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IN VIVO FUNCTIONS OF MAMMALIAN GLUTAREDOXIN 2

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Coverpage: "Grx2 goes monet"
Cerebellum from mouse stained for glutaredoxin 2, co-staining with cytoplasmic
DNase I and mitochondrial Prohibitin I.

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ABSTRACT

Oxygen is essential for all respiring life forms. However, the use of molecular oxygen as terminal electron acceptor of the respiratory electron chain leads to the generation of reactive oxygen species (ROS) as product of incomplete oxygen reduction. To different extends, ROS can react with and damage DNA, lipids and proteins. To cope with and recover from ROS-induced damage, cells have developed several antioxidant systems. Oxidative stress was once defined as an imbalance between ROS and the antioxidant systems. Today we know that several independent redox circuits exist and look at oxidative stress as an imbalance in these redox signaling and control pathways. In this respect, cysteine residues in proteins are the key regulatory units, because they are highly susceptible to oxidative modifications that can affect both activity and function of proteins. Thiol-disulfide oxidoreductases are proteins that catalyze either the formation or reduction of disulfide bonds. The focus of this thesis was the characterization of mammalian Glutaredoxin 2 (Grx2), a member of this family of proteins.

We have identified human Grx2 as the first thiol-disulfide oxidoreductase that can complex an iron-sulfur cluster. The dimeric holo-enzyme complex was enzymatically inactive. Monomerization induced by both oxidants and reductants activated the protein. Based on these findings, we have proposed a role as redox-sensor for the cluster: under normal conditions the majority of Grx2 in the cell is present in the inactive dimeric form. During oxidative stress conditions, the holo-complex dissociates and active monomeric Grx2 is released. These results were coherent with our finding that knock-down of Grx2 does not affect the viability of HeLa cells *per se*. However, loss of Grx2 dramatically sensitized the cells towards oxidative stress-induced cell death when they were challenged with doxorubicin and phenylarsine oxide. These results imply an important role of Grx2 in the response of cells to oxidative stress.

We have investigated the expression pattern of mammalian Grx2 mRNA variants and identified three different isoforms of human Grx2: the mitochondrial isoform (Grx2a) was ubiquitously expressed in all tissues and two cytosolic/nuclear isoforms (Grx2b and Grx2c) were restricted to testis in healthy tissue, but also present in several cancer cell lines, indicating a potential function in malignant transformation. In mouse we have identified five transcript variants, encoding three different protein isoforms: (1) mitochondrial Grx2a corresponds to human Grx2a, (2) Grx2c is a cytosolic protein homologous to human Grx2c but derived from different transcript variants and (3) Grx2d, an enzymatically inactive protein that lacks structurally important amino acid residues, with no counterpart in human cells. In contrast to human, mouse Grx2c was not exclusively expressed in testis, but expressed in specific cells of many organs, for instance, in enteroendocrine cells of the fundic gland and in the white pulpa of the spleen.

LIST OF PUBLICATIONS

I. Lillig, C.H., **Lönn, M.E.**, Enoksson, M., Potamitou Fernandes, A., and Holmgren, A., siRNA-mediated silencing of glutaredoxin 2 increases the sensitivity of HeLA cells towards doxorubicin and phenylarsine oxide. *Proc. Natl. Acad. Sci.* U.S.A. 101: 13227-13232 (2004)

II. Lillig, C.H., Berndt, C., Vergnolle, O., **Lönn, M.E**., Hudemann, C., Bill, E., and Holmgren, A., Characterization of human glutaredoxin 2 as new iron-sulfur protein: a possible role as redox sensor*. Proc. Natl. Acad. Sci. U.S.A.* 102: 8168-8173 (2005)

III. **Lönn, M.E.***, Hudemann, C.*, Berndt, C., Cherkasov, V., Capani, F., Holmgren, A., and Lillig, C.H., Expression pattern of human glutaredoxin 2 isoforms: Identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid. Redox Signal.* 10: 547-558 (2008)
*Contributed equally to this work

IV. Hudemann, C.*, **Lönn, M.E.*,** Godoy, J.R., Zahedi Avval, F., Capani, F., Holmgren, A., and Lillig, C.H., Identification, expression pattern and characterization of mouse glutaredoxin 2 isoforms. *Antioxid. Redox Signal.* in press (2008)
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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Aft1	Activator of ferric transcription
AP-1	Activator protein-1
ASK-1	Apoptosis signal-regulating kinase 1
dsDNA	double stranded DNA
DTNB	5,5-dithiobis-(2-nitrobenzoic acid)
E. coli	Escherchia coli
FAD	Flavin adenin dinucleotide
Fe	Iron
FUR	Ferric uptake regulator
Grx	Glutaredoxin
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
H	Hydrogen
IRE	Iron regulatory element
IRP	Iron regulatory protein
kDa	kilo Dalton
mRNA	messanger RNA
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine
NF1	Nuclear factor-1
NF-κB	Nuclear factor κB
NO	Nitric oxide
PAO	Phenylarsine oxide
PAPS reductase	3'-phosphoadenosin-5'-phosphosulfate reductase
PDI	Protein disulfide isomerase
PICOT	PKC-interacting cousin of thioredoxin
Prx	Peroxiredoxin
Ref1	redox factor1
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S	Sulfur
S. cerevisiae	Saccharomyces cerevisiae
SERCA	Sarco/endoplasmatic reticulum Ca^{2+}-ATPase
SOD	Superoxide dismutase
SRF	Serum response factor
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TSA	Thiol-specific antioxidant

1 INTRODUCTION

A chemical reaction where electrons are transferred from one chemical species to another is called a redox reaction. When an electron is gained it is called reduction and when an electron is lost we have oxidation. The transfer of electrons can be accompanied with the transfer of atoms like hydrogen (H), which is composed of an electron and a proton. Neither oxidation nor reduction can occur independently. Because different chemical compounds have different electron affinities, the electrons flow spontaneously from one compound with low electron affinity to another with higher affinity. The compound, that becomes oxidized, reduces another and is therefore called the reducing agent. The reduced compound is hence the oxidizing agent. Redox reactions play an important role in biological systems and the redox state of all compartments is tightly regulated. The cytoplasm of a cell is regarded as a reducing environment, meaning that introduced species are rather reduced. The extracellular environment is more oxidizing. Several proteins have functions as reducing and oxidizing agents regulating the redox environment in a biological system. One group of proteins that is crucial for regulating the redox environment is the family thiol-disulfide oxidoreductases. Members of this protein family can be found in all organisms where they catalyze the formation and/or reduction of disulfide bonds. Cysteines are essential amino acids important for protein folding, metal coordination and enzyme catalysis. A disulfide bond can be a structural part of a protein, essential for correct folding. In other cases, formation of disulfide bonds affects the activity of enzymes or transcription factors. The majority of the thiol-disulfide oxidoreductases belongs to the thioredoxin-family of proteins. The focus of this thesis is on one member of this family of oxido-reductases called glutaredoxin 2 (Grx2).

1.1 THE THIOREDOXIN FAMILY OF PROTEINS

Even though only four amino acids are strictly conserved between the primary structure of thioredoxin (Trx) and Grx, (Hoog, Jornvall et al. 1983), they share a similar 3D-structure with a central core of four β-strands surrounded by at least three α-helices. The characteristic Cys-Xxx-Xxx-Cys active site is located on the loop following β-strand 1 (Fig.1). Since this particular architecture was first identified in a crystal structure of a Trx from *Escherichia coli* (*E. coli*), it became known as the Trx-fold even though *E. coli* Grx1 displays the most basic representation of this fold. *E. coli* Trx1, for instance, contains an additional β-strand at the N-terminus (Holmgren, Soderberg et al. 1975; Sodano, Xia et al. 1991). Many proteins, apart from Trxs and Grxs, contain this specific fold and hence belong to the Trx-fold family. Members include other thiol-disulfide oxidoreductases like protein disulfide isomerase (PDI) (Kemmink, Darby et al. 1996), peroxiredoxins (Prx) (Choi, Kang et al. 1998) and the bacterial Dsb family of proteins (Martin, Bardwell et al. 1993) as well as functionally different proteins like glutathione–S-transferases (GST) (Reinemer, Dirr et al. 1991), glutathione peroxidases (GPX) (Epp, Ladenstein et al. 1983), members of the intracellular chloride ion channels (Harrop, DeMaere et al. 2001) and proteins involved in the cytochrome c oxidase assembly (Balatri, Banci et al. 2003).
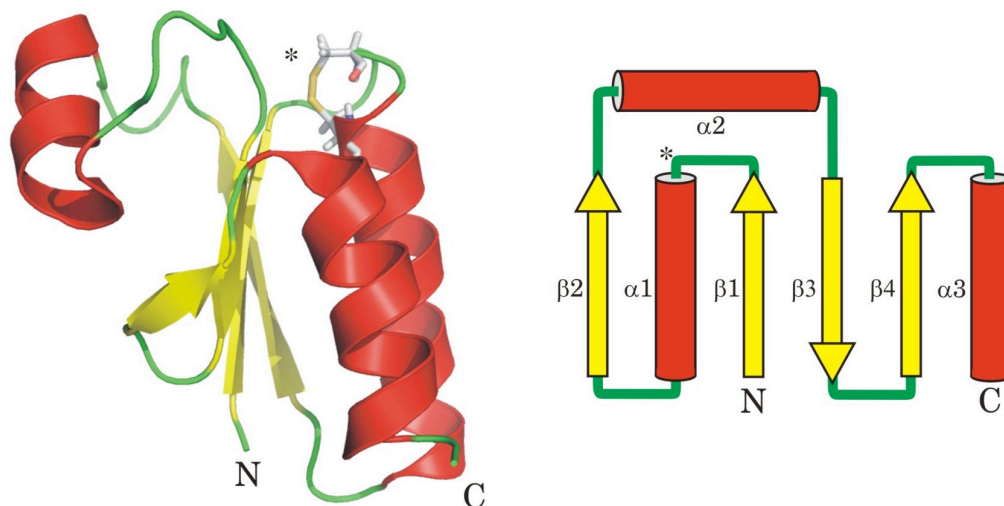
Figure 1. **The Trx-fold**. To the left, the structure of oxidized Grx1 (PDB accession number 1EGO). To the right, a schematic picture of the arrangement of α-helices (barrels) and β-sheets (arrows) in the Grx-structure. The asterix indicates the position of the active site.

