate dehydrogenase complexes, or protein H. We further showed that this electron pathway enabled Grxs to work in a reverse electron flow and thus reduce GSSG.

The lipoamide-dependent reduction of GSSG by Grx was analyzed *in vitro*, in a system with NADH, lipoamide dehydrogenase, and lipoamide, following the rate of NADH oxidation by the decreased absorbance at 340 nm. The reaction was analyzed using recombinant Grxs from human, rat, yeast and *E.coli*, and was found to be linearly dependent on the Grx concentration. A broad variation in activity was shown for the different Grxs, with the highest rate quantified for *E.coli* Grx2 followed by yeast Grx2, whereas the lowest activity was detected for the mitochondrial isoform of human Grx2 (Grx2a).

The levels of free lipoic acid in cells is low or absent, and the ability of Grxs to capture reducing power from enzyme bound dihydrolipoyl groups would require interaction of Grx with the lipoyl bearing enzymes. Those interactions were never studied in this paper but should be investigated in further experiments. Furthermore, both lipoamide and lipoic acid, have previously been demonstrated to be reduced by mammalian TrxR1 from several origins (Arner *et al*., 1996), which together with our results indicate an interesting connection between the levels of GSSG and the thioredoxin system.

2.2.3 Paper III

Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase

In paper I we showed that human Grx2 displayed a ten-fold lower specific activity in the GSH-dependent reduction of DHA and HED compared to human Grx1.

In this study we continued the enzymatic characterization of the truncated form of human Grx2, corresponding to exon II-IV, in comparison to human Grx1, and explored the non-conserved dipeptide sequence in the active site of Grx2.

Steady state kinetics of Grx1 and Grx2 were measured using two glutathionylated proteins, BSA-SG and RNase-SG, as well as the mixed disulfide formed between GSH and HED (β-ME-SG) in the presence of 1 mM GSH. The GSH-dependent reduction of these substrates revealed the same pH dependence for both Grx1 and Grx2, with a maximum rate at pH 8.5, indicative of the rate-limiting step being reduction of the Grx-GSH mixed disulfide intermediate by a second GSH molecule. The kinetic parameters were determined using non-linear regression, and demonstrated that although Grx2 reduced these substrates with a lower rate than Grx1, the apparent affinities were higher (lower K_m), which resulted in slightly higher efficiencies (k_{cat}/K_m). The highest efficiency was determined for RNase-SG followed by BSA-SG and β-ME-SG, demonstrating that Grx2 is highly specific for GSH-mixed disulfides and in particular glutathionylated proteins.

To further explore the catalytic mechanism of Grx2 and the influence of the Pro to Ser exchange in the dipeptide active site sequence, we utilized site-directed mutagenesis to construct active site mutants of both Grxs. A monothiol active site mutant, Grx2C40S, with the C-terminal active site Cys replaced by a Ser, confirmed that Grx2 followed the usual Grx mechanism, in that only the more N-terminal active site Cys was required for reduction of the glutathionylated substrates. The higher k_{cat} observed for this mutant, compared to the wild type enzyme is similar to earlier observations for the corresponding mutant proteins of Grx1 from human and pig (Yang *et al*., 1998; Yang and Wells, 1991), but different from *E. coli* Grx1, Grx3 and phage T4 Grx1, which exhibited significantly decreased activity (Bushweller *et al*., 1992; Nikkola *et al*., 1991; Nordstrand *et al*., 1999b).

Two other mutants, Grx2S38P and Grx1P23S, where we swapped the active sites of the human Grxs, were also constructed. The Grx2S38P mutant, mimicking the active site of Grx1, exhibited a two-fold increased specific activity in the GSH-dependent reduction of HED, compared to the wild type enzyme. Although this mutant catalyzed the reduction of β-ME-SG and RNase-SG with higher rates than wildtype Grx2, the affinity for both substrates was lower. Remarkably, the corresponding mutant of Grx1, Grx1P23S mimicking the active site in Grx2, showed almost the same specific

activity and catalytic properties as Grx2. The Grx1P23S mutant revealed increased affinity and decreased turnover for β-ME-SG and RNase-SG, compared to wildtype Grx1. These observations clearly demonstrated that a Pro to Ser exchange decreases the activity of the Grx but increases the affinity for glutathionylated substrates.

