REGULATION OF HUMAN THIOREDOXIN AND GLUTAREDOXIN SYSTEMS BY OXIDATION AND S-NITROSYLATION

Seyed Isaac Hashemy

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To my family
ABSTRACT

The homeostasis of intracellular redox status has a crucial role in the cell survival and different processes such as DNA synthesis, gene expression, enzymatic activity, etc. This homeostasis is achieved via an intense balance between pro-oxidants and anti-oxidants. Among anti-oxidants, there are two enzymatic systems which are also the main intracellular general protein disulfide reductases, the thioredoxin (Trx) and glutaredoxin (Grx) systems. The Trx system contains Trx, Trx reductase (TrxR) and NADPH, while the Grx system is composed of glutathione (GSH), glutathione reductase (GR), Grx and NADPH. The upregulation of Trx, TrxR, and Grx have been reported in various tumor cells that are directly correlated to the resistance to chemotherapy, aggressive tumor growth and poor prognosis. Therefore, these two systems are potential targets for anti-tumor therapy as well as some other clinical applications. Hence, it is important to know how the activity and structure of these proteins are affected via chemical compounds or by post-translational modifications.

Motexafin gadolinium (MGd), a new chemotherapeutic drug, was an NADPH oxidizing substrate for mammalian TrxRs with a Km-value of 8.65 µM (kcat/Km of 4.86 × 10⁴ M⁻¹ s⁻¹). The reaction involved redox cycling of MGd by oxygen producing superoxide and hydrogen peroxide. MGd acted as a non-competitive inhibitor (IC₅₀ of 6 µM) for rat TrxR. MGd was a better substrate (kcat/Km of 2.23 × 10⁵ M⁻¹ s⁻¹) for TrxR from E. coli and a strong inhibitor of Trx-dependent protein disulfide reduction. Direct reaction between MGd and reduced Trx from either human or E. coli was negligible. Ribonucleotide reductase (RNR) is an essential enzyme for DNA synthesis. MGd inhibited recombinant mouse RNR activity with either 3 µM reduced human Trx (IC₅₀ 2 µM) or 4 mM dithiothreitol (IC₅₀ 6 µM) as electron donors. Our results may explain the effects of the drug on cancer cells, which often overproduce TrxR and have induced RNR for replication and repair.

Besides two cysteines in the active site, human cytosolic Grx1 and mitochondrial Grx2 contain three and two additional structural cysteines, respectively. We analyzed the redox state and disulfide pairing of Cys residues upon GSSG oxidation and S-nitrosylation. Grx1 was partly inactivated both by S-nitrosylation and oxidation. Inhibition by nitrosylation was reversible under anaerobic conditions; aerobically it was stronger and irreversible, due to nitration. Oxidation of Grx1 induced a complex pattern of disulfide bonded dimers and oligomers formed between Cys 8 and either Cys 79 or Cys 83. An intramolecular disulfide between Cys 79 and Cys 83 was also identified. Grx2 retains activity upon oxidation, did not form dimers or oligomers and could not be S-nitrosylated. The dimeric iron sulfur cluster coordinating inactive form of Grx2 dissociated upon nitrosylation, leading to activation of the protein.

Besides active site cysteines in the conserved motif of -Cys-Gly-Pro-Cys-, human cytosolic Trx1 has three structural Cys residues in positions 62, 69 and 73 which upon diamide oxidation induce a second Cys 62-Cys 69 disulfide as well as dimers and multimers. After incubation with H₂O₂, only monomeric two-disulfide molecules are generated which are inactive but able to regain full activity in an autocatalytic process in the presence of NADPH and TrxR. We found that nitrosylation by GSNO at physiological pH is critically dependent on the redox state of Trx. Starting from fully reduced human Trx, both Cys 69 and Cys 73 were nitrosylated and the active site formed a disulfide; the nitrosylated Trx was not a substrate for TrxR but regained activity after a lag phase consistent with autoactivation. The reversible inhibition of human Trx1 activity by H₂O₂ and NO donors is suggested to act in cell signaling via temporal control of reduction for the transmission of oxidative and/or nitrosative signals in thiol redox control.
LIST OF PUBLICATIONS

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


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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ADF</td>
<td>Adult T cell leukemia-derived factor</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant responsive element</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DNCB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DTNB</td>
<td>5, 5'-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>GR</td>
<td>Glutathione reductase</td>
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<tr>
<td>Grx1</td>
<td>Cytosolic glutaredoxin</td>
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<tr>
<td>Grx2</td>
<td>Mitochondrial glutaredoxin</td>
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<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>GSNO</td>
<td>S-Nitrosoglutathione</td>
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<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>Jab1</td>
<td>Jun activation domain-binding protein 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
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<tr>
<td>MGd</td>
<td>Motexafin gadolinium</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
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<tr>
<td>Prx</td>
<td>Peroxiredoxin</td>
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<tr>
<td>Ref1</td>
<td>Redox factor 1</td>
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<tr>
<td>RNR</td>
<td>Ribonucleotide reductase</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
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<tr>
<td>Sec</td>
<td>Selenocysteine</td>
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<tr>
<td>S-NO</td>
<td>S-Nitrososothiol</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>TAM</td>
<td>Trx80 activated monocyte</td>
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<tr>
<td>TBP-2</td>
<td>Thioredoxin binding protein-2</td>
</tr>
<tr>
<td>TGR</td>
<td>Thioredoxin glutathione reductase</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<td>Trx1</td>
<td>Cytosolic thioredoxin</td>
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<td>Trx2</td>
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<td>Truncated thioredoxin</td>
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<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
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<tr>
<td>Txnip</td>
<td>Thioredoxin interacting protein</td>
</tr>
<tr>
<td>VDUP-1</td>
<td>Vitamin D3 upregulated protein-1</td>
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1 INTRODUCTION

The intracellular environment is kept reduced with a high content of free protein thiols. The reducing conditions are essential for the cell survival, which is in contrast to the extracellular milieu as well as the cell surface where proteins are rich in disulfide bonds. This reduced condition is a consequence of an intricate balance between pro-oxidants and anti-oxidants. If the reducing equivalents fail to counteract the effects of oxidizing factors, cells will suffer from oxidative/nitrosative stress.

Several enzymatic and non-enzymatic systems exist to maintain the redox homeostasis of cells and to defend against oxidative stress. Among the enzymatic antioxidants, the thioredoxin (Trx) and glutaredoxin (Grx) systems are two crucial cellular redox systems that by far are well characterized. These systems are implicated in numerous physiological processes in different organs and tissues, and both their activity and expression are induced under oxidative stress (Lillig and Holmgren 2007). Both Trxs and Grxs belong to the thioredoxin super family of proteins with a common structural motif known as the thioredoxin fold as well as a conserved active site motif (-Cys-X-X-Cys-) (Martin 1995). The thioredoxin fold is comprised of a central core of a four-stranded β-sheet which is surrounded by three α-helices. In Trxs and Grxs, the dithiol in the active site is utilized to catalyze the reversible reduction of disulfides.

Protein disulfide isomerases (PDIs) are the other member of this family, also involved in redox processes. However, thioredoxin superfamily contains some proteins as well which do not functionally belong to oxido-reductases, such as glutathione S-transferases and chloride intracellular channels.

Apart from the redox active proteins, cells are equipped with other enzymatic systems which are involved in the scavenging of reactive oxygen species (ROS) to achieve redox homeostasis and overcome oxidative stress. Superoxide dismutase, catalase, and glutathione peroxidases can be exemplified.

On the other hand, free radicals that are highly unstable and reactive due to their unpaired electrons tend to react with various organic compounds including lipids, proteins and DNA. Due to these reactions, the cellular organic substrates and subsequently cell organelles are susceptible to oxidative damage. Therefore free radicals at high concentrations may contribute to a variety of diseases. However, under physiological conditions, redox homeostasis is attained through the scavenging
of reactive oxygen and nitrogen species by antioxidants. In this context, non-
enzymatic antioxidants like ascorbate and glutathione play a role too (Meister 1992).

1.1 THE THIOREDOXIN SYSTEM

The thioredoxin system, comprising Trx, thioredoxin reductase (TrxR) and NADPH, is one of the major antioxidant systems applied by cells to provide and maintain reduced states in intracellular environment (Holmgren 1985) as well as defend against oxidative and nitrosative stress (Nakamura, Nakamura et al. 1997; Finkel and Holbrook 2000). Trx exerts its antioxidant effects primarily via serving as an electron donor for peroxiredoxins (thioredoxin peroxidases). In addition, some of cytosolic proteins which can be aggregated or inactivated due to the oxidative formation of intra- or inter-molecular disulfides are able to revert to their active form by accepting reducing equivalents donated by reduced Trx (Trx-(SH)₂).

The thioredoxin system is a major cellular protein disulfide reductase in which Trx, with a low redox potential (E. coli Trx1 = -270 mV), provides electrons for protein disulfides reduction (Holmgren 1985). After a non-covalent binding between a hydrophobic surface area around the active site of Trx and the target protein-disulfide, the target disulfide undergoes a nucleophilic attack by the surface-exposed N-terminal thiolate group of Trx’s active site which has a low pKa value. This reaction leads to the transient formation of a mixed disulfide intermediate which is subsequently reduced by the C-terminal thiolate group, causing the reduction of the target protein-disulfide accompanied by the oxidation of the active site in Trx (Reaction 1). Thereafter, oxidized Trx is reduced by NADPH via a reaction catalyzed with TrxR (Reaction 2) (Holmgren 1995). This thiol-disulfide exchange reaction is reversible and efficient for electron transport.

\[
\text{Trx-(SH)}_2 + \text{Protein-S}_2 \rightarrow \text{Trx-S}_2 + \text{Protein-(SH)}_2 \quad (1)
\]

\[
\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{TrxR}} \text{Trx-(SH)}_2 + \text{NADP}^+ \quad (2)
\]

In addition, the Trx system is implicated in a large and growing number of other biological functions such as DNA synthesis and cell proliferation (Laurent, Moore et al. 1964; Luthman, Eriksson et al. 1979), angiogenesis (Welsh, Bellamy et al. 2002), control and regulation of activity of numerous transcription factors (Abate, Patel et al. 1990; Matthews, Wakasugi et al. 1992), protecting cells against apoptosis (Powis, Kirkpatrick et al. 1998; Powis and Montfort 2001), as well as involvement in cell
signaling pathways through the interaction with other proteins (Saitoh, Nishitoh et al. 1998).

1.1.1 Thioredoxin

Thioredoxin was isolated for the first time by Peter Reichard and co-workers from *Escherichia coli* as the hydrogen donor for ribonucleotide reductase (Laurent, Moore et al. 1964). It is a small protein with a molecular mass of 12 kDa, ubiquitously present in various species and tissues and acts as an oxidoreductase via a dithiol/disulfide motif located in the active site (Holmgren 1968; Holmgren 1989). The active site is highly conserved (-Trp-Cys-Gly-Pro-Cys-Lys-) among species from bacteria to humans (Eklund, Gleason et al. 1991).

The three-dimensional structure of cytosolic Trx has been shown using both x-ray crystallography and nuclear magnetic resonance (NMR) (Holmgren, Söderberg et al. 1975; Dyson, Gippert et al. 1990). Thioredoxin is a compact globular protein, composed of a single twisted β-pleated sheet containing five strands which is flanked by four α helices on the external surface (Holmgren, Söderberg et al. 1975). The active site is located at the N-terminal end of the second helix. Thioredoxins from different species have a similar three-dimensional structure even though they have sequence differences in some regions (Eklund, Gleason et al. 1991; Weichsel, Gasdaska et al. 1996). The structures of reduced and oxidized forms of *E. coli* Trx show subtle conformational differences (Jeng, Campbell et al. 1994; Holmgren 1995); the distance between the Cα positions of Cys 32 and Cys 35 (numbering with respect to human Trx) is slightly shorter in the oxidized human protein (Weichsel, Gasdaska et al. 1996). Moreover, the active site of oxidized human Trx shows a less degree of mobility compared to the reduced form (Qin, Clore et al. 1996). The structures of both reduced and oxidized human mitochondrial Trx are also revealed by x-ray crystallography, showing a high degree of similarity to the cytosolic counterpart (Smeets, Evrard et al. 2005).

In most organisms, different isoforms of Trx have been identified; for example, two isoforms in *E. coli*, and three in *Saccharomyces cerevisiae*. In mammals, three different forms of Trx have been described which are encoded by different genes: Trx1, Trx2, and SpTrx which is also designated as p32TrxL (Holmgren 1985; Spyrou,
Enmark et al. 1997; Miranda-Vizuete, Ljung et al. 2001). Trx1 and Trx2 share about 35% homology in their amino acid sequences and possess similar *in vitro* catalytic properties (Spyrou, Enmark et al. 1997).