3D-structures of Grxs from various organisms, such as *E. coli* (Sodano, Xia et al. 1991; Aslund, Nordstrand et al. 1996), pig (Katti, Robbins et al. 1995) and human (Sun, Berardi et al. 1998; Johansson, Kavanagh et al. 2007) are available today. In general, the secondary structure motifs of Grxs are shorter compared to the Trxs and they lack the fifth β-strand found in some Trxs. Grxs consist of four β-strands surrounded by α-helices. The number of helices surrounding the central β-sheet ranges from three, as in bacteriophage T4 Grx (Eklund, Ingelman et al. 1992), up to five, as in human Grx2 (Sun, Berardi et al. 1998). The N-terminal active site Cys-residue is facing a hydrophobic surface region, where charged residues are absent. This area was proposed to be involved in the protein-substrate interaction (Eklund, Cambillau et al. 1984; Xia, Bushweller et al. 1992). Also part of this area is the glutathione-binding site (Bushweller, Billeter et al. 1994; Yang, Jao et al. 1998), indicating the importance of glutathione (GSH) in the Grx-reaction. Comparing the binding of GSH to human and *E. coli* Grxs reveals differences in the salt bridge and hydrogen bonding pattern, where the interactions are fewer in *E. coli* (Yang, Jao et al. 1998). The most well-conserved residue apart from the active site cysteins in the Trx-family of proteins is a cis-proline in Grxs present in the conserved Thr-Val-Pro motif. This motif preceds the third β-strand and participates in binding of both GSH and protein substrates (Bushweller, Billeter et al. 1994; Yang, Jao et al. 1998; Nordstrand, Sandstrom et al. 2000). The reduced and oxidized structures of both Trxs and Grxs display only small but significant differences (Nordstrand, Sandstrom et al. 2000).

1.1.1 Active site

Despite of profound similarities between the structures and active site of proteins from the Trx-family, different members catalyze either the oxidation, reduction or both of protein thiols and disulfides, respectively. The reason for these differences are not fully understood, but the redox properties of the active site disulfide often correlates with its action as either reductant or oxidant. A lower redox-potential of an

oxido-reductase increases its tendency to act as reductant. The amino acids between the two Cys-residues in the active site, are major determinants of the redox-potential of this dithiol-disulfide redox couple (Huber-Wunderlich and Glockshuber 1998; Mossner, Huber-Wunderlich et al. 1999). It can be more oxidizing as in the case of PDI (Lundstrom and Holmgren 1993) or more reducing as in Trx and Grx (Aslund, Berndt et al. 1997). By exchanging the active site of Trx with the active site of PDI, the redox potential increased, demonstrating the influence of these two residues on the overall redox potential of the active site disulfide (Krause, Lundstrom et al. 1991). The redox-potential also correlates with the pK_a of the more N-terminal active site thiol (Mossner, Iwai et al. 2000). This Cys-residue is solvent exposed in Grxs and has a very low pK_a value, being up to four units below the pK_a of free cysteines. A more or less tight network of hydrogen bonds stabilizes the active site thiolate ion and is thus important for the low pK_a value (Foloppe, Sagemark et al. 2001). However, the redox-potential of the active site disulfide alone does not determine the function of the Trx family of proteins. Another major contributing factor is the overall redox environment. In the cytoplasm, for instance, the majorities of the Cys-residues are kept in the reduced thiol-state. In an *E. coli* strain lacking the natural reductant of Trx called thioredoxin reductase (TrxR), however, oxidized Trx will accumulate. In this mutant strain the oxidized Trx catalyzes the formation of disulfide bonds, contrary to its usual role as a reductant of cytosolic disulfides (Stewart, Aslund et al. 1998).

1.1.2   The thioredoxin system

Trx was identified in 1964 as a small heat-stable redox protein in *E. coli* with the ability to reduce ribonucleotide reductase (RNR) (Laurent, Moore et al. 1964). Trxs have subsequently been identified in basically all life forms. The functions assigned to Trx are constantly increasing and include, for instance, the redox regulation of transcription factors like NF-κB (Matthews, Wakasugi et al. 1992), inhibition of apoptosis through sequestration of the apoptosis signal-regulating kinase 1 (Saitoh, Nishitoh et al. 1998), acting as an antioxidant removing H_2O_2 (Spector, Yan et al. 1988) and triggering the inate immune response as a secreted truncated version of Trx, called Trx80 (Pekkari, Gurunath et al. 2000). Trx catalyzes the reduction of disulfides by two cysteines in a characteristic Cys-Gly-Pro-Cys active site (Holmgren 1968). The N-terminal Cys-residue acts as a nucleophile attacking the disulfide in the target molecule leading to the formation of a mixed disulfide intermediate. This disulfide is subsequently reduced by the more C-terminal active site Cys-residue (Kallis and Holmgren 1980). Oxidized Trx is reduced by TrxR (Moore, Reichard et al. 1964) using electrons from NADPH (Fig 2).

TrxR belongs, as glutathione reductase (GR, see below), to the pyridine nucleotide disulfide oxidoreductase family of proteins. Characteristic for members of this family is the formation of homo-dimers, where both subunits contain a flavin adenine dinucleotide (FAD) molecule and a redox-active disulfide. The FAD molecule can temporarily hold electrons, before the electrons are further transported to their target. The two subunits are organized in a head-to-tail arrangement. The mammalian TrxR differs from the bacterial, plant and fungal enzymes in both structure and biochemical properties. It has a broader substrate specificity and is a larger selenium-containing protein (Luthman and Holmgren 1982; Zhong, Arner et al. 1998). Mammalian TrxR resembles GR in the overall structure and in conserved amino acids in the FAD and NADPH binding domain. The most significant difference is a C-terminal extention in

TrxR harbouring the Gly-Cys-Sec-Gly active site (Gladyshev, Jeang et al. 1996). The higher degree of homology between mammalian TrxR and GR, compared to the bacterial counterparts, suggests that it evolved from the more recent GR, rather than from prokaryotic TrxRs (Sandalova, Zhong et al. 2001). Due to the very low pK_a of the Sec-residue, the selenoate is an extremely good nucleophile. This Sec-residue is hence one of the reasons for the broader substrate specifity of mammalian TrxR compared to the bacterial enzymes. Mammalian TrxR is not species specific when reducing Trx, as in the case of bacterial TrxR, instead it can reduce Trx from various species (Holmgren 1977). Substrates for mammalian TrxR include: PDI (Lundstrom and Holmgren 1990), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Luthman and Holmgren 1982), lipoic acid (Arner, Nordberg et al. 1996), ascorbic acid, S-nitrosoglutathione (GSNO) (Nikitovic and Holmgren 1996) and ubiquinone (Xia, Nordman et al. 2003) among others. When oxidized TrxR is reduced, the active site disulfide in the FAD-binding domain is first being reduced, using electrons from NADPH, via the FAD cofactor. The electrons are then transferred to a flexible C-terminal active site in the other subunit where the Cys 479 – Sec 498 selenenylsulfide is reduced to a selenolthiol. The selenol can in turn attack the disulfide in Trx, resulting in an mixed intermediate between TrxR and Trx. The selenenylsulfide between the two compounds is attacked by the C-terminal Cys-residue in TrxR, releasing reduced Trx (Sandalova, Zhong et al. 2001).
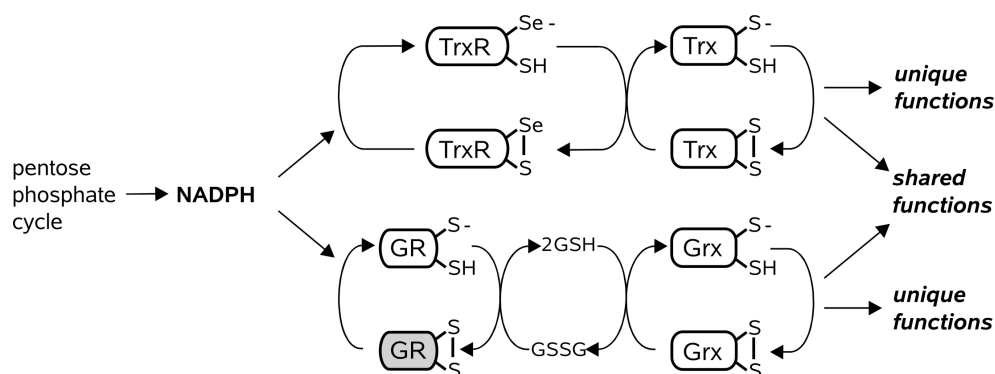

Figure 2. **The Trx- and the Grx-systems**. Oxidized Trx is reduced by TrxR using electrons from NADPH. Oxidized Grx is reduced by two molecules of GSH, generating GSSG, which in turn is reduced by GR using reducing equivalents from NADPH.

1.1.3 Glutaredoxins

Grxs were discovered in an *E. coli* strain lacking detectable levels of Trx. This strain retained RNR activity, suggesting at least one additional electron donor to be present in *E. coli*. Due to its dependance on glutathione (GSH) and action in redox reactions this protein was named glutaredoxin (Holmgren 1976). Grxs have since been assigned several other activities ranging from reduction of methionine sulfoxides and sulfate (Gonzalez Porque, Baldesten et al. 1970; Tsang 1981) and as general thiol-disulfide oxidoreductases (Holmgren 1979; Axelsson and Mannervik 1980) to regulating transcription factors (Matthews, Wakasugi et al. 1992; Pineda-Molina, Klatt et al. 2001) and involvement in apoptosis (Chrestensen, Starke et al. 2000). The

two proteins, Trx and Grx, share a number of similarities and display some overlapping and complementary activities. However, they do not act as simple backup of each other and display a series of specific differences and activities.