The significant differences in catalytic properties between human Grx1 and Grx2 prompted us to further analyze whether oxidized Grx2 was a substrate for TrxR. Surprisingly, we found that Grx2 could utilize reducing equivalents from both the cytosolic and mitochondrial isoforms of mammalian TrxR to reduce various glutathionylated substrates, including GSSG. The K_m of TrxR1 for Grx2 (20 µM) was shown to be higher than the K_m determined for Trx1 (2.5µM), and the K_{cat} represented about one third of the K_{cat} determined for Trx1. The catalytic mechanism for reduction of Grx2 by TrxR was explored using the monothiol active site mutant Grx2C40S, in a coupled reaction with various glutathionylated substrates. Those experiments revealed that TrxR was able to support both the monothiol and dithiol reactions catalyzed by Grx2. This suggested that the Grx-GSH mixed disulfide was attacked by the selenolate in TrxR, forming a Grx-TrxR mixed selenylsulfide intermediate, releasing GSH. Interestingly, this pathway resembles the electron transfer suggested for the TGR enzymes, where the monothiol or dithiol Grx domain accepts electrons from either the TrxR domain or GSH (Agorio *et al*., 2003; Alger and Williams, 2002; Sun *et al*., 2001a).

The new electron donor pathway allowed us to further investigate substrates for Grx2 in a GSH-independent manner. Using TrxR as an electron donor, we found that Grx2 could reduce both hydroxyethyl disulfide and CoA-disulfide apart from their GSH-mixed disulfides. Although reduction of the intramolecular disulfides was almost a 1000-fold lower than the GSH-mixed disulfides, it clearly demonstrated that Grx2 also is able to reduce low molecular disulfides independently of GSH.

The ability of human Grx2 to use either TrxR or GSH as a source of electrons is the first example of a cellular Grx being able to cross-react in this way. This feature together with its high specificity towards glutathionylated substrates suggested that Grx2 is particularly suited to remove and control the levels of glutathionylated proteins and low molecular weight disulfides following overproduction of reactive oxygen species during oxidative stress. During such conditions TrxR, whose activity is not

sensitive for changes in the cellular glutathione redox buffer, could facilitate an efficient backup system enabling reactivation of inactivated enzymes and reduction of GSSG to restore a reduced redox state. Hence, the results from this study suggested an important role for Grx2 in the protection and recovery of oxidative stress.

2.2.4 Paper IV (manuscript)

Human mitochondrial glutaredoxin has glutathione S-transferase activity and a redox dependent affinity for S-linked glutathione

Glutaredoxins and the N-terminal domain of glutathione S-transferases (GSTs) adopt a similar Trx fold. Despite limited sequence similarity and differences in the reactions catalyzed, these protein families have evolved a common way to bind GSH.

In this study we tested the affinity of the human Grxs for GSH-Sepharose that generally is used to purify GSTs. In this resin, the GSH moieties are coupled to the Sepharose by covalent carbon-thiol ether bonds and therefore only allow non-covalent interactions between the Grx and GSH. Surprisingly, we found that the oxidized form of Grx2 bound to the GSH-Sepharose, and subsequently could be eluted by the addition of GSH. However, bound Grx2 was most efficiently eluted by a reductant such as DTT, which has no similarity to the matrix bound GSH, indicative of a redox-controlled affinity of Grx2 towards GSH or GSH-mixed disulfides. The finding that neither pre-reduced Grx2 nor the Grx2C40S mutant revealed any affinity for the matrix reinforced this conclusion.

In contrast to Grx2, neither the oxidized nor the reduced form of Grx1 bound to the GSH-Sepharose. To explore the role of the Pro to Ser exchange in the active site of Grx2 we subsequently analyzed the affinity of the Grx2S38P and Grx1P24S mutants. Compared to the wild type enzymes, the former mutant abolished, whereas the latter increased the affinity for the matrix. Those results supported a crucial role of the Pro to Ser exchange for binding interactions with GSH.