Trx1 (gene: TXN1) is a 105-amino acid residue protein (counting the N-terminal Met), localized mainly in cytosol. However, it can be translocated to the nucleus with an unknown mechanism, maybe via the binding to another protein with a nuclear import sequence (Imamoto, Kamei et al. 1998). Importin α can be exemplified which can interact with Trx1 directly, or indirectly via thioredoxin binding protein-2 (TBP-2) (Nishinaka, Masutani et al. 2004). This translocation has been reported to happen after treatment of cells with the cancer drug cisplatin (Ueno, Masutani et al. 1999), UV irradiation (Masutani, Hirota et al. 1996), $\text{H}_2\text{O}_2$ (Makino, Yoshikawa et al. 1999), and hypoxia (Ema, Hirota et al. 1999). It is also detected in nucleus of normal cells (Maruyama, Sachi et al. 1999) as well as tumors (Nakamura, Bai et al. 2000). Interestingly, cytoplasmic and nuclear Trx1s have different redox status and they are independently controlled (Hansen, Go et al. 2006).

Human Trx2 is an 18 kDa mitochondrial protein with 166 amino acids which has the conserved catalytic active site of Trx, but lacks the structural cysteine residues. Trx2 has an N-terminal extension of 60 amino acids which plays a role as the mitochondrial translocation signal. Trx2 is essential for embryonic development and actively respirating cells (Nonn, Williams et al. 2003).

SpTrxs are tissue-specific members of this family which do not have thiol-disulfide oxidoreductase activity despite their Trx domain. Three different SpTrxs have been identified till now: SpTrx1 and SpTrx2 that are located in fibrous sheet of the sperm, and SpTrx3 which is found as a Golgi apparatus-associated protein in spermatocytes and spermatids (Miranda-Vizuete, Sadek et al. 2004).

Thioredoxins possess essential roles in cell survival due to their large and growing number of functions, to such a degree that the lack of either Trx1 or Trx2 is embryonically lethal (Matsui, Oshima et al. 1996; Nonn, Williams et al. 2003). Thioredoxin provides electrons for metabolic enzymes in different species such as sulfate reductase in yeast and methionine sulfoxide reductase (Brot and Weissbach 1983). The latter is an enzyme that is implicated in antioxidant defense and protein
repair by the reduction of methionine sulfoxide to methionine. Trx is also involved in DNA synthesis via donating electrons to ribonucleotide reductase (RNR), an essential enzyme for DNA synthesis and repair through the reduction of ribonucleotides to deoxyribonucleotides that are the building blocks of DNA (Holmgren 1989). Reduced Trx is also a general protein disulfide reductase (Holmgren 1977) which provides reducing equivalents for fibrinogen (Blombäck, Blombäck et al. 1974), choriogonadotropin (Holmgren and Morgan 1976), insulin (Holmgren 1979), thioredoxin peroxidases (peroxiredoxins), etc. Trx acts as an electron donor for peroxiredoxins (Prxs) to restore their activity via the reduction and monomerization of these proteins which are involved in reactive oxygen scavenging. The expression of Prxs is also enhanced by Trx (Berggren, Husbeck et al. 2001). The reduction and activation of Prxs is suggested to be the main mechanism by which Trx1 exerts its antioxidant effects. Peroxiredoxins are Trx-dependent peroxidases, a heterogeneous family of proteins which are ubiquitously present in all species, firstly identified as thiol-dependent antioxidants in yeast (Rhee, Chae et al. 2005). Peroxiredoxins are categorized in three different subfamilies based on the number and location of their active site cysteine residues: typical and atypical 2-Cys Prxs, and 1-Cys Prxs. Similar to Trx, Prxs are involved in antioxidant defense as well as the redox regulation of cellular signaling and differentiation (Rhee, Chae et al. 2005). Peroxiredoxins use thiol groups to scavenge ROS, and are subsequently oxidized via the formation of homo- or heterodimers with other peroxidases through the formation of disulfide bonds between their conserved cysteine residues (Berggren, Husbeck et al. 2001).

The regulation of several transcription factors and subsequently gene expression and cell function by Trx1 is another feature of this small protein. This regulation is dependent on the redox activity of Trx1 via the reduction of transcription factors which have cysteine residues in their DNA binding domains, leading to either their activation or inactivation. Nuclear factor-κB (NF-κB) is an example; a transcription factor which is involved in the cell response to oxidative stress, apoptosis and tumorigenesis and has a Cys residue (Cys 62) in the binding domain of its p50 subunit. NF-κB controls the expression of several inflammatory genes and its activity is shown to be redox-regulated by Trx1. When NF-κB is dissociated from IκB and translocates to the nucleus, Cys 62 becomes reduced by Trx1 which promotes the binding between NF-κB and DNA (Matthews, Wakasugi et al. 1992). On the other hand, oxidized Trx inhibits the binding of NF-κB to DNA. The binding between NF-
κB and DNA is also inhibited under oxidizing conditions via the formation of a disulfide bridge between two Cys 62 from two subunits (Hayashi, Ueno et al. 1993). However, in the cytoplasm Trx1 paradoxically plays a negative regulatory function for NF-κB via blocking the dissociation and degradation of the endogenous inhibitor IκB and interfering with signaling to IκB kinase (Hirota, Murata et al. 1999). Interestingly, the stimuli which activate NF-κB such as UVB irradiation and tumor necrosis factor-alpha (TNF-α) treatment promote the translocation of Trx from the cytoplasm into the nucleus (Hirota, Murata et al. 1999). Activator protein-1 (AP-1) is another transcription factor whose activity is subject to redox modulation. AP-1 regulates the expression of genes involved in cell growth in response to external stimuli such as growth factors and ionizing radiation. AP-1 has conserved Cys residues in the DNA binding domains of Fos and Jun subunits, which are involved in the DNA binding of AP-1. The reduction of these Cys residues is indirectly mediated via Trx1. In fact, Trx1 reduces another nuclear redox protein which is named redox factor 1 (Ref-1) through a direct association; and Ref-1 subsequently reduces Cys residues within the Fos and Jun subunits of AP-1 (Abate, Patel et al. 1990; Hirota, Matsui et al. 1997). The interaction between Trx and Ref-1 requires the active site cysteines of Trx as well as two cysteine residues of Ref-1 (Cys 63 and Cys 95) which are located in the N-terminal redox domain of this protein. Ref-1 has DNA-repair endonuclease activity as well which is located in its C-terminal sequences. In contrast, the activity of AP-1 can be negatively regulated by Trx via a direct interaction between Trx and Jun activation domain-binding protein 1 (Jab1) (Hwang, Ryu et al. 2004). The DNA binding activity of tumor suppressor p53 is also regulated by the redox state of its critical cysteine residues which are located in its DNA binding domain (Parks, Bolinger et al. 1997). Under oxidizing conditions, the binding of p53 to DNA is inhibited and it is shown that Trx is implicated in the regulation of the redox state of p53 via the reduction of its Cys residues in the DNA binding domain (Ueno, Masutani et al. 1999). The redox state of p53 can also be regulated by Ref-1, leading to the activation of this tumor suppressor (Jayaraman, Murthy et al. 1997). This effect can be potentiated by Trx both in vitro and in vivo which shows an indirect regulatory mechanism for Trx as well (Ueno, Masutani et al. 1999). Thioredoxin 1 is also involved in the regulation of p53-mediated p21 activation (Ueno, Masutani et al. 1999). Seemann et al recently showed an increase in DNA-binding activity of p53 after the inhibition of TrxR using RNA interference (Seemann and Hainaut 2005). Trx1 also regulates the activity of several other transcription
factors such as glucocorticoid receptor (Hutchison, Matic et al. 1991), the estrogen receptor (Hayashi, Hajiro-Nakanishi et al. 1997), hypoxia-inducible factor 1-α (Welsh, Bellamy et al. 2002), and AP-2 (Huang and Domann 1998). The oxidation of glucocorticoid receptor decreases its ligand binding activity (Chakraborti, Garabedian et al. 1992) as well as the DNA binding.

Furthermore, Trx1 is implicated in protein folding via its weak protein disulfide isomerizing activity. However, the catalysis of protein folding by Trx1 is much slower than that of protein disulfide isomerase, a protein which has two thioredoxin-like domains with the catalytic site of –Trp-Cys-Gly-His-Cys-Lys– (Freedman, Hirst et al. 1994). Thioredoxin from *E. coli* acts as a chaperone (Kern, Malki et al. 2003) and no data for human Trx is yet available.

Besides the enzymatic activities, Trx has several roles which are not dependent on its oxidoreductase activity. For example, *E. coli* Trx acts as a structural subunit of phage T7 DNA polymerase by the formation of a 1:1 complex with the DNA polymerase (Nordstrom, Randahl et al. 1981). However, this role is specific for the reduced form of Trx, and oxidized Trx-S\(_2\) is unable to bind (Huber, Tabor et al. 1987; Singha, Vlamis-Gardikas et al. 2003). *E. coli* Trx is also required for the assembly of the filamentous phages f1 and M13 (Russel and Model 1986).

In addition, there are several proteins which are known to bind to Trx1. The mechanism of the binding between these proteins and Trx is not known, but suggested to be via the formation of a mixed disulfide between a Cys residue of Trx active site and a cysteine on the other protein. Apoptosis signal-regulating kinase 1 (ASK1) is such a protein, a mitogen-activated protein (MAP) kinase kinase kinase which is implicated in apoptosis via the c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways and is involved in TNF-α-induced apoptosis (Ichijo, Nishida et al. 1997). ASK1 makes a complex with the reduced form of Trx1 both *in vitro* and *in vivo*, but not with the oxidized form or the redox inactive C32S/C35S mutant. This binding suppresses the kinase activity of ASK1 and promotes its ubiquitination and degradation, which subsequently inhibits ASK1-dependent apoptosis (Liu and Min 2002). Therefore, Trx1 has a role in the redox regulation of apoptosis (Saitoh, Nishitoh et al. 1998). Oxidation of Trx1, for example by ROS generated following exposure to cytokines or stress, leads to the dissociation and activation of ASK-1,
which subsequently results in apoptosis (Saitoh, Nishitoh et al. 1998). Trx binding protein-2 (TBP-2), which is also known as vitamin D3 upregulated protein-1 (VDUP-1) or thioredoxin interacting protein (Txnip), is another protein which binds to Trx1 and regulates its activity (Nishiyama, Matsui et al. 1999). This protein is ubiquitously present in normal cells and its expression is negatively regulated by oxidative or mechanical stress (Wang, De Keulenaer et al. 2002; Yamawaki, Pan et al. 2005). TBP-2 is involved in tumor suppression (Nishinaka, Nishiyama et al. 2004) and aging (Yoshida, Oka et al. 2003). Similar to ASK1, TBP-2 binds to the reduced Trx1 and inhibits its redox regulatory function as well as the interaction between Trx1 and ASK1 (Junn, Han et al. 2000). TBP-2 also interferes with the nuclear translocation of Trx1 which occurs after treatment of vascular smooth muscle cells with hydrogen peroxide and platelet-derived growth factor (PDGF) (Schulze, De Keulenaer et al. 2002). Therefore, TBP-2 affects the regulation of transcription factors by Trx1. The binding between TBP-2 and the reduced form of Trx1 has recently been shown to be via the formation of a stable disulfide-linked complex (Patwari, Higgins et al. 2006). Among other proteins which can bind to Trx, various isoforms of protein kinase C (Watson, Rumsby et al. 1999), p40 phagocyte oxidase (Nishiyama, Ohno et al. 1999), the cysteine protease inhibitor lipocalin (Redl, Merschak et al. 1999), and the nuclear glucocorticoid receptor (Grippo, Tienrungroj et al. 1983) can be mentioned which are regulated in a redox-dependent manner.