1.1.3.1 The Glutaredoxin system

The Grx system consists of Grx, GSH and GR. When Grx reduces a target protein, it is itself oxidized and in turn reduced by two molecules of GSH resulting in the formation of glutathione disulfide (GSSG). GSSG is in turn reduced back to two molecules of GSH by GR, using electrons from NADPH (Holmgren 1979) (Fig. 2). Grxs utilize one or two redox active cysteines in their active site to reduce different types of disulfides. In general, Grxs can reduce protein disulfide bonds, but they show strongest preference for the catalysis of reversible protein glutathionylation (Bushweller, Aslund et al. 1992; Gravina and Mieyal 1993). This type of reaction requires only the more N-terminal Cys-residue and was thus named monothiol reaction. The monothiol reaction occurs in a ping-pong reaction sequence. It is initiated by the nucleophilic attack of the N-terminal thiolate on the sulfur in GSH generating a Grx-GSH mixed disulfide, thus releasing the target protein. The disulfide formed between Grx and GSH is reduced by a second GSH, which is the rate-limiting step (Srinivasan, Mieyal et al. 1997) (Fig. 3). The second type of reaction, the dithiol mechanism, starts in the same way as the monothiol reaction, with a nucleophilic attack of the more N-terminal Cys-residue, but the substrate here is a protein disulfide. A Grx-protein mixed disulfide is formed, which in turn is attacked by the C-terminal active site thiol (Fig. 3). Since the C-terminal Cys-residue is protonated and buried in the reduced protein, a conformational change which alters the environment and hence the pK_a of this residue has been proposed (Berardi and Bushweller 1999),
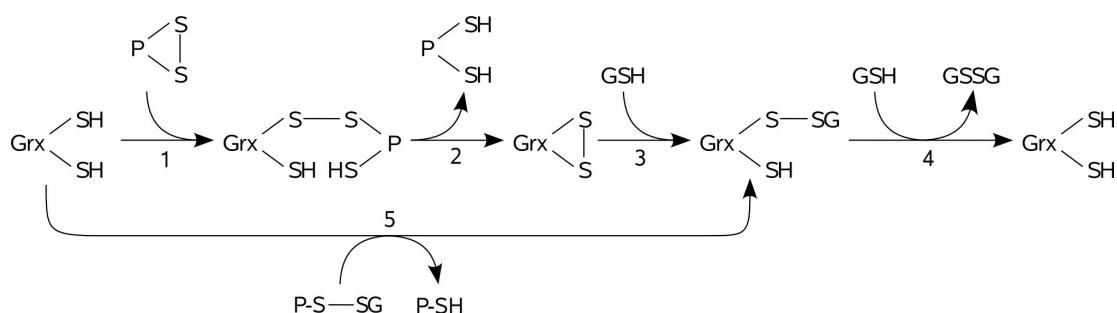
Figure 3. **The dithiol and monothiol mechanism of Grx.** Grx can reduce disulfides using either one or two Cys-residues in its active site. In the dithiol mechanism the N-terminal Cys-residue in the active site attacks the dithiol bond in the target substrate, forming a mixed disulfide between Grx and the target substrate (1). This disulfide is attacked by the C-terminal active site thiol of Grx, generating oxidized Grx and thus releasing the target substrate (2). The disulfide of Grx is reduced by GSH, generating GSSG (3,4). In the monothiol reaction, only the more N-terminal active site cysteine is required, generating a Grx-GSH mixed disulfide (5), which in turn is reduced by a second GSH.

Depending on the number of cysteines in the active site, Grxs can be divided into dithiol Grxs with a Cys-Pro-Tyr-Cys consensus active site sequence and monothiol Grxs harbouring the conserved active site sequence Gly-Cys-Phe-Ser. Despite the presence of the N-terminal active site residue in monothiol Grxs, most of these proteins do not catalyze monothiol reactions (Herrero and de la Torre-Ruiz 2007).

1.1.3.2 GSH

GSH is the major non-enzymatic antioxidant and the most abundant thiol in the cell present in millimolar concentrations (Apontoweil and Berends 1975; Griffith 1999). In the 1920s Fredrick Gowland Hopkins discovered GSH (Hopkins 1921) and it has since been thouroghly studied. GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) synthesized by gamma-glutamylcysteine synthetase and GSH synthetase from glutamate, cysteine and glycine in the cytosol (Johnston and Bloch 1951; Snoke, Yanari et al. 1953). The cysteine thiol group enables GSH to act as reductant as well as nucleophile. With its ability to scavenge free radicals, to reduce H_2O_2 and reduce disulfides, GSH acts as the cells' first line of defence against oxidative stress. The sulfur atom in GSH can accommodate the loss of a single electron, forming a thiyl radical, equation 1. Two thiyl radicals can in turn react to yield GSSG, equation 2.

$$GSH + R^{\bullet} \rightarrow GS^{\bullet} + RH \quad \text{(equation 1)}$$

$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG \quad \text{(equation 2)}$$
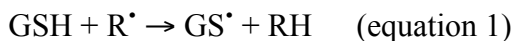
GSH, together with its oxidized counterparts GSSG and protein-SG mixed disulfides constitutes the major redox buffer of the cell (Schafer and Buettner 2001). GSH plays a central role in many redox processes, where it protects cells from oxidative and nitrosative damage. S-glutathionylation can protect critical thiol groups from irreversible oxidation and moreover, it plays an important role in signal transduction (Shelton, Chock et al. 2005). Glutathionylation will be discussed more thoroughly in chapter 1.3.1. In addition, GSH takes part in the detoxification of xenobiotics and physiological compounds. Even though GSH non-enzymatically can act directly as a nucleophile, the rates of conjugation to electrophilic compounds are highly increased by the enzymatic action of glutathione S-transferases (GST) (Tew 1994).

The pool of GSH in the cytosol, in mitochondria and the endoplasmatic reticulum are functionally separated and hence result in different redox-environments. The mitochondrial GSH pool plays an important role in detoxification of H_2O_2 from the respiratory chain (Chance, Sies et al. 1979) and is imported from the cytosol via ATP-dependant transport processes (Griffith and Meister 1985). The endoplasmatic reticulum contains a more oxidized GSH pool compared to cytosol and mitochondria (Hwang, Sinskey et al. 1992). Whether and how the nuclear GSH-pool is separated from the cytosolic, is still a matter of debate (Jevtovic-Todorovic and Guenthner 1992; Soderdahl, Enoksson et al. 2003). Low levels of GSH can also be found extracellularly, for instance in plasma (Bartoli and Sies 1978; Lang, Naryshkin et al. 1992; Kaplowitz, Fernandez-Checa et al. 1996).

1.1.3.3   GR

Like TrxR, GR belongs to the pyridine nucleotide-disulfide oxidoreductase family of proteins, that catalyzes the electron transfer from pyridine nucleotides to disulfides. The main function of GR is to maintain cellular GSH in its reduced state. The catalytic cycle starts with the binding of NADPH close to the FAD molecule in one subunit. Two electrons are transferred from NADPH to the flavin moiety, which in turn reduces the disulfide located in the same subunit. GSSG binds and a mixed disulfide is formed, the first GSH is released, followed by the reduction and release of the second GSH (Pai and Schulz 1983; Karplus and Schulz 1987; Karplus and Schulz 1989). Being the main reductant of GSH, GR is involved in the antioxidant defense. Surprisingly, GR null mutants in both *E. coli* and *Saccharomyces cerevisiae* (*S. cerevisiae*) only show mild alteration in the GSH/GSSG ratio, indicating alternative reducing pathways (Tuggle and Fuchs 1985). Mammalian GR has been detected in both cytosol and mitochondria with identical biochemical properties (Taniguchi, Hara et al. 1986). The two proteins are derived from the same gene, where a mitochondrial leader sequence is present between two in-frame start codons in the first exon (Taniguchi, Hara et al. 1986; Kelner and Montoya 2000).

1.1.4   Bacterial glutaredoxins

E. coli contains four different Grxs, three dithiol Grxs (Grx1, Grx2 and Grx3) and one essential monothiol Grx (Grx4) (Gerdes, Scholle et al. 2003). Grx2 is an unusual dithiol Grx, which is structurally similar to glutathione-S-transferases and contains a N-terminal Grx domain followed by six α-helices (Xia, Vlamis-Gardikas et al. 2001). Grx2 (24.3 kDa) is larger than the approximately 10 kDa Grx1 and Grx3. It lacks activity in reducing both RNR and 3'-phosphoadenosin-5'-phosphosulfate (PAPS) reductase, but has a very high activity in reducing mixed disulfides between GSH and both β-mercaptoethanol and arsenate reductase (Aslund, Ehn et al. 1994; Vlamis-Gardikas, Aslund et al. 1997; Lillig, Prior et al. 1999; Shi, Vlamis-Gardikas et al. 1999). Grx1 reduces general GSH-mixed disulfides with high efficiency. *In vitro* studies demonstrated substrates such as glutathionylated ribonuclease, PAPS reductase and arsenate reductase (Holmgren 1979; Lundstrom-Ljung, Vlamis-Gardikas et al. 1999; Shi, Vlamis-Gardikas et al. 1999). Grx3 has 5 % of the catalytic activity of Grx1 in reducing RNR and completely lacks activity for PAPS reductase (Vlamis-Gardikas, Aslund et al. 1997; Lillig, Prior et al. 1999). Grx3 catalyzes the reduction of GSH-mixed disulfides with the same rate as Grx1, but has a two-fold higher turn-over rate when reducing mixed disulfides between GSH and β-mercaptoethanol (Vlamis-Gardikas, Aslund et al. 1997).

The monothiol Grx4 lacks activity towards small molecular weight disulfides, insulin, PAPS reductase and HED, which are the classical targets for Grxs and Trxs. Surprisingly, just like human Grx2, which can be reduced by mammalian TrxR, Grx4 can be reduced by *E. coli* TrxR (Fernandes, Fladvad et al. 2005). The exact function of Grx4 is unknown. However, due to the regulation of Grx4 transcription by the free iron sensor ferric uptake regulator (Fur), a role in pathways using iron has been suggested (Fernandes, Fladvad et al. 2005).