Since Grx2 bound to a matrix used for purification of GSTs, we analyzed whether the human Grxs exhibited activities typical for GSTs. The GST catalyzed conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid has been extensively studied and occurs by a nucleophilic aromatic substitution, forming 1-

(S-glutathionyl)-2,4-dinitrobenzene and two diastereomeric ethacrynic acid-GSH conjugates, that absorbs at 340 nm and 270 nm, respectively (Armstrong, 1997). Thus, the reactions occur by a mechanism significantly different from the ordinary reactions catalyzed by Grxs. In contrast to Grx1, we found that Grx2 catalyzed conjugation of GSH to either CDNB or ethacrynic acid, with rates linearly dependent on the enzyme concentration. The monothiol active site mutant, Grx2C40S, revealed no activity with either of the substrates, demonstrating that both cysteines in the active site of Grx2 were required also for the GSH-conjugation activity. However, the activity detected with CDNB was approximately a 1000-fold lower than the activity previously detected for the yeast dithiol Grxs (Collinson and Grant, 2003; Collinson *et al*., 2002). Moreover, in contrast to several of the mammalian GSTs and the yeast dithiol Grxs, we showed that Grx2 reduced hydrogen peroxide only slightly more efficient than GSH itself (Collinson *et al*., 2002; Zhao *et al*., 1999). However, when we assayed that activity using TrxR as an hydrogen donor, the activity was linearly dependent on the Grx2 concentration, but still approximately a 1000-fold lower that the rate determined for the yeast Grxs.

The results from this study indicated that Grx2 represents a protein in the intersection of the Grx and the GST protein families. The redox dependent affinity of Grx2 for GSH-Sepharose was unexpected and could have important mechanistic implications for the role of Grx2 *in vivo*.

2.2.5 Paper V (manuscript)

Analysis of the redox state upon oxidation of human glutaredoxins and identification of a native structural disulfide in glutaredoxin 2

Human Grx1 and Grx2 contain three and two cysteines, respectively, in addition to those present in their active site. These cysteines, which are conserved in higher eukaryotes but not between the two isoenzymes, may provide a mechanism for redox control of Grx function. Exposing Grx1 to oxidizing conditions, with or without glutathione present, leads to partial inactivation of the protein, which is reversed by incubation with a strong reductant like dithiotreitol (DTT). In contrast, the GSH-mixed disulfide reducing activity of Grx2 is virtually unaffected by pretreatment with oxidants or reductants, demonstrating significant differences between the human Grxs. In this

paper we investigated the redox state of the additional cysteines upon oxidation and reduction of the two proteins.

The numbers of thiols in GSSG oxidized or reduced Grx1 and Grx2, were quantified by 5,5´dithiobis-2nitrobenzoic acid (DTNB). In addition, the proteins were analyzed using thiol specific alkylation with the fluorescent probe 5-iodoacetamidofluorescein (5-IAF) by non-reducing SDS-PAGE. Grx1 revealed a complex pattern of dimers and oligomers upon GSSG oxidation and only a weak fluorescence was detected from the oxidized forms, indicating that only a small fraction of the protein contained free thiols. Incubation with DTT resulted in complete reduction of the protein, only one band was observed on SDS-PAGE with a strong fluorescence, and the number of free thiols determined with DTNB corresponded to the total number of cysteines in the protein. In contrast, neither of the cellular reductants GSH, Trx or TrxR was able to fully reduce the protein. Grx2, on the contrary, did not show any tendency to form disulfide bonded dimers or oligomers upon oxidation. Analysis of the thiol content, by 5-IAF and DTNB, revealed no free thiols in the oxidized protein, which suggested the presence of either intramolecular disulfides or GSH-mixed disulfides. Similar to Grx1, oxidized Grx2 could only be fully reduced by DTT, whereas GSH and TrxR reduced approximately one disulfide per Grx2 (paper III).

To further analyze and map intra- and intermolecular disulfides, both GSSG oxidized and DTT reduced, Grx1 and Grx2 were trypsin digested and analyzed by mass spectrometry (MALDI-TOF and ES-TOF). Those results indicated that the basis for oligomerization of Grx1 were formation of intermolecular disulfide bonds between Cys8 and either Cys79 or Cys83. This is consistent with previous observations, showing that mutation of Cys8 significantly decreased tendency of Grx1 to oligomerize (Padilla *et al*., 1996b). Mass spectrometry data also indicated formation an intramolecular disulfide between Cys79 and Cys83. These cysteines are separated by a distance of approximately 12 Å in the reduced protein (Sun *et al*., 1998), and formation of a disulfide would substantially alter the structure of the protein. GSH-mixed disulfides were further identified with Cys8 and presumably also with Cys79 and Cys83. Formation of such mixed disulfides might either prevent formation of the intramolecular disulfide, or promote it by providing an intermediate for its formation. In the case of Grx2, mass spectrometry data revealed only the presence of an

intramolecular disulfide in the active site of the oxidized protein, and no masses could confirm the precence of a second disulfide or mixed disulfides with GSH.