Trx1 can be secreted from a variety of normal cells such as hepatocytes, fibroblasts, and activated monocytes and lymphocytes as well as transformed and cancer cells. For example, adult T cell leukemia-derived factor (ADF) which had been first identified as a growth factor secreted by human T lymphotropic virus I-transformed leukemic cell lines was later shown to be identical to human Trx (Tagaya, Maeda et al. 1989). The precise mechanism of Trx1 secretion is unknown, but it is probably independent of the endoplasmic-Golgi exocytotic pathways since the protein does not have a typical amino-terminal signal sequence that usually exists in proteins secreted through this pathway (Rubartelli, Bajetto et al. 1992). Secretion of Trx mainly occurs under oxidizing conditions and inflammations (Rubartelli, Bajetto et al. 1992). For example, elevated level of Trx in plasma has been reported during cardiovascular diseases such as heart failure and cardiomyopathy (Kishimoto, Shioji et al. 2001), airway-related disorders like asthma (Yamada, Nakamura et al. 2003), patients with human immunodeficiency virus (HIV) infection (Nakamura, De Rosa et
al. 1996), rheumatoid arthritis (Yoshida, Katoh et al. 1999), Sjogren’s syndrome (Kumagai 1998), and type II diabetes (Kakisaka, Nakashima et al. 2002). The elevated level of Trx1 is also reported in patients with tumors, such as hepatocellular carcinoma (Miyazaki, Noda et al. 1998) and pancreatic ductal carcinoma (Nakamura, Bai et al. 2000). The measurement of serum/plasma levels of Trx1 to evaluate the oxidative stress and prognosis in a number of diseases has been studied. For example, it is shown that the plasma level of Trx1 in HIV infection is negatively correlated with the levels of intracellular glutathione in lymphocytes and prognosis (Nakamura, De Rosa et al. 2001). The serum levels of Trx can also be utilized to estimate the oxidative stress in chronic liver diseases such as nonalcoholic steatohepatitis (Nakashima, Sumida et al. 2005) and hepatitis C virus infection (Sumida, Nakashima et al. 2000), as well as cardiovascular disorders like acute ischemic heart disease (Miyamoto, Kawano et al. 2004) and chronic heart failure (Shioji, Nakamura et al. 2003).

When Trx1 is secreted, it is involved in a variety of physiologic and pathophysiologic functions. For example, secretory Trx1 acts as a co-cytokine and chemokine for immune cells, stimulating the growth of lymphocytes and in this way it is involved in immunomodulation (Nakamura, Nakamura et al. 1997; Bertini, Howard et al. 1999). Bertini et al showed that the redox activity of Trx and the presence of active site cysteines are required for the chemotactic activity. Secreted Trx1 has also been suggested to be an autocrine growth factor for normal fibroblasts and several tumor cell lines (Gasdaska, Berggren et al. 1995). There is not any evidence showing the presence of receptors for Trx1 and the mechanism for growth stimulatory effect of this protein is not known. However, it is proposed that Trx1 can enhance the expression and/or activity of a variety of cytokines and growth factors which are produced by the cell itself (Gasdaska, Berggren et al. 1995; Schenk, Vogt et al. 1996). Recently, it is shown that extracellular thioredoxin-1 is involved in redox regulation of cytokine receptor signalling (Schwertassek, Balmer et al. 2007).

In addition, a truncated form of Trx (Trx80) which has the first 80 or 84 N-terminal amino acids is identified which has been found to be secreted or located to the surface of monocytic cell lines (Pekkari, Gurunath et al. 2000). Trx80 is present in human plasma, enhancing eosinophilic cytotoxic activity compared to wild type Trx1 (Silberstein, Ali et al. 1989; Pekkari, Gurunath et al. 2000). It also has the
chemotactic activities for monocytes and polymorphonuclear neutrophiles as Trx1 possesses (Bizzarri, Holmgren et al. 2005). Recently, Pekkari et al showed the differentiation of human CD14+ monocytes into a novel cell type, designated Trx80 activated monocytes (TAMs) which is induced by the truncated thioredoxin (Pekkari, Goodarzi et al. 2005). Trx80 lacks the oxidoreductase activity of Trx1 even though it has the conserved active site, and it is not a substrate for TrxR. However, Trx80 can be reduced by Trx1 (Pekkari and Holmgren 2004).

Different factors are involved in the regulation of Trx activity, including its expression level, localization, protein-protein interaction, and posttranslational modifications. In a very recent study, it is shown that Trx activity can also be affected by a mechanical force. Wiita et al showed the inhibition of Trx activity and diminished antioxidant properties of the enzyme following the application of the increased mechanical forces (Wiita, Perez-Jimenez et al. 2007). Regarding the expression of Trx1, there are different regulatory elements which affect the promoters of TXN1 gene and induce the transcription. For instance, antioxidant responsive elements (ARE) can induce the expression of Trx1 upon oxidative stress (Kim, Masutani et al. 2001). The promoter region of TXN1 gene also contains other stress-responsive elements such as oxidative response element (Taniguchi, Taniguchi-Ueda et al. 1996) and heat shock responsive element (Leppa, Pirkkala et al. 1997). The promoter region of the human TXN1 gene has regulatory binding motifs compatible with both constitutive or inducible expression (Kaghad, Dessarps et al. 1994). The expression of Trx is reported to increase because of different stress stimuli in cells such as O$_2$ (Das, Guo et al. 1999), H$_2$O$_2$ (Higashikubo, Tanaka et al. 1999), photochemical oxidative stress, hypoxia (Berggren, Gallegos et al. 1996), viral infections, lipopolysaccharide (Ejima, Koji et al. 1999), X-radiation and UV irradiation (Hoshi, Tanooka et al. 1997). The expression of Trx is also upregulated by TNF-$\alpha$, estrogen, and prostaglandin E1, and following the treatment with certain drugs such as adriamycin and suberoylanilide hydroxamic acid (SAHA) that is a histone deacetylase inhibitor (Ungerstedt, Sowa et al. 2005), and during some diseases like heart failure, myocarditis and in atherosclerotic plaques (Ago and Sadoshima 2007). On the other hand, the expression of Trx is reported to be downregulated by cathepsin D (Haendeler, Popp et al. 2005), SAHA (Butler, Zhou et al. 2002) and hypertension (Tanito, Nakamura et al. 2004). In fact under some certain conditions, the expression of Trx is either negatively or positively regulated, which
can be explained by differences regarding cell types, cell conditions, or strength of stimulation. Posttranslational modifications of Trx1 will be discussed in details later.

### 1.1.2 Thioredoxin reductase

Thioredoxin reductases are the only enzymes which are able to reduce the active site of Trxs. Similar to Trxs, they are ubiquitously present in all living cells. They belong to the family of pyridine nucleotide-disulfide oxidoreductases which also contain glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase (Mustacich and Powis 2000). All members of this family form homodimers with a redox active dithiol/disulfide bond in each subunit. In mammalian TrxRs, each subunit contains an FAD binding domain, an NADPH binding domain, and an interface domain (Waksman, Krishna et al. 1994; Sandalova, Zhong et al. 2001). These two subunits are packed in a head-to-tail arrangement (Zhong, Arner et al. 2000).

Thioredoxin reductases from lower organisms such as bacteria and fungi, as well as TrxRs from plants are flavoproteins, composed of two identical subunits with 320 amino acids and a molecular mass of about 35 kDa for each subunit. TrxR from *E. coli* has been extensively studied and characterized. It has a redox active site with a conserved sequence of –Cys-Ala-Thr-Cys- which undergoes reversible oxidation reduction reactions comparable to dithiol/disulfide exchange reactions of Trx.

Mammalian TrxRs are dimeric selenoproteins which in contrast to *E. coli* TrxR have a larger molecular mass of about 112 kDa. Their conserved –Cys-Val-Asn-Val-Gly-Cys- N-terminal active site is also different from the corresponding site in TrxRs from lower organisms in two aspects. First, two cysteine residues in the active site are separated from each other with 4 amino acids, in contrast to two residues which are between redox active cysteines of TrxRs from lower organisms. And the second difference is the location of this active site which is in the central NADPH binding domain in TrxRs from lower organisms while it is located in the N-terminal FAD binding domain in mammalian TrxRs. In fact, the amino acid sequence of mammalian TrxRs shows a high homology to glutathione reductase (GR), especially regarding the identical sequence motif within the FAD-binding domain in which the redox active site is located (Zhong, Arner et al. 2000). Another prominent feature of mammalian TrxRs is their C-terminal selenium-containing active site which is present neither in
TrxRs from lower organisms nor in GR. This extra active site shows a homology to the -Cys-Cys-containing elongation in another flavoprotein, mercuric reductase (Zhong, Arner et al. 1998). The penultimate carboxyl-terminal –Gly-Cys-Sec-Gly motif is essential for the redox activity of mammalian TrxRs (Zhong, Arner et al. 2000), particularly regarding the Sec residue. Replacement by Ser results in a complete loss of activity. However, replacement by Cys gives a major loss of kcat but also a lower Km (Zhong and Holmgren 2000). In all organisms, selenocysteine is encoded by a UGA codon which usually confers the termination of translation. The C-terminal active site undergoes an exchange reaction comparable to the dithiol/disulfide reaction in Trx active site as well as N-terminal active site of TrxR, i.e. the formation of a selenenylsulfide in the oxidized enzyme and a selenolthiol in the reduced form (Lee, Bar-Noy et al. 2000; Zhong, Arner et al. 2000). In mammalian TrxRs, the active site disulfide in the FAD binding domain of each subunit is first reduced to a dithiol by electrons from NADPH and via the enzyme-bound FAD. Thereafter, the electrons are transferred from this buried N-terminal active site to the conserved C-terminal Cys497-Sec498 motif of the other subunit which acts as a second redox center. The reduction of the C-terminal active site leads to its movement to the surface where it finally transports the reducing equivalents to the substrate (Sandalova, Zhong et al. 2001).

A selenol group of a Sec residue has a high nucleophilic reactivity and it is ionized under physiological conditions due to its low pKa value. The localization of the Sec residue and the redox properties of selenium may explain why mammalian TrxRs have broad substrate specificity. The conformational characteristics of the reduced C-terminal active site of mammalian TrxR lead to its open accessibility and the penultimate Sec accounts for the broad substrate specificity of the enzyme (Sandalova, Zhong et al. 2001). In contrast to TrxRs from the lower organisms which are specific to their corresponding Trxs, mammalian TrxRs can reduce Trxs from different species (Holmgren 1977). Other substrates which are characterized include both disulfide bonds such as Trx-like 1 (Txl-1) (Lee, Murakawa et al. 1998), PDI (Lundström and Holmgren 1990), lipoic acid (Arner, Nordberg et al. 1996), and 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB) (Luthman and Holmgren 1982), as well as non-disulfide low molecular weight substrates such as lipid hydroperoxides and hydrogen peroxide (Björnstedt, Hamberg et al. 1995), S-nitrosoglutathione (GSNO) (Nikitovic and Holmgren 1996), selenodiglutathione (Björnstedt, Kumar et al. 1992),
selenite (Kumar, Bjornstedt et al. 1992), selenocysteine (Björnstedt, Kumar et al. 1995), methylseleninate (Gromer and Gross 2002), alloxan (Holmgren and Lyckeberg 1980), vitamin K, and dehydroascorbate (vitamin C) (May, Mendiratta et al. 1997). Furthermore, TrxR can reduce ubiquinone to ubiquinol (Xia, Nordman et al. 2003) as well as other quinones (Cenas, Nivinskas et al. 2004).

Three isoenzymes for mammalian TrxRs have been introduced: TrxR1 with a molecular weight of 54.400 for each subunit which is mainly targeted to cytosol (Gasdaska, Gasdaska et al. 1995), TrxR2 which is in mitochondria (Lee, Kim et al. 1999), and the testis specific thioredoxin glutathione reductase (TGR) (Sun, Kirnarsky et al. 2001). These isoenzymes, with an almost similar three-dimensional structure, show more than 50 % identity regarding their amino acid sequences.

Mitochondrial TrxR2 has a molecular weight of 56.200 for each subunit and a 33-aminoacid N-terminal extension as its mitochondrial import sequence (Lee, Kim et al. 1999; Miranda-Vizuete, Damdimopoulos et al. 1999). TrxR1 and TrxR2 have similar kinetic properties (Miranda-Vizuete, Damdimopoulos et al. 2000).

In contrast to TrxR1 and TrxR2, TGR has an N-terminal extension in form of a monothiol glutaredoxin domain and it can reduce both Trx and GSSG. In addition to its functions as both thioredoxin reductase and glutathione reductase, it also possesses low Grx activity. However, this activity is not GSH-dependent since the C-terminal selenocysteine-containing active site probably donates electrons to the Grx domain (Sun, Kirnarsky et al. 2001; Rendon, del Arenal et al. 2004). TGR is mainly located in testis, implicated in disulfide bond formation and sperm maturation (Su, Novoselov et al. 2005).

Mammalian TrxR1 and TrxR2 have numerous alternative splicing variants (Sun, Zappacosta et al. 2001; Rundlof and Arner 2004). For example, five different 5′ cDNA isoforms of human TrxR1 have been identified which have alternative N-terminal domains. The importance of these splicing variants is under investigation. The expression and intracellular concentration of TrxR1 can increase under prolonged oxidizing conditions, such as exposure of cells to H₂O₂ (Sun, Wu et al. 1999).
1.1.3 Modifications of thioredoxin system

The activity, structure, and/or localization of proteins can be modulated through the post-translational modifications of their amino acid residues. These modifications can be reversible or irreversible; however the former is the mechanism of choice for cellular regulation and signal transduction. There are a variety of proteins which are subjected to different kinds of post-translational modifications, and some can be regulated by more than one type of modifications. Human Trx1 is an example that can be modulated via different modifications, which are suggested to be involved in signaling pathways.