1.1.5 Yeast glutaredoxins

In *S. cerevisiae* at least seven Grxs have been identified. Grx1 and Grx2 contain the dithiol Cys-Pro-Tyr-Cys active site and Grx3, Grx4 and Grx5 are monothiols with the Cys-Gly-Phe-Ser active site. Grx6 and Grx7 are unusual monothiol Grxs with Cys-Pro-Tyr-Ser and Cys-Ser-Tyr-Ser active sites, respectively (Izquierdo, Casas et al. 2008). Grx1 resides in the cytosol, whereas two different isoforms of Grx2 are localized in the cytosol or mitochondria (Pedrajas, Porras et al. 2002). Grx1 and Grx2 have, beside their normal thiol-disulfide activity, the ability to directly reduce H_2O_2 and to conjugate GSH to electrophilic compounds (glutathione-S-transferase activity) (Collinson, Wheeler et al. 2002). The activity of Grx1 *in vitro* is approximately two fold higher compared to Grx2's. Contradictionary, results from mutational studies showed that Grx2 accounts for the majority of Grx activity *in vivo*. The total Grx activity was unaffected in Grx1 mutants, but decreased by 80 % in Grx2 mutants. A double negative mutant of the dithiol Grxs in yeast is viable, but it is more sensitive to oxidative stress. Grx1 mutants are more sensitive to oxidative stress induced by superoxide anion, whereas Grx2 mutants are sensitized towards H_2O_2 (Luikenhuis, Perrone et al. 1998). Yeast cells lacking Grx2 are less susceptible to apoptosis induced by cadmium, but accumulate mitochondrial mutations at high frequency (Gomes, Pereira et al. 2008).

Neither of the monothiol Grxs in *S. cerevisae* can compensate for the oxidase-reductase activity of the dithiol Grxs since the double Grx1/2 mutant lacks heat stable oxidoreductase activity (Luikenhuis, Perrone et al. 1998). On the other hand, the dithiol Grxs cannot compensate for the activity of the monothiols, since the triple mutant of Grx3/4/5 is lethal (Rodriguez-Manzaneque, Ros et al. 1999). No clear phenotype has been ascribed yeast cells lacking either Grx3 or Grx4, but a double Grx3/4 mutant displays cell cycle progression defects, intracellular iron accumulation and sensitivity to certain compounds inducing oxidative stress (Pujol-Carrion, Belli et al. 2006). Recent studies have demonstrated that Grx3 and Grx4 can regulate the localization of the transcription factor activator of ferric transcription (Aft1). Both proteins regulate the nuclear localization of Aft1 and form a functional complex (Ojeda, Keller et al. 2006; Pujol-Carrion, Belli et al. 2006). Grx5 is the only monothiol Grx that shows a specific phenotype in single mutational studies. The absence of Grx5 leads to defects in the iron-sulfur assembly machinery, increased levels of carbonylated proteins and increased sensitivity towards oxidative stress (Rodriguez-Manzaneque, Ros et al. 1999; Rodriguez-Manzaneque, Tamarit et al. 2002).

1.1.6 Mammalian glutaredoxins

At present, four different mammalian Grxs have been identified in the mammalian cell. Two classical dithiol Grxs, Grx1 and Grx2, and two monothiol Grx5 and Grx3 (also known as the PKC-interacting cousin of thioredoxin (PICOT) and thioredoxin-like 2 (TXNL-2)). A schematic picture of the different mammalian Grxs in the cell is shown in Figure 4. The monothiol Grxs can be divided into two different subgroups, depending on their composition of domains. They can exist either with a single Grx domain or with a Trx domain followed by one or several Grx domains (Herrero and de la Torre-Ruiz 2007; Lillig, Berndt et al. 2008).

8

1.1.6.1   Dithiol glutaredoxins

1.1.6.1.1   Glutaredoxin 1

The most well characterized mammalian Grx is Grx1 (official gene name: GLRX1), which is a 12 kDa protein mainly residing in the cytosol. Grx1 from a number of different species, including rat (Axelsson, Eriksson et al. 1978), bovine (Luthman, Eriksson et al. 1979), rabbit (Hopper, Johnson et al. 1989) and human (Mieyal, Starke et al. 1991), show 80 % homology and the conserved Cys-Pro-Tyr-Cys active site. Grx1 from pig contains a tyrosine instead of the phenylalanine in the active site (Gan and Wells 1987; Gan and Wells 1987).

Although the main proportion of Grx1 is localized in the cytosol, the protein can translocate to the nucleus upon certain stimuli (Padilla, Martinez-Galisteo et al. 1992; Rozell, Barcena et al. 1993). The GLRX1 gene is localized on chromosome 5q14, but the gene does not contain any nuclear localization signal and the mechanism for nuclear transport is unknown (Padilla, Bajalica et al. 1996; Park and Levine 1997). Despite of this, the nuclear localisation may play an important role, since *in vitro* studies have shown that Grx1 may regulate several transcription factors, including nuclear factor 1 (NF1), nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (Bandyopadhyay, Starke et al. 1998; Hirota, Matsui et al. 2000; Pineda-Molina, Klatt et al. 2001). Grx1 has also been detected in the mitochondrial intermembrane space, where it was proposed to regulate mitochondrial function by catalyzing reversible S-glutathionylation (Pai, Starke et al. 2007). Detection of Grx1 in the plasma of healthy persons and patients indicates secretion of the protein under both physiological and pathological conditions (Nakamura, Vaage et al. 1998; Lundberg, Fernandes et al. 2004; Wahlgren and Pekkari 2005). The extracellular function of Grx1 is unknown, but Grx1 can reduce glutathione peroxidase 3 (GPX3) residing in the plasma (Bjornstedt, Xue et al. 1994). Besides being secreted, Grx1 can be taken up by neuronal cells. Thus Grx1 is able to pass membranes in both directions by unknown mechanisms (Daily, Vlamis-Gardikas et al. 2001).

Both bovine and human Grx1 can serve as hydrogen donors for RNR. The K_M for bovine Grx1 is lower compared to bovine Trx1 (0.6 µM and 1.8 µM, respectively) (Luthman and Holmgren 1982; Padilla, Martinez-Galisteo et al. 1995). However, neither the tissue distribution of Grx1 and RNR, nor Trx1 and RNR, correlates, suggesting additional electron donors for RNR. Several additional functions have been assigned to Grx1, based on both *in vitro* and *in vivo* experiments. Grx1 can reduce dehydroascorbate to ascorbate (vitamin C) via the monothiol mechanism *in vitro* (Wells, Xu et al. 1990; Washburn and Wells 1999). The ability of Grx1 to deglutathionylate proteins may regulate the activity of several proteins like HIV-1 asparyl protease (Davis, Newcomb et al. 1997), NF1 (Bandyopadhyay, Starke et al. 1998) and NF-κB (Pineda-Molina, Klatt et al. 2001). Both *in vivo* and *in vitro* studies have linked Grx1 to actin polymerisation by reversible glutathionylation (Wang, Boja et al. 2001).

A role of Grx1 in the regulation of apoptosis has been suggested because over-expression of Grx1 prevented cadmium-induced apoptosis (Chrestensen, Starke et al. 2000), Grx1 can act as a negative regulator of apoptosis signal regulating kinase 1 (ASK-1) . Reduced Grx1 can bind to the C-terminal part of ASK-1 and thereby

inhibit the ASK-1's kinase activity and the subsequent signal transduction pathway (Song, Rhee et al. 2002). In Parkinson's disease the dopaminergic neurons degenerate and eventually die, causing movement disorders with symptoms such as rigidity and tremour. Dopamine is a neurotransmittor, but can also be a strong oxidant damaging the cell. Treatment with human Grx1, as well as *E. coli* Grx2, resulted in the protection of dopaminergic neurons against dopamine-induced cell death. The effect was proposed to be based on activation of NF-κB via the intranuclear redox factor 1 (Ref1) (Daily, Vlamis-Gardikas et al. 2001). Treatment of 1–methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) is a widely used animal-model for Parkinson's disease. In the cell MPTP is activated to MPP^+, which inhibits complex I and induces Parkinson's disease like symptoms. Grx1 was suggested to regulate the activity of complex I since downregulation of Grx1 prevented the recovery of complex I after MPTP treatment (Kenchappa and Ravindranath 2003). Results indicating a protective role of Grx1 in the pathogenesis of Parkinson's disease. In a study performed by Saeed et al. the downregulation of Grx1 in Neuro-2a cells resulted in an increase of ROS as well as alteration of mitochondrial membrane potential. These effects were proposed to be induced by oxidation of the thiol-groups in the voltage dependent anion channel (Saeed, Durgadoss et al. 2008). Comparing mRNA levels of Grx1 in tangle bearing neurons versus healthy neurons in brain sections from patients with Alzheimer's disease and control patients demonstrated reduced levels of Grx1 in tangle-bearing neurons (Ginsberg, Hemby et al. 2000). Immunohistochemistry of brains from Alzheimer's patients displayed upregulation of Grx1 in healthy neurons. Overexpression of Grx1 protected neuroblastoma SH-SY5Y cells from amyloid β-induced toxicity. The protective effect was proposed to be mediated by inhibition of the ASK-1 signaling pathway via Daxx (Akterin, Cowburn et al. 2006).
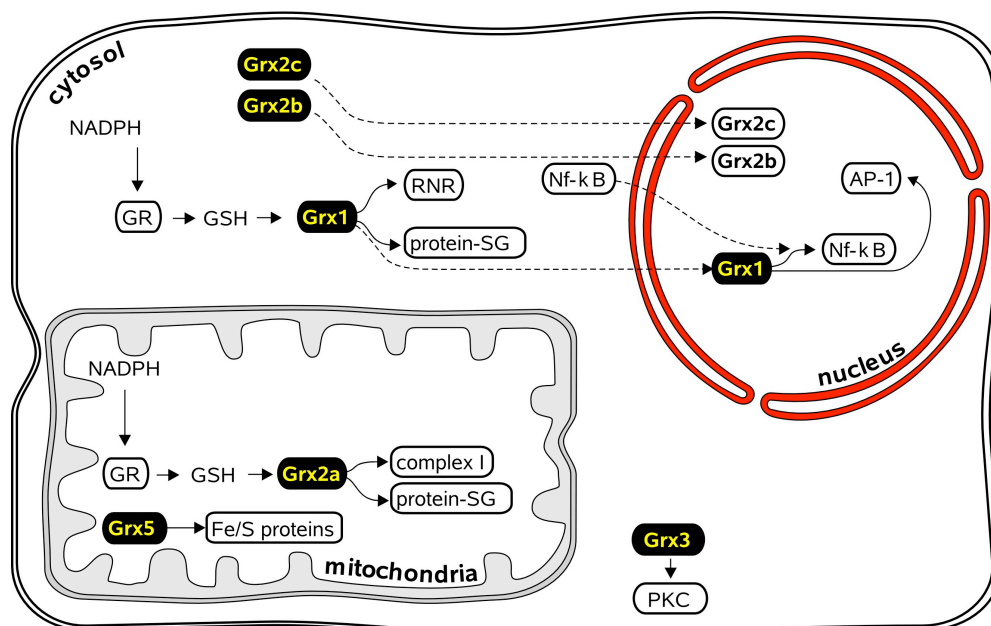
Figure 4. **The mammalian Grxs**. There are four Grxs in the mammalian cell. Grx1 and Grx3 are cytosolic proteins, where Grx1 can reduce disulfides as well as serve as an electron donor for RNR. Grx1 also has the ability to translocate to the nucleus, where it can regulate the activity of certain transcription factors. Three different isoforms of Grx2 exist in either the mitochondria, cytosol and nucleus. Grx5 resides in the mitochondria and a role in the [Fe-S]-biogenesis has been proposed. Grx1 and Grx2 are dithiol Grxs, whereas Grx3 and Grx5 are monothiol Grxs.