The three-dimensional structure of Grx2 has not been determined but a structural model of Grx2, based on known Grx structures, indicates that the two additional cysteines present in Grx2 (Cys28 and Cys113) are located on loop regions only 5.4 Å apart. Formation of a disulfide bond between these cysteines would only require small conformational changes of the loops. Hence, this model suggests that there are no structural impediments to the formation of a second disulfide in Grx2. Furthermore, conformational stability measurements using urea denaturation, revealed a greater stability of fully oxidized Grx2, with a magnitude of 5.4 kcal/mol. This large difference in stability can, in view of the available data, best be explained by the presence of a structural disulfide. The importance of the structural disulfide in Grx2 was also highlighted by the instability of a double mutant Grx2C28SC113S, where the two cysteines were replaced by serines. This mutant was prone to precipitation during all steps of the purification procedure, which prevented its characterization.

The significant differences between the human Grxs upon oxidation demonstrated in this paper might be an adaptation to different redox environments or reactions catalyzed. It is not clear which of the cysteine modifications in oxidized Grx1 that caused the decreased activity. Oligomerization of the protein could mask the active site, structural changes by formation of an intramolecular disulfide might inactivate the protein, but also glutathionylation has been shown to deactivate a number of proteins. This oxidation “pattern”, is very similar to the behavior of human cytosolic Trx1, which upon oxidation forms covalently linked homodimers and oligomers, that are predicted to alter both the interactions with its substrates and TrxR. Trx1 has also been demonstrated to be inactivated by glutathionylation (Casagrande *et al*., 2002). Moreover, the retained deglutathionylase activity of oxidized Grx2 together with the demonstrated stabilization of the oxidized protein ensures a high pool of active protein during oxidative redox conditions.

3 CONCLUSIONS

- A second, ubiquitously expressed Grx of approximately 18 kDa, was identified in mammalian cells. The protein, termed Grx2, showed 34 % sequence identity to the previously characterized cytosolic Grx1, with all regions characteristic for a Grx conserved. However, Grx2 contained the CSYS active site sequence instead of the consensus CPYC typically found in Grxs (paper I).

- Two isoforms of human Grx2, with mitochondrial and putative nuclear localization (Grx2a and Grx2b, respectively) were identified. Analysis of the genomic sequence revealed that Grx2 is localized on chromosome 1 and that alternative first exon splicing is likely to generate the two isoforms. Both isoforms of Grx2 were shown to exhibit GSH-dependent HED and DHA reducing activities, but with a ten-fold lower specific activity than Grx1 (paper I).

- Both Grx1 and Grx2, as well as the Grxs from *E. coli* and yeast, were shown to reduce GSSG utilizing electrons from dihydrolipoamide. This demonstrated that Grxs are able to work in a reverse electron flow (paper II).

- Steady state kinetics demonstrated that Grx2 catalyzed the reduction of glutathionylated substrates with lower rates than Grx1, but with apparent higher affinities. Two active site mutants, Grx2S38P and Grx1P23S, revealed that these properties are linked to the non-conserved CSYC active site of Grx2. A monothiol mutant, Grx2C40S demonstrated that the reactions proceeded by a monothiol mechanism, similar to Grx1 (paper III).

- Grx2 was shown to be a substrate for both the cytosolic and mitochondrial isoforms of TrxR. Utilizing this electron pathway, Grx2 reduced both low molecular weight disulfides and GSH-mixed disulfides, including GSSG. Thus, a total of three different relevant reductants have been identified for Grx2 in the mitochondrion: GSH, dihydrolipoamide and TrxR (paper III).

- Grx2 was, in contrast to Grx1, found to catalyze conjugation of GSH to both CDNB and ethacrynic acid. The reaction was shown to be dependent on both active site cysteines and could not be explained by the non-conserved active site sequence in Grx2 (paper IV).