Besides two Cys residues in the redox-regulatory domain of Trx1 which are common in all kingdoms of life, mammalian cytosolic Trxs have additional structural cysteines which are first reported to be involved in protein aggregation and inactivation via their oxidation (Engstrom, Holmgren et al. 1974). Human Trx1 has three additional Cys residues at positions 62, 69 and 73 which have unknown biological functions; however, it is suggested they can be modified leading to the regulation of Trx1 activity. Among these structural cysteine residues, Cys 73 is the closest one to the active site in the three-dimensional structure of Trx, protruding from the surface.

Posttranslational modifications of human Trx1 which are involved in the regulation of its activity can happen via modification of Cys residues including thiol oxidation, glutathionylation and S-nitrosylation or it may occur via modification of other amino acid residues such as nitration of Tyr 49.
An intermolecular disulfide bond has been identified via Cys 73 residues of two Trx1 molecules, suggested to be involved in homodimerization (Ren, Björnstedt et al. 1993); though it is not essential for dimerization (Weichsel, Gasdaska et al. 1996). Homodimerization of human Trx1 in solution occurs especially under strong oxidizing conditions or when it is stored at high concentrations. After homodimerization of Trx1 via a disulfide bond between two Cys 73, the active site is inaccessible to TrxR, and Trx activity is therefore inhibited (Holmgren 1977; Weichsel, Gasdaska et al. 1996). The physiological importance of Trx1 dimerization is not known. Interestingly, the homodimerization of Trx in the oxidizing extracellular environment limits the growth-stimulating effects of this protein (Gasdaska, Kirkpatrick et al. 1996).

Under relatively mild oxidizing conditions, a second disulfide bond may form between Cys 62 and Cys 69; This is not a substrate for TrxR and prohibits the reduction of active site disulfide by TrxR (Luthman and Holmgren 1982; Watson, Pohl et al. 2003). This inhibitory effect is probably mediated via attenuation the accessibility of TrxR to Trx1. The disulfide between Cys 62 and Cys 69 can be reduced by the active site of Trx1. This modification needs a substantial structural rearrangement such as local unfolding and disruption of the helical structure of α3 helix since there is a distance of about 10 Å between Cys 62 and Cys 69 (Watson, Pohl et al. 2003). The formation of this intramolecular disulfide bond between two structural cysteines by diamide has been identified by mass spectrometry, suggested to be involved in providing a redox mechanism for control of Trx1 function as well as providing more time for redox-dependent signaling processes (Watson, Pohl et al. 2003).

S-glutathionylation is another kind of oxidative post-translational modification of proteins which is a reversible process. This modification is suggested to be involved in the buffering of oxidative stress, as well as the protection of proteins against irreversible oxidation and regulation of protein activity (Cotgreave and Gerdes 1998). S-glutathionylation of Trx1 is shown to occur at Cys 73 both in vitro as shown by mass spectrometry and in vivo after treatment of cells with diamide which is a strong oxidant generating GSSG (Casagrande, Bonetto et al. 2002). It happens during oxidative stress via formation of a mixed disulfide between the protein and GSH (Casagrande, Bonetto et al. 2002). Even though glutathionylation may occur under
physiological conditions, Trx1 could only become glutathionylated under oxidative stress. The enzymatic oxidoreductase activity of Trx1 was shown to be inhibited because of this modification; however, it is reversible via a process of auto-activation with sigmoidal kinetics. Glutathionylation of thioredoxin is also demonstrated in plants and again under conditions of oxidative stress. In plants, it occurs at cysteine 60, leading to a reduction in the enzymatic activity (Michelet, Zaffagnini et al. 2005).

Trx1 can also be modified by reactive nitrogen species, leading to S-nitrosylation of the protein. For the first time, S-nitrosylation of Trx1 was reported by Haendeler et al in 2002. They reported that human cytosolic Trx is nitrosylated on Cys 69 under basal conditions, a modification which was necessary for its anti-apoptotic function, scavenging reactive oxygen species as well as the redox regulatory activity via enzymatic activation (Haendeler, Hoffmann et al. 2002). Thereafter, this modification has been extensively studied, leading to controversial results, showing the complexity of this area of research. In another study which was performed by Mitchell and Marletta, they studied the S-nitrosylation of Trx1 by means of mass spectrometry, immunological methods and site-directed mutagenesis. They reported that after treating His-tagged Trx1 with 50 molar equivalents of GSNO, a nitrosylating agent, they could not detect any modification of Cys 73; Cys 69 was fully nitrosylated and a disulfide bridge was formed between two cysteine residues in the active site (Mitchell and Marletta 2005). In this study, they also showed a transnitrosation process between caspase-3 and Trx1, which was proved later to be required for S-nitrosation of pro-caspase-3 and the inhibition of apoptosis in Jurkat cells (Mitchell, Morton et al. 2007). In addition to caspase-3, Trx is involved in the denitrosylation of several other proteins such as metallothionein and albumin (Stoyanovsky, Tyurina et al. 2005) as well as low molecular weight compounds containing nitrosothiols such as GSNO (Nikitovic and Holmgren 1996), S-nitroso-L-cysteine (CysSNO), S-nitroso-L-homocysteine (HCysSNO), and S-nitroso-N-acetylpenicillamine (SNAP) without any significant substrate selectivity (Sengupta, Ryter et al. 2007). Sengupta et al showed that all HepG2 cell-derived S-nitrosoproteins with a molecular mass of 23-30 kDa are substrates for the Trx system.

Recently, the first crystal structural study of nitrosylated Trx1 was done by Weichsel et al (Weichsel, Brailey et al. 2007). In this study, GSNO-treated human Trx1 did not exhibit any modification of Cys 73. However, a disulfide bond was formed between
Cys 32 and Cys 35, and Cys 62 and Cys 69 were nitrosylated. This nitrosylation was relatively stable, even in the presence of 1 mM glutathione, and showed a pH-dependent pattern, i.e. at pH 7.0, 1 mol of S-NO/mol of Trx was found which was corresponding to the nitrosylation of Cys 62, while both Cys 62 and Cys 69 became nitrosylated at pH 9. At pH 5.6, no nitrosylation was detected.

The physiological and pathophysiological significance of S-nitrosylation of mammalian Trxs as well as its kinetics remain to be fully explored. The further insight into this modification of Trx1 as well as the formation of the second disulfide between Cys 62 and 69 is one of the goals of the research presented in this thesis.

Moreover, Trx1 is reported to be affected by peroxynitrite via nitration of Tyr 49. Mammalian Trx1 has only one tyrosine which is located within a region that is essential for its folding. Nitration of this tyrosine residue leads to an irreversible inhibition of Trx1 redox regulatory activity through a conformational change. Nitrated Trx1 loses its antiapoptotic and cardioprotective effects as well. The nitration of Trx1 also results in its dissociation from ASK-1, leading to ASK-1 activation and apoptosis (Tao, Jiao et al. 2006). Recently, the decrease of Trx activity in the aging heart was reported which is due to the posttranslational nitrative modification and results in apoptotic cardiomyocyte death. According to this study, the expression of Trx is increased in the aging heart; and therefore, it does not contribute in the reduced Trx activity (Zhang, Tao et al. 2007). Peroxynitrite is also shown to inhibit the activity of TrxR in vivo via a reaction with the selenocysteine residue, a reaction which is irreversible (Park, Fujiwara et al. 2002).

The importance of other amino acids in the structure of Trxs and their effects on the activity and/or structure of these proteins has not been investigated as much as the Cys residues (Holmgren 1985; Krause and Holmgren 1991). However, there are several studies in this field which are mainly based on the mutagenesis of Trxs to investigate the effects on protein characteristics. Krause et al showed a 35 mV increase in the redox potential of Trx with the mutation of Pro 34 to His which mimics the active site of PDI (Krause, Lundström et al. 1991). An even more extensive study has been shown that two amino acids which are located in the active site between two redox active cysteines are crucial for the redox properties of Trx (Mossner, Huber-Wunderlich et al. 1998). In a recent study, the role of a conserved
cis-proline which is located in the thioredoxin fold, immediately upstream of β3 strand (Pro 75 in human Trx1) was studied (Su, Berndt et al. 2007). Mutation of this cis-proline led to the formation of a Fe$_2$S$_2$ cluster between the first cysteine in the active site and a cysteine in the C-terminal region of Trx. Results show that this conserved cis-proline is involved in preventing formation of an iron-sulfur complex, a role in positioning of the substrate in Trx and Grx structures has been suggested for this Pro which is essential for the redox reactions (Su, Berndt et al. 2007).

1.1.4 Inhibitors of thioredoxin system and clinical applications

Because of the numerous functions of the thioredoxin system, the inhibition of this oxidoreductase system which mainly happens via the targeting TrxR can result in major cellular consequences, e.g., less total antioxidant capacity, impairment of the whole reductive capacity of cells, increased levels of ROS and the subsequent oxidative stress, decreased GSH levels and increased GSSG and glutathionylated proteins, increased expression and secretion of Trx, increased expression of TrxR, and increased secretion of NF-κB-dependent proteins (Nordberg and Arner 2001). Because of these effects which are potentially pro-oxidant in nature, inhibition of the Trx system results in cell death via necrosis or apoptosis if overexpression of Trx and other antioxidative enzymes can not recuperate the cell response.

On the other hand, elevated levels of Trx and/or TrxR have been reported in many different human malignancies such as lung, hepatic, colorectal, pancreatic, and cervical cancer (Berggren, Gallegos et al. 1996; Gladyshev, Factor et al. 1998; Lincoln, Ali Emadi et al. 2003). The level of Trx and TrxR is positively correlated with aggressive tumor growth, poor prognosis and decreased patient survival (Kakolyris, Giatromanolaki et al. 2001; Raffel, Bhattacharyya et al. 2003). However, a relationship between Trx catalytic activity and tumor growth and stage is less clear. Recently, Yoo et al showed a direct role in carcinogenesis for TrxR1 in vivo, implicating that the enzyme is essential for the growth of a murine tumor (Yoo, Xu et al. 2006).

Moreover, several studies suggest that Trx may confer resistance to the cytotoxic effects of anti-cancer drugs. In one study with several human bladder and prostatic cancer cell lines resistant to cis-diamminedichloroplatinum(II) (cisplatin), all drug-resistant cell lines had much higher levels of thioredoxin than drug-sensitive cells. By
introducing thioredoxin antisense expression plasmids into drug-resistant cell lines, increased sensitivity to cisplatin and also to other superoxide-generating agents, i.e., doxorubicin, mitomycin C, etoposide, and hydrogen peroxide, as well as to UV irradiation was observed (Yokomizo, Ono et al. 1995). Tumor sensitivity to cisplatin is also negatively correlated with mRNA levels of Trx in hepatocellular carcinoma (Kawahara, Tanaka et al. 1996) and human cervical carcinoma cell lines (Sasada, Nakamura et al. 1999). Resistance to adriamycin is also reported in various T-cell leukemia cell lines including adult T-cell leukemia (ATL) cell lines with high expression of thioredoxin (Wang, Kobayashi et al. 1997). In a recent study, the positive correlation between thioredoxin expression and resistance to docetaxel therapy in breast cancer patients was reported (Kim, Miyoshi et al. 2005). This effect may be due to the role of Trx in cell survival and its anti-apoptotic functions, or may be derived from the scavenging of ROS which are increased during chemotherapy or radiation therapy, leading to the apoptosis.