10

Knock-out mice with a targeted disruption of the Grx1 gene are viable. Mouse embryonic fibroblasts derived from these mice are more susceptible to cell damage induced by diquat and paraquat and show alterations in the formation and reduction of glutathionylated proteins after treatment with H_2O_2. However, no significant differences were demonstrated after ischemia/reperfusion-induced heart injury or hypoxia-induced lung injury (Ho, Xiong et al. 2007). These findings contradict studies where Grx1 has been proposed to contribute to disease pathology in a mouse model of allergic airway disease (Reynaert, Wouters et al. 2007).

Contradictory results have also been published regarding the role of Grx1 in myocardial ischemia-reperfusion injury. In a different study, knock-out of Grx1 depressed the functional recovery and increased infarct size (Malik, Nagy et al. 2008). Future research will have to address these points.

1.1.6.1.2   Glutaredoxin 2

The second mammalian Grx was identified in 2001 (official gene namne: GLRX2) (Gladyshev, Liu et al. 2001; Lundberg, Johansson et al. 2001). Two different isoforms were proposed, differing in their first exon. Grx2a contains a mitochondrial translocation signal and a nuclear localization was proposed for Grx2b (Lundberg, Johansson et al. 2001). The GLRX2 gene consists of five exons, with two alternative first exons. Exon II-IV encode the Grx core domain including the active site. The human and mouse isoforms of Grx2 will be presented later in this thesis (paper III and IV).

There is only 34 % sequence homology between Grx1 and the mitochondrial Grx2. Yet, Grx2 displays the Trx-fold, with a hydrophobic surface area and GSH binding site, but with a Cys-Ser-Tyr-Cys active site sequence (Gladyshev, Liu et al. 2001; Lundberg, Johansson et al. 2001). The exchange of a serine instead of a proline in the active site sequence results in an increased affinity for glutathionylated proteins (Johansson, Lillig et al. 2004). Glutathionylation of complex I in the mitochondria correlates with increased production of $O_2^{\cdot-}$ (Taylor, Hurrell et al. 2003). Grx2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane proteins, including complex I and may hence be involved in mitochondrial redox regulation (Beer, Taylor et al. 2004). Downregulation of Grx2 in mouse brain resulted in inactivation of complex I activity (Karunakaran, Saeed et al. 2007). Furthermore, Grx2 is not only dependent on GSH for its reduction, but is also a substrate for mammalian TrxR. This might be an important pathway, under conditions when the ratio GSH/GSSG is low, i.e. during oxidative stress (Johansson, Lillig et al. 2004). Results identifying Grx2 as an iron-sulfur cluster protein will be presented in paper II. A protective effect of Grx2 in cardiovascular disorder has been proposed. Myocardial overexpression of the mitochondrial Grx2 protects cardiac cells from ischemia and reperfusion induced cell death. Increased levels of Grx2a preserved a higher GSH/GSSG ratio and prevented the loss of cardiolipin, cytochrome c release and activation of caspases (Nagy, Malik et al. 2008).

1.1.6.2 Monothiol glutaredoxins

1.1.6.2.1 Grx3/PICOT

Grx3 or PICOT for PKC-interacting cousin of thioredoxin (official gene name GLRX3) was identified in two hybrid analysis aiming for the identification of novel protein kinase C θ (PKCθ) interacting proteins. The protein consists of a N-terminal Trx domain, lacking the C-terminal active site cysteine followed by two repeats of highly conserved monothiol Grx domains, also named PICOT-homology domains with the Cys-Gly-Phe-Ser active site (Isakov, Witte et al. 2000; Witte, Villalba et al. 2000). Involvement of PICOT in cardiac hypertrophy has been demonstrated. PICOT was upregulated upon hypertrophic stimuli and a cardiac specific overexpression in mice inhibited cardiac hypertrophy induced by pressure overload (Jeong, Cha et al. 2006). When chromatin immunoprecipitation was used to identify targets regulated by serum response factor (SRF), Grx3 was verified as a target. SRF is a transcription regulator which recruits accessory factors for the fos promotor and is essential for development of mesoderm tissue. Since these studies were performed in a cardiac cell model, identification of Grx3 as a target for SRF implies a role in embryonic formation of cardiac tissue (Zhang, Garcia-Gras et al. 2005).

1.1.6.2.2 Glutaredoxin 5

Human Grx 5 (official gene name GLRX5) is a homologue of yeast Grx5 and was henced named Grx5 although no Grx4 has been identified in human cells so far (Molina-Navarro, Casas et al. 2006). In *S. cerevisae*, Grx5 is essential for the mitochochondrial biogenesis of [Fe-S]-clusters (Rodriguez-Manzaneque, Tamarit et al. 2002). Homologues of this monothiol, single Grx domain, exist in all eukaryotic cells. The human gene contains a predicted mitochondrial targeting sequence and a mouse homologue has been found inside the mitochondria in a proteomic study (Mootha, Bunkenborg et al. 2003). A zebrafish mutant, called shiraz, suffering from hyperchromic anemia and defective hemoglobin production, was caused by the loss of Grx5 (Wingert, Galloway et al. 2005). A similar condition was described in human and a mutation in the Grx5 gene leading to decreased levels of Grx5 was demonstrated as the cause (Camaschella, Campanella et al. 2007). The human protein can rescue yeast Grx5 mutants as well as the shiraz phenotype.

1.1.7 Proteins related to glutaredoxins

1.1.7.1 Glutathione-S-transferases

GSTs constitute a multi-gene family of enzymes involved in the cell's defence against toxic compounds, due to their ability to conjugate GSH to various electrophiles (Strange, Spiteri et al. 2001). The addition of GSH forms a conjugate which usually is less reactive and more water soluble and hence more easily eliminated from the cell (Armstrong 1997). GSTs have the ability to conjugate GSH to a wide variety of chemical structures, and important substrates are polycyclic aromatic hydrocarbon epoxides as well as several by-products of oxidative stress

(Strange, Spiteri et al. 2001). GSTs can also conjugate GSH to endogenous substances like leukotriene A4 hydrolase and prostglandin A1 (Christ-Hazelhof, Nugteren et al. 1976; Bach, Brashler et al. 1984). There are several cytosolic isoforms of GSTs and one membrane bound microsomal GST. The microsomal GST differs from the cytosolic isoform by being a trimer, instead of a dimer (Morgenstern, DePierre et al. 1985). The different isoforms can be divided into seven distinct groups based on their biochemical and immunological properties: alpha, pi, theta, zeta, sigma, kappa and omega (Strange, Spiteri et al. 2001). The omega class GSTs can form a disulfide with GSH and show low activity towards classical GST substrates. However, proteins from this GST family show activity in reactions typical for Grxs, like the reduction of GSH mixed disulfides. The omega class of GSTs is structurally similar to *E. coli* Grx2 (Board, Coggan et al. 2000). Together, these proteins form a bridge between these two classes of GSH-utilizing enzymes, the GSTs and the Grxs. Crystal structures of several cytosolic GSTs have been solved. Even though there is a general low sequence identity across the various classes, they do follow a similar fold, consisting of two domains. The structure of the first domain follows the Trx-fold, with four β-strands and three α-helices. This is a highly conserved domain, which provides the GSH binding surface. The second domain consists of various numbers of α-helices. The big variation of the structure in this domain might reflect the different substrate specificities (Dirr, Reinemer et al. 1994).

1.1.7.2 Glutathione peroxidases

GPX was the first selenoprotein identified in mammals (Flohe, Gunzler et al. 1973). Using GSH as reducing agent, GPX reduces H_2O_2, organic peroxide as well as lipid peroxides, thereby protecting the cell from oxidative stress. Five mammalian selenium-containing GPXs have been identified (GPX I-IV,VI), as well as one non-selenium GPX (GPXV). GPXV has a Cys-residue instead of a Sec and is less reactive compared to other GPXs (Vernet, Rigaudiere et al. 1996). The different GPXs differ in their cellular distribution. GPXI and GPXII are cytosolic proteins, whereas GPXIII and GPXV are extracellular proteins (Brigelius-Flohe 1999). GPXIV is a phosphlipid peroxide, that prevents lipidperoxydation by reducing membrane bound hydroperoxides (Ursini, Maiorino et al. 1982; Ursini, Maiorino et al. 1985; Brigelius-Flohe 1999). GPXI^{-/-} knock-out mice are viable, but are sensitized to paraquat induced oxidative stress (Cheng, Ho et al. 1998). The reason for this higher sensitivity is the protection of lipids and proteins from oxidative destruction (Cheng, Fu et al. 1999). GPX harbour, like GSTs, a Trx-fold domain and a GSH binding site (Martin 1995).