- Grx2 showed in contrast to other Grxs high affinity for S-linked GSH-Sepharose. The binding was specific for the oxidized form of Grx2, implying a redox dependent affinity for S-linked GSH. The Grx2S38P mutant completely abolished the affinity for the GSH-Sepharose, supporting a crucial role of the dipeptide active site sequence for GSH binding (paper IV).

- Human Grx1 was shown to form disulfide bonded dimers and oligomers, between Cys8 and either Cys79 or Cys83 upon oxidation. In addition, an intramolecular disulfide between Cys79 and Cys83 was indicated, likely to have a profound effect on the structure. GSSG oxidation was also shown to induce GSH-mixed disulfides with all three structural cysteines, which is likely to prevent formation of either the intra- or intermolecular disulfides. These cysteine modifications might all explain the partial inactivation, observed upon oxidation of the protein (paper V).

- The activity of Grx2 was in contrast to Grx1 unaffected by oxidation or reduction, and the protein revealed no tendency to oligomerize. Analysis of the thiol content and molecular modelling indicated the precence of a second intramolecular disulfide in Grx2, formed between Cys28 and Cys113, which was neither reduced by TrxR nor by GSH. Conformational stability measurements revealed a greatly increased stability of fully oxidized Grx2, supporting the precence of a structural disulfide (paper V).

4 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The general goal with this thesis project was to identify novel mammalian Grxs. Initially we tried to purify novel Grxs from various mammalian tissues following the Grx specific reduction of GSH-mixed disulfides. When these attempts turned out to be unsuccessful we switched to an bioinformatic approach, and identified Grx2.

As demonstrated in paper I, the identified expressed sequence tags of human Grx2 differed in the 5´-sequence, delineating both mitochondrial and potential nuclear isoforms. Consistent with this, subfractionation of Jurkat cells showed nuclear and mitochondrial immunoreactivity for Grx2. These results provided the first evidence for subcellular localization of Grx2. The mitochondrial localization of Grx2 has later been confirmed in isolated rat mitochondria using affinity purified rabbit anti-Grx2 antibodies (unpublished data). In addition, Grx-like activity ascribed to Grx2 has been detected in isolated mitochondria from mouse brain and liver (Ehrhart *et al*., 2002). Although attempts to purify the mitochondrial enzyme and to determine its N-terminal processing site have been unsuccessful, there is little reason to doubt the presence and cleavage site of the mitochondrial targeting signal in Grx2. In contrast, the potential nuclear or perinuclear localization of human Grx2 requires further investigation since some of the assays have yielded conflicting results. Homologues to this isoform of Grx2 were not found in the mice and rat libraries, indicating that it might be specific for humans. Thus, since only the mitochondrial isoform of Grx2 is well confirmed in mammalian cells, paper I-V focused mainly on Grx2 as a mitochondrial protein.

Interestingly, Jurado et al. (Jurado *et al*., 2003) recently analyzed the absolute gene expression pattern of Grx2 in mouse. These studies showed a ubiquitous expression of total Grx2 mRNA in different organs with highest expression in testis. Analysis of the different splice forms revealed that the mitochondrial transcripts accounted for approximately 40 % of all available Grx2 mRNAs in each of the organs examined, however in testis the mitochondrial transcripts represented only 1%. The total Grx2 mRNA levels were also analyzed in whole mouse embryos at three different developmental stages yielding no substantial changes during mouse gestation. However, the mitochondrial Grx2 isoform was elevated 3-fold on day 11, when the mitochondrial ultra structure becomes characteristic of actively aerobically respirating

cells (Jurado *et al*., 2003). This study strongly indicated the precence of yet additional isoforms of Grx2, which indeed requires further investigation.

In paper III we demonstrated that Grx2 is highly specific for GSH-mixed disulfides, and in particular glutathionylated proteins. Since glutathionylation has been shown to act as a reversible switch in much the same way as phosphorylation, this feature implies important roles for Grx2 *in vivo*, both in a protective and a regulatory mode. In the mitochondria, the GSH redox status modulates the activity of number of proteins including α-ketoglutarate dehydrogenase, succinate dehydrogenase, and NADH-ubiquinone oxidoreductase (Kenchappa and Ravindranath, 2003; Nulton-Persson *et al*., 2003; Taylor *et al*., 2003). The importance of Grx2 for the reactivation of these enzymes remains to be studied, as well as the identification of other candidate substrates.