The Trx system provides tumor cells with a survival advantage through different mechanisms, such as its anti-apoptotic effects, role as a growth factor, stimulating angiogenesis, etc. Hence, this enzymatic system is an appealing target for anticancer therapy (Smart, Ortiz et al. 2004; Urig and Becker 2006). Since thioredoxin reductase has a crucial role in the thioredoxin system and it has a highly reactive C-terminal active site which can be targeted by different electrophilic compounds, TrxR has been particularly considered as a target of anticancer agents (Nguyen, Awwad et al. 2006). Another feature of TrxR which makes it a good target for antitumor therapy is its inducible NADPH-oxidase activity which leads to the formation of ROS. DNCB (1-chloro-2,4-dinitrobenzene) is an electrophilic compound which irreversibly inhibits the activity of mammalian TrxRs with second order kinetics (Arner, Bjornstedt et al. 1995). This effect is due to the alkylation of both Cys and Sec residues in the C-terminal active site of NADPH-reduced TrxR (Nordberg, Zhong et al. 1998). But upon alkylation, DNCB induces the NADPH oxidase activity of TrxR about 30-fold of the enzyme. This effect is dependent on the presence of oxygen, leading to an aerobic redox cycling and the formation of ROS (Arner, Bjornstedt et al. 1995; Nordberg, Zhong et al. 1998). It is also shown certain anticancer drugs such as cisplatin as well as the chemopreventive agent curcumin form selenium-compromised thioredoxin reductase with an enhanced NADPH oxidase activity, leading to the production of more ROS and causing oxidative stress (Sasada, Nakamura et al. 1999).
In fact, these compounds switch TrxR from an antioxidant to a prooxidant. Curcumin irreversibly inhibits TrxR activity via the formation of a 1:2 covalent adduct by alkylation of both Cys and Sec residues in the catalytically active site of the NADPH-reduced enzyme. This inhibition is dose- and time-dependent and the curcumin-modified enzyme shows a strongly induced NADPH oxidase activity producing ROS (Fang, Lu et al. 2005). The role of ROS in the induction of apoptosis and their involvement in anti-tumor therapy is discussed later.

There are several anticancer compounds which are in clinical use and they can inhibit TrxR, like retinoic acid (Becker, Gromer et al. 2000), nitrosoureas such as carmustine (bis-chloroethyl-nitrosurea, BCNU) and fotemustine (Schallreuter, Gleason et al. 1990), platinum compounds such as cisplatin (Sasada, Nakamura et al. 1999; Arner, Nakamura et al. 2001), and quinones (Mau and Powis 1990; Mau and Powis 1992; Mau and Powis 1992).

The inhibition of TrxR by BCNU and other nitrosourea drugs is irreversible and happens through carbamoylation of a cysteine in the catalytic site of reduced TrxR; hence oxidized enzyme is not a target for these compounds (Gromer, Schirmer et al. 1997). Similar to nitrosourea drugs, cisplatin irreversibly inhibits the NADPH-reduced (but not oxidized) TrxR; however via covalent modification of the reduced selenocysteine residue. Recently, new platinum compounds have been synthesized which inhibit TrxR irreversibly with nanomolar concentrations directed to the C-terminal active site and micromolar affinities for the N-terminal active site (Urig and Becker 2006). Phosphole complexes containing Au- and Pt-phospholes are new drug candidates which inhibit TrxR both in vitro and in cell culture (Urig, Fritz-Wolf et al. 2006). TrxR is also a target for arsenic trioxide (ATO) which is an effective anticancer drug for acute promyelocytic leukemia with potential therapeutic applications in a wide range of solid tumors (Lu, Chew et al. 2007).

The reducing activity of the Trx system can also be inhibited by an organotellurium analog of vitamin E which is able to inhibit the growth of tumor cells (Engman, Al-Maharik et al. 2003). Azelaic acid is another example of a suggested TrxR inhibitor with growth inhibitory effect on skin cells. Azelaic acid was proposed to competitively inhibit the electron transfer from the enzyme’s active site (Schallreuter and Wood 1987). 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE) is
a novel organoselenium compound which was shown to be another thioredoxin 
reductase inhibitor with the inhibitory effect on the growth of a variety of human 
cancer cells (Shi, Yu et al. 2003). In a very recent study, Wang et al showed the 
inhibition of TrxR in vivo as a molecular mechanism for ifosfamide, an 
oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity in 
both adults and children (Wang, Zhang et al. 2007). In this study, a transient 
inactivation of TrxR with an unknown mechanism was detected which was followed 
with a dramatic deceleration in tumor progression. This inhibitory effect was 
relatively specific to TrxR, since other antioxidants such as catalase, superoxide 
dismutase and glutathione S-transferase were not affected. The in vivo inhibition of 
TrxR has also been shown to be involved in antitumor effects of cyclophosphamide 
(Wang, Zhang et al. 2007).

Mammalian TrxRs can also be inhibited by a variety of low molecular weight 
electrophilic compounds such as thiol-alkylating compounds including 4- vinyl 
pyridine (Nordberg, Zhong et al. 1998) and iodo-acetic acid (Nordberg, Zhong et al. 
1998), arslenicals (Lin, Cullen et al. 1999), nitroaromatic compounds such as tetryl 
(Cenas, Prast et al. 2006), quinone compounds (Cenas, Nivinskas et al. 2004), 
flavonoids (Lu, Papp et al. 2006), gold compounds (Gromer, Arscott et al. 1998), and 
dinitrohalobenzenes, e.g., DNCB (Nordberg, Zhong et al. 1998). Gold is well-known 
for its affinity to thiols. Two gold-containing antirheumatic drugs, auranofin and 
aurothioglucone, can inactivate the NADPH-reduced form of TrxR at nanomolar 
concentrations via binding to the Sec residue (Gromer, Arscott et al. 1998; Becker, 
Gromer et al. 2000). Besides gold(I) compounds, there are several other compounds 
with different oxidation levels of gold which can inhibit TrxR (Omata, Folan et al. 
2006). As mentioned above, DNBC is an irreversible inhibitor of human TrxR which 
modifies the selenocysteine residue and is uniquely capable of inducing the NADPH 
oxidase activity of TrxR simultaneously, leading to the formation of superoxide 
(Arner, Bjornstedt et al. 1995). The fluoride analoge to DNBC, 1-fluoro-2,4-
dinitrobenzene (DNFB), is also an inhibitor of TrxR and induces the NADPH oxidase 
activity as well. Recently, Wataha et al showed the potent inhibition of TrxR1 
activity by Hg(II) in both cell-free and intracellular assays using monocytes. This 
inhibition was dose-dependent and assumed to be mediated via thiol or selenol 
dependent mechanism. This mercurial compound is also shown to result in a transient
decrease in Trx1 level which is suggested to happen because of Trx1 secretion (Wataha, Lewis et al. 2007).

Since the Trx system has crucial roles in cell survival, the inhibition of this system leads to different side effects. In order to partially overcome this problem, the inhibition of TrxR can be directed specifically to one of different isoenzymes. For example, mitochondrial TrxR2 can be selectively inhibited by organogold(III) complexes, leading to $\text{Ca}^{2+}$-dependent mitochondrial membrane permeability and cytochrome C release (Pia Rigobello, Messori et al. 2004).

On the other hand, several compounds have been reported to affect the Trx system via the modification of Trx. PX-12 is such a drug, a substituted 2-imidazolyl disulfide which is in phase I clinical trial in patients with advanced metastatic cancer. PX-12 affects the activity of Trx via thioalkylation of Cys 73, and the effect is irreversible (Welsh, Williams et al. 2003). Pleurotin is another inhibitor of Trx which decreases angiogenesis in cancer cells via the inhibition of hypoxia-induced factor-1$\alpha$ (HIF-1$\alpha$) and vascular endothelial growth factor (VEGF) formation (Welsh, Williams et al. 2003).

The anti-oxidative and anti-apoptotic effects of Trx have been a reason for some investigators to study the clinical application of Trx as a drug. This approach seems especially appealing because of the small size and stable structure of Trx, which can function both intracellularly and extracellularly. This goal can be achieved by means of exogenous Trx which can enter into cells or via the induction of Trx1 expression. The administration of recombinant human Trx and its therapeutic advantages have been studied for acute lung injury (Hoshino, Nakamura et al. 2003), and cerebral ischemia (Hattori, Takagi et al. 2004). The protective effect of exogenous Trx in cardiovascular diseases such as autoimmune myocarditis (Liu, Nakamura et al. 2004), reperfusion-induced arrhythmias (Aota, Matsuda et al. 1996), myocardial apoptosis and infarct size (Tao, Gao et al. 2006), and age-induced cardiac hypertrophy and fibrosis (Ago and Sadoshima 2006) has also been shown.

1.2 THE GLUTAREDOXIN SYSTEM

The glutaredoxin system comprising glutaredoxin (Grx), glutathione reductase (GR), glutathione and NADPH is the other major intracellular protein disulfide reductase.
This enzyme system is particularly important since it encompasses reduced glutathione (GSH), the most abundant thiol-containing compound in cells with a concentration of up to 10-20 mM. Glutathione needs NADPH and GR to stay in the reduced form.

The reduction of protein-disulfides is catalyzed by the glutaredoxin system via two different but functionally connected mechanisms. One is the dithiol mechanism in which a disulfide bond in the target protein is reduced at the expense of two electrons from active site cysteinyl thiolates of Grx. This reaction generates a disulfide in the active site of Grx which in turn is reduced to a dithiol by two molecules of GSH, generating GSSG. GSSG is subsequently reduced to two GSH molecules by glutathione reductase using electrons from NADPH.

\[
\begin{align*}
\text{Grx-(SH)}_2 + \text{Protein-S}_2 & \rightarrow \text{Grx-S}_2 + \text{Protein-(SH)}_2 & (3) \\
\text{Grx-S}_2 + 2 \text{GSH} & \rightarrow \text{Grx-(SH)}_2 + \text{GSSG} & (4) \\
\text{GSSG} + \text{NADPH} + \text{H}^+ & \xrightarrow{\text{GR}} 2 \text{GSH} + \text{NADP}^+ & (5)
\end{align*}
\]

In contrast to Trxs, Grxs also have another unique mechanism of disulfide reduction in which only the N-terminal thiolate group of the active site is required (Bushweller, Åslund et al. 1992). In this monothiol mechanism which is implicated in de-glutathionylation and reduction of proteins, the protein-GSH disulfide is attacked by the N-terminal cysteine residue (S\text{\textsuperscript{N}}), leading to the formation of a mixed disulfide between Grx and GSH. The latter is then reduced by another molecule of GSH which is the rate-limiting step of the monothiol mechanism (Srinivasan, Mieyal et al. 1997). The GSSG produced is subsequently reduced to two molecules of GSH by GR.

\[
\begin{align*}
\text{Grx-(SH)}_2 + \text{Protein-S-SG} & \rightarrow \text{GS-S}^{\text{N}}\text{-Grx-S}^{\text{C}}\text{H} + \text{Protein-(SH)} & (6) \\
\text{GS-S}^{\text{N}}\text{-Grx-S}^{\text{C}}\text{H} + \text{GSH} & \rightarrow \text{Grx-(SH)}_2 + \text{GSSG} & (7)
\end{align*}
\]

The last reaction is suggested to be the rate limiting step in the reduction of glutathione mixed disulfides by Grx (Srinivasan, Mieyal et al. 1997).

### 1.2.1 Glutathione

The tripeptide glutathione (L-\(\gamma\)-glutamyl-L-cysteinylglycine) is synthesized within the cells via two ATP dependent enzymatic reactions; First, \(\gamma\)-glutamyl-cysteine is
synthesized through a reaction catalyzed by γ-glutamyl-cysteine synthetase, which is then followed by the addition of glycine catalyzed by glutathione synthetase. The first reaction is the rate-limiting step in the biosynthesis of GSH. These reactions occur in the cytosol; thereafter, GSH is compartmentalized to different cellular organelles, even though most of the cellular GSH (85-90 %) remains in the cytosol. The GSH pools in these different compartments are separated from each other, providing different redox environments within a cell. For example, GSH can be imported into mitochondria using specific ATP-dependent transport processes (Griffith and Meister 1985). This leads to a higher level of GSH in mitochondria compared to the nucleus and cytosol; this pool of GSH also has a longer half-life (Söderdahl, Enoksson et al. 2003). The separation of nuclear and cytosolic GSH pools is still not clear and can be regarded as controversial. The redox environment in the endoplasmic reticulum is more oxidized in comparison with the cytosol, and glutathione is more oxidized with a ratio of GSH/GSSG of 3:1 (Hwang, Sinskey et al. 1992). Glutathione can also be transported between cells. The concentration of extracellular GSH is much lower than intracellularly. For example, the concentration of glutathione in plasma is about 2-20 µM; but, there is an exception that is bile acid which may contain up to 10 mM GSH (Griffith 1999; Jones 2002).

Glutathione has a major role in the determination of cellular redox potential which is correlated with the biological status of cells; that is -240 mV during proliferation, -200 mV during differentiation for growth arrested cells, and -170 mV during apoptosis (Watson, Chen et al. 2003). These redox potentials are suggested to provide the optimal redox environment for the activity of enzymes, transcription factors and other proteins which are involved in each cellular process (Watson, Chen et al. 2003). The ratio between GSH and GSSG is a crucial indicator for the cellular redox state. In a resting normal cell, glutathione is mainly in the reduced form with less than 1% as GSSG. Under oxidative stress, the ratio of GSH/GSSG approaches to a value of about 1 instead of the normal ratio of around 100 (Gilbert 1995). It means that oxidative stress leads to a more positive cellular redox potential (Klatt and Lamas 2000).