1.1.7.3 Peroxiredoxins

Prx was first identified in yeast as a 25 kDa enzyme involved in the protection against oxidative stress (Kim, Kim et al. 1988). Since the enzyme reduced H_2O_2 supported by a variety of thiols, but not by other reductants like ascorbic acid, it was named thiol-specific antioxidant (TSA) (Chae, Robison et al. 1994). When Prx reduces H_2O_2 an intermolecular disulfide is formed. Trx, together with TrxR can reduce this disulfide. To reflect Trx as electron donor the enzyme was renamed to thioredoxin peroxidase. The protein family, including members which are not reduced by Trx, was named

Prxs (Chae, Chung et al. 1994). Prxs are present in organisms from all kingdom of life and exist in multiple isoforms in eukaryotic cells. Depending on the number of conserved Cys-residues, Prx can be divided into two groups,: 2-Cys Prx, containing a N-terminal as well as a C-terminal cysteine and 1-Cys Prx which only contain the N-terminal cysteine (Chae, Robison et al. 1994; Kang, Baines et al. 1998). There is also an atypical Prx, which contains the more N-terminal cysteine, but require an additional non-conserved cysteine for its activity. Mammalian Prx 1-IV are 2-Cys Prxs, PrxV is an atypical 2-Cys Prx and PrxVI is the only mammalian 1-Cys Prx. Prx I-IV exist as a homo-dimer, arranged in a head-to-tail fashion. When Prx reduces H_2O_2, the N-terminal Cys-residue is oxidized to sulfenic acid, which in turn reacts with the C-terminal Cys-residue of the second Prx molecule, forming an intermolecular disulfide. In Prx V the sulfenic acid of the N-terminal Cys-residue, reacts with a second Cys-residue in the same molecule, forming an intramolecular disulfide bond. The exact mechanism for Prx VI is not yet clear (Rhee, Kang et al. 2001). The six different Prxs are distributed in various compartments in the cell. PrxI, II and VI are cytosolic, PrxIII resides in mitochondria and PrxIV has been identified in endoplasmatic reticulum and extracellularly. PrxV can be found in the cytosol, peroxisomes and mitochondria (Rhee, Kang et al. 2001). Grxs have been identified as possible reductants of plant Prxs *in vitro* (Rouhier, Gelhaye et al. 2002).

1.1.7.4   Protein disulfide isomerase

In 1963 PDI was isolated from liver and its ability to catalyze the refolding of ribonuclease A via rearranging disulfides was characterized (Goldberger, Epstein et al. 1963; Goldberger, Epstein et al. 1964). The structure of PDI contains four domains with Trx-fold, where only two of the domains hold the Cys-Xxx-Xxx-Cys motif. A disulfide formed in the active site can be reduced by TrxR *in vitro* (Lundstrom and Holmgren 1990). PDI acts as a thiol-disulfide isomerase using a Cys-Gly-His-Cys active site sequence. The bulk of PDI is localized in the endoplasmatic reticulum and PDI contains both a signal sequence and an endoplasmatic reticulum retention signal. However, PDI has been demonstrated at the plasma membrane, in the cytosol and nuclei (Turano, Coppari et al. 2002). The primary function of PDI is to catalyze oxidation and isomerisation of disulfide bonds, thus assisting in protein folding. Additional functions like ER-associated degradation, trafficking, calcium homeostasis, antigen presentation and virus entry have been proposed (Appenzeller-Herzog and Ellgaard 2008).

1.2   IRON-SULFUR CLUSTERS

Iron-sulfur centers exist in all forms of life and are cofactors to a wide range of proteins, where they can undergo reversible redox reactions, determine protein structure and act as catalytic centers. Several iron-sulfur cluster proteins are involved in the mitochondria respiratory chain, for instance. In proteins, iron-sulfur clusters are usually coordinated by cysteine residues, but also by histidine or other amino acid residues. However, as outlined below, iron is highly reactive and can generate hydroxyl radicals by the Fenton reaction. Therefore, cells are equipped with a complex system for the biosynthesis of [Fe-S]-clusters to avoid high levels of redox free iron. To control

the iron status in the cell organisms have evolved a number of regulatory circuits. In mammals, the levels of iron-uptake and iron-binding proteins are regulated by iron regulatory protein (IRP) 1 and 2. When the levels of free iron in the cell decrease, IRPs bind to hairpin structures present on the mRNA of IRP regulated genes called the iron regulatory elements (IRE). Depending on whether the IRPs bind on the 5'UTR or 3'UTR, the mRNA translation is repressed or stabilized, respectively (Hentze, Muckenthaler et al. 2004). Dysregulation of iron has been implied in several pathological conditions such as Alzheimer's disease (Adlard and Bush 2006; Magaki, Raghavan et al. 2007), Friedrich ataxia (Wilson 2006) and Parkinson's disease (Bharath, Hsu et al. 2002).

1.2.1 Iron-sulfur cluster biogenesis

Three different systems for the biosynthesis of [Fe-S]-clusters exist: the Isc-, the Nif- and the Suf-system. In mammals the isc-system is present (Muhlenhoff, Gerber et al. 2003). Even though iron-sulfur proteins exist in various compartments of the cell, mitochondria are essential for the maturation of cytosolic and nuclear located [Fe-S]-proteins as well. Moreover, it was shown that localization of the iron-sulfur biosynthesis in mitochondria is the primary reason why this organelle is essential. The core machinery consists of a still unknown protein providing iron, a cysteine desulfurase providing sulfide and a scaffold protein assembling and transferring the newly synthesized cofactors. For the last step, the transfer of the cluster to an apo-protein, the help of chaperones of the DnaK and DnaJ type is required (Lill and Muhlenhoff 2006). Depletion of Grx5 in yeast resulted in iron accumulation, inactivation of [Fe-S]-proteins and an increase of the levels of iron loaded scaffold proteins. Overexpression of the chaperones complemented these effects. These results indicate a crucial role of Grx5 during iron-sulfur biosynthesis, especially during the insertion of the cofactor into an apo-protein (Muhlenhoff, Gerber et al. 2003).

1.3   OXIDATIVE STRESS AND REACTIVE OXYGEN AND NITROGEN SPECIES

Cells constantly produce reactive oxygen species (ROS) as by-products of their normal metabolism and sometimes even intentionally. The major endogeneous contributor of ROS is the respiratory chain in the mitochondria, especially complex I, where electrons leak out from the electron transport chain causing partial reduction of oxygen (Droge 2002). In the cytosol enzymes like cytochrome p-450, xantine oxidase and cyclooxygenase generate ROS. Exogenous sources such as redox active chemicals and UV radiation can also induce ROS formation. The major ROS derivate is the superoxide anion ($O_2^{\cdot-}$), which in the cell is quickly disproportionated by superoxide dismutase (SOD) to H_2O_2 and O_2 (McCord and Fridovich 1969). H_2O_2 can cross membranes and is relatively unreactive. However, in the presence of reduced transition metals such as Fe^{2+}, it can be reduced to the very reactive hydroxyl

radical (OH$^\bullet$) in a reaction called the Fenton reaction (McCord and Day 1978), equation 3.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^- \qquad \text{(equation 3)}$$

Due to the presence of an unpaired electron in the valence shell, radicals are very reactive. ROS can damage basically all cellular structures, i.e. proteins, lipids and DNA (Beckman and Ames 1998). When ROS react with a biomolecule, a chain reaction of radical formation is initiated. To be able to stop the reaction two radicals must react, eliminating the unpaired electrons, or one radical has to react with a radical scavenger terminating the reaction. To be able to cope with the constant production of ROS, the cell has developed several systems which are able to detoxify ROS. Non-enzymatic antioxidants like vitamin C, vitamin E and thiol antioxidants like GSH or enzymes like SOD, catalase or GPXs, as well as members of the Trx and Grx family of proteins prevent ROS-induced cell damage. Under normal conditions there is a fine balance between ROS and the antioxidant systems. A classical definition of oxidative stress is the damage that occur when this balance is disturbed, either by an excess production of ROS or insufficient antioxidant protection. But, since the redox status cannot be seen as a single entity, new concepts of oxidative stress are emerging. A better definition is an imbalance in specific redox circuits (Jones 2006). Oxidative damage caused by ROS has been linked to several pathological conditions such as cancer, atherosclerosis and neurodegenerative diseases (Andersen 2004). Furthermore, ROS-induced damage has been suggested to participate in ageing (Cadenas and Davies 2000). However, ROS are not always harmful. In low concentration ROS can play a regulatory role and several cytokines, growth factors, hormones and neurotransmitters use ROS as a second messenger in cell signaling or in signal transduction mechanisms for apoptosis (Jacobson 1996; Finkel 1998). Nitric Oxide (NO) is a gas synthezised from L-arginine and serves as a second messenger. Since NO is a very small molecule it can easily penetrate membranes. When NO reacts with ROS a series of reactive nitrogen species (RNS) can be formed. One example is the reaction between NO and $O2^{\bullet\bullet}$ generating peroxynitrite (ONOO$^-$). Irreversible modification of tyrosine residues through nitration with ONOO$^-$ has been implied in physiological and pathological signaling (Turko and Murad 2002).