Furthermore, in paper III we demonstrated that the Pro to Ser exchange in the non-conserved CSYS active site of Grx2 was directly linked to the catalytic properties of the enzyme. Previous studies have shown that a Pro residue in this position favor formation of hydrogen bonds to the N-terminal active site thiolate, which stabilize the thiolate and decrease its pK_a value (Foloppe *et al*., 2001). Determination of the pK_a value of the N-terminal cysteine thiol as well as the redox potential of human Grx1, Grx2 and active site mutants is an ongoing project. Those results will provide information of the redox properties of the human Grxs, as well as the importance of the Pro to Ser exchange in the active site of Grx2.

Despite major differences in the primary structure of Grx1 and Grx2 it was unexpected that Grx2 was a direct substrate for TrxR. The Trx and Grx systems are considered as parallel in terms of electron flow, and the ability of human Grx2 to use either TrxR or GSH as a source of electrons is the first example of a cellular Grx being able to cross-react in this way. The only previous example of this interaction comes from virus infected *E. coli* where bacteriophage-coded T4 Grx1 is an equally good substrate for *E. coli* TrxR as the host Trx1 (Berglund and Holmgren, 1975). It is also interesting to note the Grx-like protein NrdH in *E. coli*, that does not interact with GSH but only with *E. coli* TrxR (Jordan, 1997). *In vivo* experiments, with cells lacking either GSH or TrxR will further elucidate the importance of the different electron pathways.

The ability of Grx2 to regenerate GSH using reducing equivalents from either TrxR or dihydrolipoamide, together with its retained activity upon oxidative conditions, further supported a key role for Grx2 in the protection and recovery of oxidative stress. It is known that oxidation of the mitochondrial glutathione and protein thiols correlate with the mitochondrial permeability transition and with apoptosis (Borutaite *et al*., 2000). In addition, superoxide formation in the mitochondria during both normal and altered respiration is detoxified by Mn-SOD and glutathione peroxidases, resulting in oxidation of GSH to GSSG. Since mitochondria cannot efflux GSSG, excess must be reduced to GSH. Hence, the regeneration of GSSG to GSH by Grx2 might be highly relevant in the mitochondria.

In paper IV we demonstrated that Grx2, in contrast to Grx1 possessed features typical for GSTs, which is intriguing since GSTs are suggested to have evolved from a common Grx progenitor. The low GSH conjugating activity detected for Grx2 is most probably of no biological relevance, but reflects, together with the redox dependent affinity of Grx2 for S-linked GSH, significant differences in the way Grx1 and Grx2 interact with GSH. The finding that the affinity of Grx2 for the GSH-Sepharose was dependent on an intramolecular disulfide in the active site is in agreement with its reduction by GSH. However, if the resin resembles a GSH moiety in a GSH-mixed disulfide, then a higher affinity of the oxidized protein is intriguing with regards to its function as a reductant of glutathionylated proteins. An interesting aspect of this finding is that Grx2 during oxidative conditions could form rather stable complexes with glutathionylated proteins that would ensure fast reduction once the number of reducing equivalents increases. Obviously, the interaction between Grxs, and particularly Grx2, and GSH-mixed disulfides needs to be further investigated.

Despite the increasing number of reports on proteins being regulated by various cysteine modifications, it is still unknown how different cellular redox status conditions affect the properties of the human Grxs. In paper V we started to investigate the role of the additional cysteines present in both human Grx1 and Grx2, and substantial differences between the Grxs upon oxidation and reduction were demonstrated. The cysteine modifications identified in this paper could provide a mechanism for redox control *in vivo*. Further studies are being undertaken to confirm the existence of the additional intramolecular disulfides, indicated to form in both Grxs upon oxidation, both *in vitro* and in *vivo*.

Furthermore, the three-dimensional structure of Grx2, modeled in paper V, resembled a typical Trx/Grx fold. However, in order to understand the unusual properties of Grx2, an experimental structure will be determined and compared to other proteins in the Trx-fold superfamily.

Finally, it remains to be tested whether Grx2, similar to human Grx1, can function as a hydrogen donor for ribonucleotide reductase. Furthermore, the electron donor for the p53 induced ribonucleotide reductase involved in DNA repair, is currently unknown, and the role of both human Grxs as reductants for this enzyme remains to be elucidated.

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