In addition to the role in protecting cells from oxidative and nitrosative stress, glutathione is involved in many cellular processes through a posttranslational modification of proteins which is referred to as S-glutathionylation. This modification happens through the reversible covalent binding of glutathione to cysteine residues of
target proteins. Via S-glutathionylation, glutathione regulates the activity of a large number of proteins which are involved in numerous physiological processes such as cell growth, differentiation, metabolism, etc (Lind, Gerdes et al. 2002). With most of proteins such as protein tyrosine phosphatase-1 B and phosphofructokinase, glutathionylation is inactivating; however, this modification increases the activity of some other proteins such as HIV-1 protease and microsomal glutathione S-transferase (Shelton, Chock et al. 2005). Glutathionylation is involved in the regulation of transcription factors in vitro via the inhibition of their DNA binding activity. AP-1 and NF-κB are such transcription factors which can be regulated by their redox state and can be modified via glutathionylation (Klatt, Molina et al. 1999; Pineda-Molina, Klatt et al. 2001). Human Trx1 is another example which can be regulated through the formation of a mixed disulfide between its Cys 73 and glutathione (Casagrande, Bonetto et al. 2002) as described before. Therefore, S-glutathionylation is a potential mechanism by which cells transduce the oxidative signals into functional responses. This modification of proteins may also protect them from irreversible oxidations such as oxidation of cysteine residues to sulphinic or sulphonic acid (Fratelli, Gianazza et al. 2004).

S-glutathionylation may happen via a thiol/disulfide exchange reaction between cysteine residues and GSSG. It means that the redox potential of cells and the ratio between GSH and GSSG can affect the ratio of this reaction. However, glutathionylation is not always dependent on the cellular redox state and it may happen under normal conditions. Different mechanisms have been demonstrated for the latter including one electron oxidation of either the protein thiol or GSH which is followed by the formation of a mixed disulfide, a reaction between GSH and nitrosylated proteins, a reaction between GSNO and protein thiols, and a reaction between GSH and sulfenic acid (Huang and Huang 2002). The latter explains one of the mechanisms by which glutathione exerts its homeostatic protective effects, since protein-sulfenates are not stable and easily oxidized to sulfinates and sulfonates that are considered irreversible. It is shown that a significant amount of intracellular glutathione (up to 15 %) is bound to proteins (Sies 1999).

Moreover, glutathione is implicated in metabolism and detoxification of electrophilic xenobiotics. After conjugation of GSH to these compounds, the product will be more
metabolized or excreted out of the cell. GSH is also implicated in the metabolism of nitric oxide via a conjugation reaction and formation of S-nitrosoglutathion.

1.2.2 Glutaredoxin

Glutaredoxins are glutathione-dependent oxidoreductases and members of the Trx fold family. Grx is a small protein which was first discovered as a GSH-dependent electron donor for RNR in an E. coli mutant lacking Trx (Holmgren 1976; Holmgren 1979; Luthman, Eriksson et al. 1979); Grx constitutes a family of proteins with four molecules in E. coli classically with the active site –Cys-Pro-Tyr-Cys- (Fernandes and Holmgren 2004). In mammalian cells, three different Grxs have been described: cytosolic Grx1, mitochondrial Grx2, and Grx5 which is named because of the homology to yeast Grx5. In contrast to Grx1 and Grx2 which are classical dithiol Grxs, mammalian Grx5 is a monothiol Grx which may be targeted to mitochondria (Molina-Navarro, Casas et al. 2006).

Cytosolic Grx1 is composed of 105 amino acids with a molecular mass of 12 kDa. The structure comprises the Trx fold with a central core of four-stranded β-sheet which is surrounded by five α-helices (Sun, Berardi et al. 1998). There is a sequence identity of more than 80 % between different mammalian species, such as rat (Axelsson, Eriksson et al. 1978), bovine (Luthman, Eriksson et al. 1979), rabbit (Hopper, Johnson et al. 1989) and human (Padilla, Martinez-Galisteo et al. 1995). The three-dimensional structures of both reduced and oxidized Grx1s have been revealed, showing only some minor differences which are mainly around the active site (Xia, Bushweller et al. 1992). The conserved active site motif (–Cys-Pro-Tyr-Cys-) in Grx1 with two redox active cysteines, located at the N-terminal part of helix 2 in mammalian Grx1. In pig, however, Tyr is replaced by Phe (Gan and Wells 1987). The N-terminal active site cysteine is more exposed on the surface and has a very low pKa of about 3.5 in pig Grx1 (Yang and Wells 1991). The intervening amino acids are also involved in determination of the redox potential of active site. This protein, that is widely distributed in different species, also has two other highly conserved area revealed in the three-dimensional structure; First, there is a glutathione binding site which explains the high specificity of Grx toward GSH and GSH-mixed disulfides (Bushweller, Billeter et al. 1994). Second, Grx has a hydrophobic surface area where the interaction between Grx and its substrate happens (Xia, Bushweller et al. 1992). The binding between Grx1 and either GSH or its substrates leads to
significant changes of the helices in the three-dimensional structure which are suggested to have significant mechanistic implications for the effect of glutaredoxins (Berardi and Bushweller 1999; Nordstrand, Aslund et al. 1999). Similar to Trx1, Grx1 which is mainly a cytosolic protein can be translocated to the nucleus with an unknown mechanism, where it is implicated in the regulation of transcription factors such as NF1, NF-κB and AP1.

Human Grx2 has two alternatively spliced Grx2 isoforms that differ in their 5' region, Grx2a and Grx2b, which are encoded with a same gene but alternative exons. Grx2a is localized in mitochondria while Grx2b is targeted to the nucleus (Lundberg, Johansson et al. 2001). Mitochondrial Grx2, with only 34 % sequence homology to Grx1, has a molecular mass of 14 kDa and a conserved –Cys-Ser-Tyr-Cys- active site (Lundberg, Johansson et al. 2001). It is the first member of the Trx superfamily identified as an iron-sulfur cluster-containing protein (Lillig, Berndt et al. 2005). In the dimeric holoenzyme, two molecules of Grx2 via their N-terminal active site thiols and two molecules of GSH are bridged via a (2Fe-2S) cluster (Berndt, Hudemann et al. 2007). Due to the role of GSH in the stability of the cluster and their equilibrium with glutathione in solution, Grx2 is in dimeric form under reducing conditions and is enzymatically inactive. But the cluster is degraded during oxidative stress, leading to the monomerization and activation of the protein as an oxidoreductase. This process suggests a redox sensor activity for Grx2 (Lillig, Berndt et al. 2005). In contrast to Grx1 which exclusively needs GSH to be reduced, the oxidized active site of Grx2 can also be reduced by TrxR and NADPH (Johansson, Lillig et al. 2004). Moreover, the Grx2-S-SG intermediate which is formed during the reduction of glutathionylated proteins is also a substrate for TrxR. This feature enables Grx2 to be involved in the reduction of protein disulfides as well as glutathionylated proteins even during oxidative stress (Johansson, Lillig et al. 2004). Glutaredoxin 2 is involved in mitochondrial redox regulation and antioxidant defence via the catalysis of reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins (Beer, Taylor et al. 2004).

Glutaredoxin 5 is a second mitochondrial Grx which has only one cysteine residue in its highly conserved active site (-Cys-Gly-Phe-Ser-), and no significant redox activity has been reported for this protein. It is shown that Grx5 is involved in the synthesis of iron-sulfur clusters and regulation of the activity of iron/sulfur enzymes, a function
which is conserved even in higher eukaryotes (Rodriguez-Manzaneque, Tamarit et al. 2002; Molina-Navarro, Casas et al. 2006).

Glutaredoxins have some functions which are overlapping with Trxs such as the role as an electron donor to ribonucleotide reductase (Holmgren 1976; Luthman, Eriksson et al. 1979), cellular differentiation/proliferation (Takashima, Hirota et al. 1999), anti-apoptotic functions (Daily, Vlamis-Gardikas et al. 2001), as well as the reduction of dehydroascorbate to ascorbate (vitamin C) \textit{in vitro} (Wells, Xu et al. 1990). The latter probably occurs via a monothiol mechanism (Washburn and Wells 1999).

Glutaredoxins play important roles in protecting cells from apoptosis by different mechanism. For example, Grx1 has been shown to be involved in the regulation of the redox state of the serine/threonine kinase Akt through a GSH-dependent mechanism which leads to the dephosphorylation and inactivation of Akt (Murata, Ihara et al. 2003). Regulation of ASK1 by Grx1 has also been reported which is comparable to the Trx1 effect (Song, Rhee et al. 2002). But in contrast to Trx1 which binds to the N-terminal portion of ASK1, Grx1 binds to its C-terminal domain and negatively regulates its kinase activity (Song and Lee 2003). Moreover, it has been shown that overexpression of Grx2 decreases apoptosis in HeLa cells treated by doxorubicin or 2-deoxy-D-glucose through preventing the release of cytochrome c and subsequent caspase activation (Enoksson, Fernandes et al. 2005). In contrast, the downregulation of Grx2 by RNA interference makes HeLa cells more sensitive to apoptosis towards oxidative stress-inducing compounds (Lillig, Lonn et al. 2004).

Glutaredoxins are also implicated in the regulation of transcription factors (Hirota, Matsui et al. 2000). For instance, NF-κB can be activated by Grx via Ref-1, a mechanism by which Grx1 is involved in the protection of cerebellar granule neurons from dopamine-induced apoptosis (Daily, Vlamis-Gardikas et al. 2001). Grx1 also controls the DNA binding activity of the transcription factor Nuclear factor I (NFI) through the reduction of a mixed disulfide between NFI and glutathione (Bandyopadhyay, Starke et al. 1998). Moreover, Grxs can catalyze the formation of GSH-mixed protein disulfides. Even though the reduction of these disulfide bonds is the favourable reaction for Grxs, under oxidative stress and in the presence of more GSSG, Grxs can be involved in the formation of GSH-mixed disulfides (Ruoppolo, Lundstrom-Ljung et al. 1997).
On the other hand, there are some functions which are specific to Grxs. For example, the reduction of GSH-mixed disulfides which are not substrates for Trx can be catalyzed by glutaredoxins. The de-glutathionylation of proteins can be catalyzed by Grx2 more efficiently than Grx1, with a $k_{cat}/K_m$ about 1.5-3 fold higher (Johansson, Lillig et al. 2004). Since glutathionylated proteins have been implicated as switches in many biochemical pathways, Grxs are crucial players within cells because of their specificity for reducing GSH-mixed disulfides. Nuclear factor 1 is an example; a protein with a conserved cysteine residue (Cys 3) in its DNA-binding domain which can be glutathionylated, leading to the decrease in its DNA binding activity. This protein can be de-glutathionylated by human Grx1, restoring the DNA-binding activity (Bandyopadhyay, Starke et al. 1998). Furthermore, G actin can be modified via the glutathionylation of its Cys 374, decreasing the rate of polymerization. Human Grx1 can reduce this GSH-mixed disulfide, enhancing growth factor-induced actin polymerization (Wang, Boja et al. 2001).

Similar to Trx1, Grx1 can be secreted from cells into plasma (Björnstedt, Xue et al. 1994; Nakamura, Vaage et al. 1998). Daily et al showed that Grx1 can be translocated through membranes through an unknown mechanism which enables the protein to penetrate neuronal cells and protect them from apoptosis (Daily, Vlamis-Gardikas et al. 2001). Secreted Grx1 is suggested to be a potential electron donor for plasma glutathione peroxidase (Björnstedt, Xue et al. 1994). Mitochondrial Grx2 can not be found in plasma (Lundberg, Fernandes et al. 2004).

Apart from the similarities between Trxs and Grxs regarding their structures and functions, the upregulation of Grx1 in tumor cells has also been reported (Nakamura, Bai et al. 2000). Increased Grx1 is shown to be involved in drug-resistance (Meyer and Wells 1999).

### 1.2.3 Glutathione reductase

Glutathione reductases are flavoenzymes responsible to maintain a supply of reduced glutathione; however, they are not essential in maintaining the glutathione redox status at least in some species (Tuggle and Fuchs 1985). Glutathione reductases belong to the pyridine nucleotide disulfide oxidoreductase family, with homology to mammalian TrxRs with an N-terminal -Cys-Val-Asn-Val-Gly-Cys- active site which is highly conserved in different species. The active enzyme is in dimeric form,
composed of two identical subunits which are organized in a so-called “head-to-tail” pattern. Each subunit of human GR has a molecular mass of 51 kDa, and contains three domains including an NADPH binding domain, an FAD binding domain, and an interface domain (Karplus and Schulz 1987). In order to reduce glutathione, two electrons are transferred from NADPH to FAD, and subsequently to the active site of GR which is located in the FAD binding domain. After a disulfide/dithiol exchange reaction, electrons from the reduced GR are transferred to GSSG to reduce it to GSH. In contrast to TrxR which has two distinct isoenzymes which are localized in different cellular compartments and are encoded by different genes, there is a single gene to encode both cytosolic and mitochondrial GRs which have indistinguishable biochemical properties (Taniguchi, Hara et al. 1986).