1.3.1 Modifications of thiols

The thiol group of cysteines is sensitive to both ROS and RNS. In an oxidized environment thiol groups can be modified to disulfide bonds, sulfenic (-SOH), sulfinic (-SO$_2$H) and sulfonic (-SO$_3$H) acid. Oxidation to disulfide bonds and sulfenic acid are regarded as reversible modifications, whereas sulfinic and sulfonic acid formation is irreversible and with the exception of the sulfiredoxin/peroxiredoxin redox couple these modifications cannot be reduced by biological reductants under physiological conditions (Biteau, Labarre et al. 2003; Woo, Chae et al. 2003). Disulfide bonds can be intra- as well as intermolecular. Glutathionylation is an intermolecular mixed disulfide between a protein thiol and GSH. Since this is a reversible modification, it was proposed that it may protect certain thiols from irreversible oxidation (Cotgreave and Gerdes 1998; Linke and Jakob 2003; Biswas, Chida et al. 2006). Today, the function of glutathionylation goes beyond a protective modification, and the concept of glutathionylation as a redox regulatory switch is

16

strongly emerging. Glutathionylation can affect proteins in several ways. It can, for instance, inhibit enzymes and transcription factors (Pineda-Molina, Klatt et al. 2001; Shenton and Grant 2003). Glutathionylation activates the Sarco/endoplasmatic reticulum Ca^{2+}-ATPase (SERCA) transporter (Adachi, Weisbrod et al. 2004), and glutathionylation on Cys 95 of HIV-1 protease inhibits the enzyme, whereas glutathionylation of Cys 67 stabilizes the protein (Davis, Dorsey et al. 1996). Glutathionylation on active site cysteines, is not the only mechanism to target substrates activity. Glutathionylation can also change the physico-chemical properties, e.g. the isoelectric point of a protein or create a steric hindrance on the surface of the target protein (Klatt, Molina et al. 1999; Wang, Boja et al. 2001; Cao, Kambe et al. 2005). Grxs efficiently catalyze both glutathionylation as well as the reversed reaction called deglutathionylation (Gravina and Mieyal 1993; Lind, Gerdes et al. 1998)

The most thoroughly characterized modification of thiols induced by NO, e.g. after treatment with NO-donors like S-nitrosoglutathione (GSNO), is called S-nitrosylation. S-nitrosylation is a reversible modification where a nitrosothiol is formed and can regulate ion channel activitiy, enzyme catalysis and cofactor binding (Hess, Matsumoto et al. 2005). The exact mechanism for S-nitrosylation *in vivo* is a matter of debate, but a mechanism where a thiolate anion reacts with NO in the presence of an electron acceptor has been proposed (Gaston, Carver et al. 2003). Nitrosylation can also be observed after treatment with S-nitrosoglutathione (GSNO) (Padgett and Whorton 1995). NO is involved in protein glutathionylation in several ways. Glutathionylation is proposed as a mechanism for denitrosylation, where a nitrosylated protein reacts with GSH to form a glutathionylated protein (West, Hill et al. 2006). Glutathionylation also occurs via an $ONOO^-$-dependent pathways, where either the cysteine residue or GSH is oxidized to oxy-sulfenic acid (Radi, Beckman et al. 1991), which subsequently form a mixed disulfide between GSH and the protein (Poole, Karplus et al. 2004). Furthermore, protein thiols can be either specifically nitrosylated or gluthionylated by GSNO (Padgett and Whorton 1995; Mohr, Hallak et al. 1999).

1.3.2 Modifications of human glutaredoxins

Apart from the active site cysteines, human Grxs contain additional cysteines. Human Grx1 contains three additional Cys-residues (Cys 8, Cys 79 and Cys 83). Neither of these cysteines are necessary for its activity, but their redox state influence the enzymatic activity of the protein (Yang, Jao et al. 1998; Hashemy, Johansson et al. 2007).
Grx1 and Grx2 show remarkable differences upon oxidation. Grx1 is inhibited by treatment with H_2O_2 and GSSG (Starke, Chen et al. 1997), whereas Grx2 remains active after GSSG treatment (Lundberg, Johansson et al. 2001). Oxidation of Grx1 by GSSG induced both inter- and intramolecular disulfide bonds. Cys 79 and Cys 8 can form an intramolecular bond and disulfides between Cys 8 and either Cys 79 or Cys 83 lead to homodimers or higher oligomers. Grx2, on the other hand, does not form any intermolecular bonds upon GSSG treatment. Furthermore, opposite to Grx2, Grx1 is inhibited by nitrosylation (Hashemy, Johansson et al. 2007). Human Grx2 contains two additional Cys-residues (Cys 28 and Cys 113). These two non-active site thiols form a disulfide bond even under most reducing conditions, contributing to the overall stability of the protein (Sagemark, Elgan et al. 2007).

2 PRESENT INVESTIGATION

2.1 AIM OF STUDY

Human Grx2 was identified in 2001 as a mitochondrial homologue of the cytosolic Grx1. At first, two different isoforms of human Grx2 were proposed, with mitochondrial (Grx2a) and nuclear (Grx2b) localization, respectively. The mouse gene encoded an isoform corresponding to mitochondrial Grx2, but no equivalent to human Grx2b. However, additional studies suggested additional isoforms for mouse Grx2 as well. Thus, the specific aims of this study were:

- To study the role of human Grx2 in cellular models of oxidative stress-induced cell death.

- To biochemically and biophysically characterize the recombinant protein.

- To identify isoforms of human and mouse Grx2.

- To characterize the different isoforms of human and mouse Grx2 with respect to subcellular localization, enzymatic activity and expression pattern.

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

siRNA-mediated silencing of glutaredoxin 2 increases the sensitivity of HeLa cells towards doxorubicin and phenylarsine oxide

The introduction of double stranded RNA (dsRNA) can be utilized to induce specific degradation of mRNA homologous to the sequence of the delivered dsRNA (Fire, Xu et al. 1998; Elbashir, Harborth et al. 2001). Since we were interested to learn about *in vivo* function of human Grx2, we established a short interfering RNA approach to knock-down Grx2 expression in the cervix cancer cell line HeLa. We were able to decrease the Grx2 levels to below 3 % of the original levels. The knock-down of Grx2 did not influence the cell viability when the cells were propagated under optimal conditions. No changes in the levels of carbonylated proteins, which is a possible modification of proteins during oxidative stress, were detected. However, cells depleted of Grx2 were sensitized dramatically towards the anti-cancer drug doxorubicin and phenylarsine oxide (PAO). The ED_{50} dropped from 40 to 0.7 µm and 200 to 5 nM, respectively. PAO attacks vicinal dithiols, targeting both Trxs and Grxs and especially TrxR. Doxorubicin induces oxidative stress by redox cycling with mitochondrial complex I producing superoxide. Treatment with cadmium, a known inhibitor of Grx1, did not result in any significant differences. The results indicate a prominent role for Grx2 in defence against apoptosis induced by oxidative stress.

2.2.2 Paper II

Characterization of human glutaredoxin 2 as new iron-sulfur protein: a possible role as redox sensor

We have purified large quantities of recombinant human Grx2 for biochemical analysis and surprisingly found the protein to contain a brownish chromophore. When applied to gel filtration chromatography, the purified protein solution separated into two fractions: one colorless fraction of approximately 17 kDa and a brownish fraction of approximately 34 kDa. When the fractions were applied to reducing SDS page, both fractions resulted in bands of 16 kDa, identified as Grx2 by Western blotting. The spectral properties of the high molecular weight fraction indicated an iron-sulfur cluster. Mössbauer spectroscopy revealed a $[2Fe-2S]^{2+}$-cluster bridging the holo-dimer. Mutational analysis suggested that the Cys 28 and Cys 113 residues, i.e. the two non-active site cysteines, were the residues coordinating the [2Fe-2S]-cluster. The holo-enzyme was enzymatically inactive as oxidoreductase using glutathionylated RNase and a mixed disulfide between GSH and 2-mercaptoethanol as substrates. Treatment with both reductants like dithionite and oxidants like ferricyanide resulted in monomerization and enzymatic activation of the protein. GSSG treatment resulted in the loss of the chromophore, whereas GSH stabilized the cluster. Co-immunoprecipitation of Grx2 and Fe in a Burkitt lymphoma cell line and HeLa cells strongly advocated for the existence of the cluster *in vivo*. We therefore proposed a role as a redox-sensor for the cluster. In resting cells the majority of Grx2 is present in the inactive dimeric form. Changes of the intracellular redox state and thereby of the GSH/HSSG ratio may lead to monomerization and hence activation of Grx2.

2.2.3 Paper III

Expression pattern of human glutaredoxin 2 isoforms: Identification and characterization of two testis/cancer cell-specific isoforms

When human Grx2 was identified in 2001, two different isoforms were suggested: a mitochondrial (Grx2a), and a proposed nuclear (Grx2b) isoform. Here, we have analyzed the expression pattern of these isoforms in various human tissues and cancer cell lines. Grx2a-encoding mRNAs were identified in all tissues and cell lines analyzed. Grx2b expression, on the other hand, was restricted to testis and approximately 60 % of the cancer cell lines. In addition, a new third isoform was identified, named Grx2c. Grx2c was derived from alternative splicing of Grx2b and also exclusively present in testis and cancer cell lines. The Grx2c protein is essentially identical to processed, mature Grx2a. Grx2a, Grx2b and Grx2c are enzymatically active, but only Grx2a and Grx2c can hold the [2Fe-2S]-cluster. Using TrxR as an electron donor, Grx2b reduced GSSG with about half the specific activity of Grx2c. The subcellular localization of all isoforms was analyzed using both N- and C-terminally GFP-tagged proteins as well as HeLa cells overexpressing the different isoforms without tags. The results confirmed the mitochondrial localization of Grx2a. However, no exclusive nuclear localization could be confirmed for Grx2b. Instead, both Grx2b and Grx2c, displayed a more diffuse staining, indicating both nuclear and cytosolic staining. The expression of Grx2b and Grx2c in testis and transformed cell lines indicate potential functions in cellular differentiation and tumorgenesis.

2.2.4 Paper IV

Identification, expression pattern and characterization of mouse glutaredoxin 2 isoforms

In 2003 Jurado et al confirmed the ubiquitous expression of mouse Grx2a and proposed the existence of additional isoforms in mouse. Based on bioinformatical screening of expressed sequence tag database, we screened the mouse transcriptome for additional isoforms. RT-PCR and rapid amplification of cDNA ends experiments were performed to confirm in total five different transcript variants encoding three different proteins: (1) mitochondrial Grx2a. (2) mouse Grx2c resembles human Grx2c, but is derived from different transcript variants. (3) Grx2d, encoded by a transcript variant containing an additional single casette exon. Grx2c was enzymatically active and able to donate electrons to RNR and mixed disulfides of GSH and 2-mercaptoethanol. Grx2d, on the other hand, completely lacked enzymatic activity. Grx2a was expressed in all tissues, but testis, whereas Grx2d was exclusively expressed in testis. Grx2c-encoding transcripts were identified in most tissues investigated, in particular in testis. By using immunofluorescence techniques cytosolic staining of Grx2 could be detected in specific cells of testis, spleen and stomach, confirming the presence of the non-mitochondrial isoform.

3 CONCLUSIONS

- Knock-down of Grx2 sensitized HeLa cells towards doxorubicin and phenylarsine oxide, indicating a crucial role of Grx2 in protection against oxidative stress-induced cell death (paper I).
- Grx2 was identified as the first [Fe-S]-protein of the Trx family. It can coordinate a $[2Fe-2S]^{2+}$-cluster in a dimeric holo-complex. In contrast to most other [Fe-S]-proteins, holo-Grx2 is enzymatically inactive (paperII)
- Oxidation as well as single electron reduction of the cluster induces dissociation of the holo complex generating enzymatically active monomeric apo-Grx2. Hence a role as a redox-sensor for the [Fe-S]-cluster has been proposed (paper II).
- Three different isoforms of Grx2 have been identified in human. Mitochondrial Grx2a is ubiquitously expressed and cytosolic/nuclear Grx2b and Grx2c are testis/cancer specific isoforms (paper III).
- Grx2b is constitutively active with half the specific activity compared to Grx2c using TrxR as electron donor (paper III).
- Mouse contains three different isoforms (Grx2a, Grx2c and Grx2d), derived from five transcript variants. Mitochondrial Grx2a is expressed in all tissues but testis. Cytosolic Grx2c and Grx2d are expressed in testis. Grx2c is also present in specific cells of several other organs (paper IV).
- Mouse Grx2c has general Grx-activity and can reduce RNR. Grx2d, on the other hand, lacks enzymatic activity (paper IV).
- No homologue of human Grx2b is present in mouse and no homologue of mouse Grx2d is present in human (paper III and IV).

4 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The general goal of this thesis was to investigate the *in vivo* function and expression of mammalian Grx2. Grx1 has been extensively studied and possible functions in protection against oxidative stress, regulation of transcription factors and apoptosis have been suggested. To study the role of Grx2 in cells, siRNA was used to knock down the levels of Grx2. The knock-down of Grx2 did not affect the cells *per se*, but when they were challenged with doxorubicin and phenyarsine oxide dramatic effects were seen. The sensitivity of cells against these ROS inducing agents increased up to 40 times when Grx2 levels were reduced. Additional studies, using cells overexpressing Grx2 demonstrated the opposite effect. Increased levels of Grx2 protected the cells against apoptosis induced by 2-deoxy-D-glucose and doxorubicin, by preventing cytochrome c release and cardiolipin oxidation (Enoksson, Fernandes et al. 2005). A protective effect of Grx2 against various forms of challenges has been demonstrated (Karunakaran, Saeed et al. 2007; Nagy, Malik et al. 2008). Grx2 catalyzes reversible glutathionylation of several mitochondrial membrane proteins, including the 75 kDa and 51 kDa subunit of complex I. Since cleavage of the 75 kDa subunit is an early event in apoptosis, a role of Grx2 catalyzed glutathionylation of complex I in apoptosis was proposed (Beer, Taylor et al. 2004). Further studies to unravel the molecular mechanism of the protective function of Grx2 are necessary.

Grx2 was the first protein of the Trx-family to be identified as an iron-sulfur protein. In principle, Trx-fold proteins are able to coordinate metal cofactors in three ways (Lillig 2009): (i) by additional cysteines, (ii) replacement of the *cis*-proline, (iii) and as in the model we described for Grx2. In our early studies molecular modeling indicated that the active site cysteines and the two additional cysteines were located on opposite sides of the protein, offering two possible cofactor coordination sites. By mutational studies the two additional cysteines were proposed as ligands at first. Today we know that these cysteines form a structural disulfide and that the loss of the cluster in mutants missing one of these cysteines is caused by instability of the overall protein. Further mutational studies revealed that the two N-terminal active site cysteines of the two Grx2 molecules together with two GSH-molecules coordinate the cluster (Berndt, Hudemann et al. 2007). This model easily explained the inactivity of the dimer and the stabilization of the holo complex by GSH and was confirmed by the crystal structure of the dimeric holo enzyme (Johansson, Kavanagh et al. 2007). The GSH coordinating the cluster is in constant exchange with free GSH, explaining the stabilization of the cluster by GSH (Berndt, Hudemann et al. 2007). The lack of the proline of the active site is essential for formation of the holo complex. Exchanging the active site of Grx1, to the Cys-Ser-Tyr-Cys active site of Grx2, permitted Grx1 to bind a [Fe-S]-cluster (Berndt, Hudemann et al. 2007). The GrxC1 from poplar, containing a Cys-Gly-Tyr-Cys-active site, has also the ability to incorporate a [Fe-S]-cluster in essentially the same manner as Grx2 (Feng, Zhong et al. 2006; Rouhier, Unno et al. 2007). Recent data demonstrate that many monothiol Grxs with the active site Cys-Gly-Tyr-Ser motif are able to bind a [Fe-S]-cluster in the same way (Picciocchi, Saguez et al. 2007; Mesecke, Mittler et al. 2008). We proposed that the cluster serves as redox sensor (Fig. 5). Under oxidizing

22

conditions when the GSH/GSSG ratio is decreased, the cluster is disassembled and Grx2 turns into an active monomer. Under these conditions electrons can be received from TrxR. Since monothiol Grxs are suggested to be involved in iron metabolism and iron-sulfur cluster biosynthesis, a role in transfer of the cluster from the scaffold proteins to apo-proteins has been proposed (Bandyopadhyay, Gama et al. 2008).
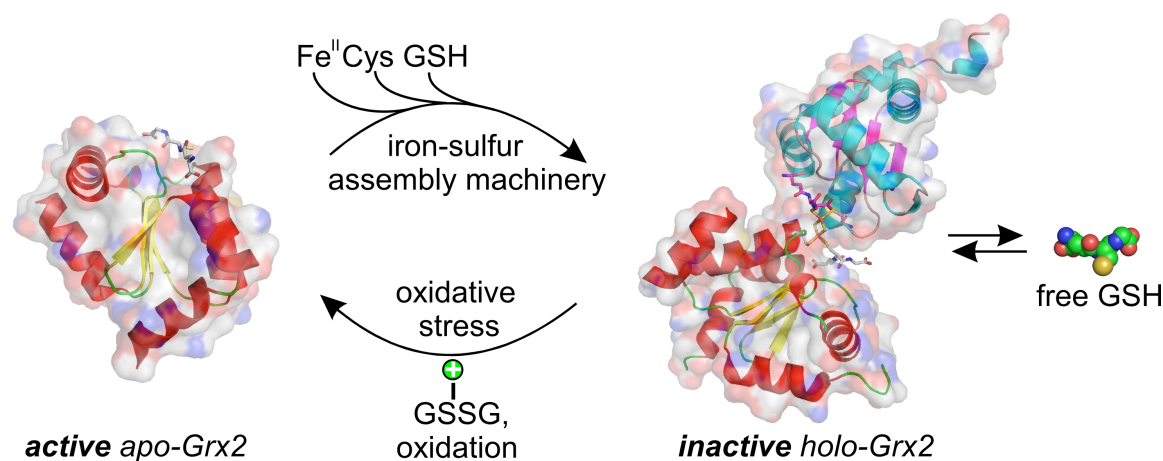

Figure 5. **The cluster of Grx2 as a redox sensor**. The cluster is coordinated by the two N-terminal active site Cys-residues of two Grxs and two molecules of GSH. The holo-protein is inactive, but upon oxidative conditions the dimer monomerizes generating active Grx2.

Three different isoforms of Grx2 exist in human. Mitochondrial Grx2a is ubiquitously expressed demonstrating its general importance. Grx2b and Grx2c are restricted to testis and transformed cells. The environment of the testis is special, with respect to oxygen availaibility as well as the high degree of proliferation, conditions similar to the ones for cancer cells. A whole gene-family restricted to testis and cancer has been described (Zendman, Ruiter et al. 2003). A splice-variant of TrxR1 containing a N-terminal Grx domain is, although not strictly restricted to, predominantly expressed in testis. Similar to the testis/cancer specific isoforms of Grx2, this splice variant was also identified in several cancer cell lines. Treatment with estradiol and testosterone induced the expression of this transcript variant in HeLa cells (Dammeyer, Damdimopoulos et al. 2008). In contrast, no significant induction of transcription by treatment with steroid sex hormones was found for the trancript variants encoding Grx2b and Grx2c. Understanding the function of Grx2b and Grx2c and their involvement in transformation is of great interest. The generation of transgenic mice expressing Grx2a and Grx2b will provide a powerful tool, to understand the isoform's specific functions.

Mice do not contain a counterpart to human Grx2b, but five different transcript variants encode three different isoforms. Grx2a corresponds to the mitochondrial Grx2a in human and is expressed in all tissues, apart from testis. Grx2c is mainly expressed in

testis, but also in other tissues. Grx2d, a mouse specific isoform, is localized in testis and lacks enzymatic activity. The identification of two additional isoforms is in agreement with the studies performed by Jurado et al, which suggested additional isoforms of mouse Grx2 (Jurado, Prieto-Alamo et al. 2003). Both mouse and human contain a testis specific isoform of Grx2. However, Grx2c in mouse is not restricted to testis, as it is in human. The reason for the more general distribution of a cytosolic Grx2 in mouse is at this point not clear. It is tempting to speculate that this cytosolic Grx2 acts as a back up for Grx1, since mice with a target disruption of the Grx1 gene are viable (Ho, Xiong et al. 2007; Malik, Nagy et al. 2008). Further studies are needed to unravel the specific functions of the different isoforms of Grx2 in mouse.

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32

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