1.2.4 Modifications of glutaredoxin system

Besides the two cysteine residues located in the active site, mammalian Grxs contain additional cysteine residues which are not conserved between Grx1 and Grx2. In the structure of Grx1 in most animals such as bovine and rabbit, there are two additional cysteines which are located closer to the C-terminus (Cys 79 and Cys 83 in human Grx1). In human Grx1, there is also a third structural cysteine which is located N-terminal of the active site (Cys 8). Grx2s from human, mouse, and rat contain two additional cysteines, one located N-terminal (Cys-28, human Grx2 numbering starting at exon II) and the other one C-terminal (Cys-113) to the active site thiol/disulfide pair. Since these additional cysteines are exposed on the surface, they are presumably involved in the post-translational modifications and subsequently the regulation of the activity.
The activity of Grx1 is partially inhibited due to the oxidation with \( \text{H}_2\text{O}_2 \) or GSSG (Starke, Chen et al. 1997). On the other hand, GSSG does not affect the activity of Grx2 (Lundberg, Johansson et al. 2001). Moreover, it is shown that peroxynitrite can inactivate Grx1 through an unknown mechanism. This inhibitory effect is irreversible and DTT cannot restore the activity (Aykac-Toker, Bulgurcuogelu et al. 2001). The activity of GR can also be inhibited by peroxynitrite, with GR from the malarial parasite plasmodium falciparum being more sensitive than human GR (Savvides, Scheiwein et al. 2002).

A second intramolecular disulfide bond can form in Grx2 structure which is between two structural cysteine residues, implicated in the stabilization of the protein (Sagemark, Elgan et al. 2007).

The posttranslational modifications of Grxs have not been studied as extensively as for Trx. Therefore, one of our aims in this thesis work was to investigate further how the activity and structure of human glutaredoxins are affected through the oxidative modifications as well as S-nitrosylation of their cysteine residues.

### 1.2.5 Inhibitors of glutaredoxin system

As mentioned above and for Trx, the upregulation of Grxs has been reported in tumor cells with a positive correlation to the resistance to chemotherapy. For example, pancreatic cancer cells contain more Grx1 (Nakamura, Bai et al. 2000); and overexpression of Grx1 is involved in the resistance of some tumor cells to doxorubicin/adriamycin (Meyer and Wells 1999). Moreover, it has been shown that the downregulation of Grx2 mRNA by short interfering RNA increases the sensitivity of HeLa cells to doxorubicin (Lillig, Lonn et al. 2004). Therefore, the Grx system has been studied to understand its role in apoptosis as well as its clinical applications specially as a target for tumor therapy (Enoksson, Fernandes et al. 2005).

The glutaredoxin system can be inactivated by carmustine (BCNU) and other nitrosourea compounds through irreversible inhibition of reduced form (but not oxidized form) of GR (Gromer, Schirmer et al. 1997). Comparable to TrxR, the inhibition is probably mediated through carbamoylation of an active-site cysteine residue. Intracellular GSH adduct of cisplatin, GS-Pt, is an inhibitor of glutaredoxins.
as well as of TrxR (Arner, Nakamura et al. 2001). But in contrast to the thioredoxin system, glutaredoxin system is not targeted directly by cisplatin.

The glutaredoxin system can also be inhibited by some low molecular weight compounds. Similar to TrxR, GR is inhibited by auranofin and aurothioglucose; but with a more than 1000-fold lower sensitivity (Gromer, Arscott et al. 1998). Phosphole complexes which are TrxR inhibitors can inhibit the activity of human GR as well. The affinity of Au-containing phospholes for GR and TrxR is similar; but the effect of Pt-containing compounds on GR is about 400-times weaker compared to the effect on TrxR (Deponte, Urig et al. 2005). GR as well as Grx can be inactivated by the exposure to cadmium in a range of micromolar. Cadmium also has an inhibitory effect on Trx and TrxR (Chrestensen, Starke et al. 2000).

Grx1 can selectively be inhibited by sporidesmin which is a natural product, belonging to the class of epidithiopiperazine-2,5-dione (ETP) fungal toxins (Srinivasan, Bala et al. 2006). Sporidesmin and its analogue gliotoxin which exhibit antibacterial, antiviral, immunosuppressive, and antineoplastic effects have a disulfide bridge in their structure which is implicated as a mechanism of action via the formation of a mixed disulfide between the ETP moiety and cysteine residues of some proteins (Hurne, Chai et al. 2000). It is shown that sporidesmin is a substrate for Grx1 in the presence of GSH; however, oxidized sporidesmin as well as gliotoxin and other ETPs inactivate reduced Grx1 in the absence of GSH in a concentration-, time-, and oxygen-dependent fashion that is possibly through the formation of a mixed disulfide with a Grx1 cysteine residue. Interestingly, this inhibitory effect was selective for Grx1 since yeast GR, E. coli Trx and TrxR, as well as bovine Trx were not affected by sporidesmin in this study (Srinivasan, Bala et al. 2006).

Interestingly, the resistance to chemotherapy can be correlated to an increased cellular concentration of glutathione (Rudin, Yang et al. 2003). Therefore, the depletion of cellular glutathione is another mechanism to apply in treatment of cancers, and it has been shown that the depletion of GSH can enhance the cytotoxicity of platinum compounds and alkylating agents (Skapek, Colvin et al. 1988). Buthionine sulfoximine (BSO) is a drug which decreases the concentration of cellular GSH via the inhibition of \( \gamma \)-glutamylcysteine synthetase, enhancing the effect of chemotherapy (Rudin, Yang et al. 2003). BSO has been in phase I clinical trial as an
adjuvant in chemotherapy of tumors (O'Dwyer, Hamilton et al. 1996; Bailey, Ripple et al. 1997).

In addition to the targeting of the Grx system in tumor therapy, glutathione reductase has become an attractive drug target for antimalarial drug development since the malarial parasite plasmodium falciparum is known to be sensitive to oxidative stress (Sarma, Savvides et al. 2003).

1.3 MOTEXAFIN GADOLINIUM

Motexafin gadolinium (MGd, Xcytrin®) is a representative of a new series of synthetic metal-coordinating expanded porphyrins, known as texaphyrins (Sessler and Miller 2000). The complexes between these macrocycles and metal cations, including the trivalent lanthanide series are highly stable. MGd contains Gd$^{3+}$ in the central cavity of the macrocycle. Same to the porphyrins, MGd is fully aromatic and highly coloured (Sessler and Miller 2000). This feature leads to the most frequently reported adverse effect of MGd which is the transient discoloration of skin and urine. Grade 1 and 2 hypertension and nausea are the other frequent adverse events attributed to MGd.

MGd has a high electron affinity with a half-wave potential of approximately -50 mV versus normal hydrogen electrode (Sessler, Tvermoes et al. 1999); that is, it is easily reduced. *In vitro* MGd catalyzes the oxidation of intracellular reducing metabolites such as ascorbate, GSH, NADPH, dihydrolipoate and protein thiols under aerobic conditions in a process called futile redox cycling leading to the generation of ROS (Magda, Lepp et al. 2001; Sessler, Tvermoes et al. 2001).

Another appealing feature of MGd is its detectibility by magnetic resonance imaging (MRI) due to the paramagnetic gadolinium ion (Young, Sidhu et al. 1994; Young, Qing et al. 1996; Viala, Vanel et al. 1999). This characteristic of the drug enables the noninvasive control of drug delivery and kinetics as well as response monitoring of tumors to the treatment. In addition, as with some naturally occurring porphyrins (Zawirska 1979), MGd has tumor selectivity. The mechanism of this selectivity is unknown, probably based on the anaerobic glycolysis which is the primary metabolic pathway of tumor cells and the altered redox state of these tissues. It may also be a consequence of the higher rate of metabolism in tumor cells since MGd also
accumulates in other highly metabolic tissues such as atherosclerotic plaques as demonstrated very recently (Chris Brushett 2008). Selective accumulation and retention of MGd in tumors and not considerably in adjacent normal tissue is confirmed in animal models (Miller, Woodburn et al. 1999) and in patients using MRI (Rosenthal, Nurenberg et al. 1999; Carde, Timmerman et al. 2001; Mehta, Shapiro et al. 2002) which may persevere for several months after administration of several doses. Furthermore, the subcellular localization of MGd has been studied in two different cancer cell lines using interferometric Fourier fluorescence, indicating that it primarily collects in lysosomes and endoplasmic reticulum, and to a lesser extent in the Golgi apparatus and mitochondria; Nucleus is not a main target for MGd (Woodburn 2000; Woodburn 2001).

MGd is also a radiation sensitizer demonstrated in vivo in three murine tumor models (Young, Qing et al. 1996; Miller, Woodburn et al. 1999). Different mechanisms have been suggested which are involved in the enhancement of radiation therapy by MGd, such as the sensitization of tumor cells by the generation of ROS as a consequence of futile redox cycling (Magda, Lepp et al. 2001), inhibition of energy metabolism (Xu, Zakian et al. 2001), and selective improvement of tumor oxygenation (Donnelly, Liu et al. 2004). Generated ROS lead to the depletion of GSH, decreased GSH/GSSG ratio, and induction of single-strand DNA damage (Donnelly, Liu et al. 2005). MGd has also been shown to enhance cytotoxic response of tumor cells to several chemotherapeutic drugs, such as bleomycin and doxorubicin in a dose-dependent fashion (Miller, Woodburn et al. 2001). The mechanism is suggested to be the inhibition of potentially lethal damage repair. Moreover, MGd has a direct and independent dose-dependent cytotoxic effect in myeloma cell lines due to the disruption of redox balance (Evens, Lecane et al. 2005). In this study, it was shown that this direct cytotoxicity effect is mediated by the alteration in mitochondrial membrane potential, accompanied with increased formation of ROS since it can be reversed with catalase. Through the loss of mitochondrial membrane potential, MGd triggers the mitochondrial apoptotic pathway in the HF-1 lymphoma cell line via the release of cytochrome c from mitochondria, activation of caspase-9 prior to caspase-8, and cleavage of PARP and annexin V binding (Chen, Ramos et al. 2005). In addition to myeloma, MGd alone can induce apoptosis when added to cell cultures of lymphoma or leukemia cells (Adis-International-Limited 2004). The direct cytotoxicity of MGd can also be via the disruption of zinc metabolism, a mechanism
by which MGd affects the activity of transcription factors, cell signaling pathways and gene expression. MGd disrupts the metabolism of zinc through the oxidation of zinc metallothionein, leading to the formation of thionein and increased intracellular free zinc levels (Lecane, Karaman et al. 2005; Magda, Lecane et al. 2005). Moreover, MGd inhibits the activity of heme oxygenase-1 \textit{in vitro}, an enzyme that protects against oxidative stress and is antiapoptotic. This inhibitory effect is through the interaction of MGd with NADPH-cytochrome P450 reductase, the electron donor for heme oxygenase-1 (Evans, Xu et al. 2007). Another mechanism for the cytotoxicity of MGd is to synergize with inhibitors of Akt phosphorylation such as celecoxib or docetaxel. However, MGd treatment as a single agent increases the levels of phosphorylated Akt/protein kinase B, a serine/threonine kinase that acts in cell survival pathways to suppress apoptosis (Ramos, Sirisawad et al. 2006).

MGd is in phase III clinical trial in lung cancer patients with brain metastases as an adjuvant to whole brain radiation therapy, demonstrated to improve neurologic and neurocognitive functions (Mehta, Shapiro et al. 2002; Mehta, Rodrigus et al. 2003; Meyers, Smith et al. 2004). Moreover, MGd is in phase I or phase II clinical trials either as a single agent or in combination with radiation and/or chemotherapy in several other tumors; for example as a single-dose in patients with incurable primary or metastatic cancer (Rosenthal, Nureenberg et al. 1999) or with multiple doses in patients with glioblastoma multiforme who were receiving whole brain radiation therapy (Ford, Seiferheld et al. 2007). It is also in phase I clinical trial in combination with docetaxel and cisplatin for the treatment of non-small cell lung cancer (William, Zinner et al. 2007). The effect of MGd has also been studied in other tumors such as hematologic malignancies, pancreatic carcinoma, renal cell carcinoma, etc. In clinical trials, MGd was well tolerated by patients, with dose-limiting reversible acute renal failure at high doses after single administration (Rosenthal, Nureenberg et al. 1999), and reversible hepatotoxicity after repeated administration (Carde, Timmerman et al. 2001).

Besides the application of MGd as an antitumor drug, it shows therapeutic utility as an anti-HIV agent which is able to selectively target and remove HIV-infected cells through the induction of apoptosis in an infected host (Perez, Nolan et al. 2002).
1.4 REACTIVE OXYGEN SPECIES

Reactive oxygen species such as superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the highly reactive hydroxyl radical (OH) are normal by-products of cellular metabolism which are physiologically generated during aerobic metabolism or inflammations. During respiration in mitochondria, some electrons leak out from the electron transport chain, leading to the partial reduction of oxygen and subsequently the formation of ROS. Moreover, cells contain several enzymatic systems which are implicated in the generation of ROS, such as plasma membrane NADPH-oxidase, cytoplasmic xanthine oxidase, and peroximal cytochrome P-450 oxidases. ROS can form exogenously due to the environmental stress such as ionizing radiation and heavy metals.

Under physiological conditions, ROS exist at low concentrations and they have regulatory functions and are involved in a wide variety of cellular processes, such as cell proliferation and differentiation. They have been shown to participate in cell signaling as intracellular second messengers, especially for a multitude of cytokines and growth factors (Finkel 1998). Most of these effects are exerted via the reversible modification of the structure and/or activity of thiol-containing molecules. In fact, covalent modification of reactive cysteine residues in proteins is a plausible mechanism of growing importance for signal transduction. Among these oxidative post-translational modifications which are induced by ROS and reactive nitrogen species (RNS), inter- or intramolecular disulfide bond formation, sulfenic acid formation, S-glutathionylation, and S-nitrosylation are the prominent ones. Moreover, ROS participate in the signal transduction mechanism for apoptosis as signaling intermediates (Jacobson 1996).

If ROS can not be detoxified by antioxidants, depolarization of mitochondrial membrane occurs, leading to its permeabilisation and the release of soluble mitochondrial intermembrane proteins such as cytochrome c and apoptosis-inducing factor (AIF) (Costantini, Jacotot et al. 2000; Jiang and Wang 2004). These events lead to the activation of downstream caspases and subsequent apoptotic cell death. Because of this feature, the generation of ROS is considered as a therapeutic mechanism in the treatment of cancer and to enhance the effect of radiation in antitumor therapy (Renschler 2004). Procarbazine is such a drug which increases DNA degradation via a ROS-dependent mechanism. Doxorubicin is another example
which increases the generation of ROS via a redox-cycling mechanism through interactions with trace metals, such as iron or copper (Hasinoff and Davey 1988). The formation and accumulation of ROS in transformed cells is also seen after treatment with SAHA which is in phases I and II clinical trials against both hematologic and solid tumors. The selectivity of histone deacetylase inhibitors to kill tumor cells but not normal cells is reported which has an unknown mechanism. The increased levels of Trx is also observed after treatment with SAHA which occurs selectively in normal cells, and it can provide a mechanism for preventing the accumulation of ROS and subsequently cell death in normal cells (Ungerstedt, Sowa et al. 2005). Interestingly, SAHA leads to a decreased level of Trx in cancer cells which is accompanied by the upregulation of TBP-2 (also called Txnip), subsequently arresting the cell growth of the tumor cells (Butler, Zhou et al. 2002).

Under oxidizing conditions when ROS are excessively produced or when antioxidants defenses are insufficient to eliminate the increased amount of ROS, the normal metabolism and physiology of cells is affected, leading to pathogenesis of diseases such as cancers, neurodegenerative diseases (Andersen 2004), cardiovascular diseases, etc. as well as aging (Dröge 2002). In the nervous system, neurons can be damaged and lost due to oxidative stress; an effect which is suggested to be associated with the pathogenesis of several neurodegenerative diseases, such as Alzheimer’s disease, Parkinson disease, and amyotrophic lateral sclerosis (ALS). Interestingly, oxidative stress is also reported to be a consequence of neuronal cell death.

1.5 REACTIVE NITROGEN SPECIES

Nitric oxide is a highly reactive free radical which is synthesized within cells from L-arginin; a reaction which is catalyzed by 3 different mammalian NO synthases (NOS), dependent on the presence of cofactors NADPH, tetrahydrobiopterin, and flavin for electron transfer (Alderton, Cooper et al. 2001). Thereafter, NO leads to the formation of other RNS via different reactions with ROS. For example, NO reacts with \( \text{O}_2^- \) under biological conditions and with the extremely rapid rate constant, close to the diffusion limit, to form peroxynitrite (\( \text{ONOO}^- \)). NO is involved in a variety of cellular functions, such as cell growth, cell death, regulation of blood pressure, tissue development, memory function, etc. For example, NO is involved in the regulation of transcription factors and thereby in gene expression (Bogdan 2001).
On the other hand, NO contributes in pathophysiological conditions such as septic shock and neurodegeneration when it is produced at a high level (Levy, Prince et al. 2005; Uehara, Nakamura et al. 2006).

NO exerts its effects via different mechanisms which can be categorized into two distinct groups: cGMP-dependent and cGMP-independent mechanisms. The former is very well characterized in which NO binds to the heme of soluble guanylate cyclase, stimulating the protein to convert GTP to cyclic GMP, that subsequently leads to a cGMP signaling cascade (Cary, Winger et al. 2006). This mechanism is for instance adopted by NO as a signaling molecule in neurotransmission and smooth muscle relaxation. The cGMP-independent mechanisms can be exemplified by nitration of proteins, S-nitrosylation of thiol groups, as well as reaction with metal-ions centers.

S-nitrosylation is a selective and reversible posttranslational modification of certain proteins via the formation of a nitrosothiol (S-NO) between a cysteine residue and nitric oxide (NO). The exact mechanism by which S-Nitrosylation of proteins happens is not known yet, however, it is assumed to occur when a thiolate anion reacts with NO in the presence of an electron acceptor such as oxygen or metal centers to form NO$^+$ and subsequently an S-NO bond (Gaston, Carver et al. 2003). A reaction between a thyl radical and NO is another mechanism which is proposed for the formation of S-nitrosothiols. Higher oxides of nitrogen such as N$_2$O$_3$ are also proposed to mediate S-nitrosylation. Certain cysteine residues can be targeted by this modification, and the biochemical factors which are involved in this selectivity are unknown. However, the exposure and availability of Cys residues on the surface as well as a low pKa which makes them more reactive to electrophilic compounds are suggested to affect this selectivity. The reactivity of Cys residues also depends on their microenvironment and adjacent amino acids (Stamler, Toone et al. 1997). The proximity between target proteins and NO generators is also another factor that is involved (Handy and Loscalzo 2006).

There are numerous proteins which can be nitrosylated in vitro, and some of these such as G proteins, kinases, receptors and proteases have been shown to be subjected to this modification in vivo as well (Hess, Matsumoto et al. 2005). Therefore, a lot of different functions of cells can be regulated by S-nitrosylation. For instance, NO is involved in the regulation of gene expression via the modification of transcription
factors (Pineda-Molina and Lamas 2001). NO can also influence apoptotic cell death via the modification of some proteins which are involved in apoptosis such as caspases (Li, Billiar et al. 1997) and ASK-1 (Park, Yu et al. 2004). Under basal condition and in resting cells, caspases which are a family of cysteine proteases are S-nitrosylated. This modification inhibits the activity of these proteins which have crucial roles in the initiation and execution of apoptosis. Different apoptotic stimuli can lead to denitrosylation and activation of caspases, promoting apoptotic cell death (Mannick 2007). Human Trx1 is another example of a protein which is a target of S-nitrosylation, as discussed above in details. Besides the regulatory effects of this modification, nitrosothiols are suggested to function as the reservoir of the short-lived NO (Butler and Rhodes 1997).

Nitrosothiols are not stable and can be decomposed spontaneously; in fact, nitrosothiols are suggested to be intermediate, not endproducts which are dynamically sensitive to the immediate chemical environment (Lancaster 2006). However, there are several enzymatic systems which are involved in the catabolism of nitrosothiols, including the Trx system (Nikitovic and Holmgren 1996; Stoyanovsky, Tyurina et al. 2005), protein disulfide isomerase (Sliskovic, Raturi et al. 2005), and alcohol dehydrogenase class III (Jensen, Belka et al. 1998). The subcellular localization of these enzymatic systems affects the level of protein S-nitrosylation which is also dependent on the rate of NO production and S-nitrosothiol formation.

In addition to S-nitrosylation, NO can also be involved in intracellular regulation of protein functions via protein glutathionylation (Ramana, Chandra et al. 2003), disulfide bond formation, and the formation of cysteine oxy-sulfur acids such as sulfenic, sulfenic, or sulfonic acid derivatives (Singh, Wishnok et al. 1996; Klatt and Lamas 2000). Protein glutathiolation induced by exposure to either exogenous (autocrine mechanism) or endogenous (paracrine mechanism) NO is shown to be specific for some proteins as well as saturable and reversible (West, Hill et al. 2006); resulting from direct modification of protein thiols by GSNO (reaction 1) (Mohr, Hallak et al. 1999; Zech, Wilm et al. 1999; Xian, Chen et al. 2000) or GSH-assisted protein denitrosylation (reaction 2) (West, Hill et al. 2006).

\[
\text{GSNO} + \text{PSH} \rightarrow \text{PSN-(OH)-SG} \rightarrow \text{PSSG} + \text{HNO} \quad (8)
\]
Intracellular proteins can also be glutathionylated via peroxynitrite-dependent pathways in which cysteine residues of proteins or GSH could be oxidized to oxy-sulfenic acids (Radi, Beckman et al. 1991), which readily form mixed disulfides (Poole, Karplus et al. 2004). However, peroxynitrite (ONOO\(^{-}\)) usually irreversibly modifies proteins through the nitration of tyrosine residues (addition of a nitro [-NO\(_2\)] group), a selective posttranslational modification by which it is implicated in different physiological and pathological signalings (Turko and Murad 2002; Ischiropoulos 2003). For instance, manganese superoxide dismutase (MnSOD) which is a key mitochondrial antioxidant enzyme can be nitrated by peroxynitrite to form 3-nitrotyrosine in its active site leading to its inactivation under conditions of oxidative stress (Guo, Adachi et al. 2003). Prostacyclin synthase is another example which can be nitrated and inactivated by peroxynitrite, a modification which is suggested to be involved in the development of diabetic vascular diseases (Zou, Shi et al. 2002). Peroxynitrite is also a weak nitrosylation molecule which needs a high concentration to be able to result in this modification.
2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

The thioredoxin and glutaredoxin systems are two of the enzymatic systems which are used by cells to maintain their redox homeostasis and to defend against oxidative stress. They are also involved in numerous other functions to such a degree that the lack of them is embryonically lethal. Moreover, the upregulation of these two systems has been reported in different tumors, providing a survival advantage for tumor cells through different mechanisms. Hence, these enzymatic systems are considered as potential targets for antitumor therapy, and their clinical applications in other disease have also been investigated. Therefore, it is important to know more about the regulation of the thioredoxin and glutaredoxin systems via physiological modifications as well as drugs, and the specific aims of this project work then were to:

- Characterize the reactivity of MGd with thioredoxin and thioredoxin reductase as well as the interaction with ribonucleotide reductase.

- Elucidate the function of the additional structural cysteine residues present in the mammalian thioredoxins and glutaredoxins.

- Investigate the post-translational modifications of the human thioredoxins and glutaredoxins by reactive oxygen and reactive nitrogen species.

- Characterize the effects of oxidation as well as S-nitrosylation of the structural cysteine residues of human thioredoxins and glutaredoxins on their structure and activity.
2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

Motexafin Gadolinium, a Tumor-selective Drug Targeting Thioredoxin Reductase and Ribonucleotide Reductase.

Addition of increasing amounts of MGd resulted in a saturable oxidation of NADPH in the presence of either rat or E. coli TrxR, indicating that MGd is a substrate for the enzyme. An apparent Km-value of 8.65 µM, a kcat of $0.42 \times s^{-1}$ and a kcat/Km of $4.86 \times 10^4 M^{-1} s^{-1}$ for the rat TrxR1 was calculated. MGd acted faster with the E. coli enzyme and a Km-value of 22.1 µM and a kcat of $4.93 \times s^{-1}$ and a kcat/Km of $2.23 \times 10^5 M^{-1} s^{-1}$ was calculated. Only in the presence of oxygen, significant MGd-mediated oxidation of NADPH occurred which led to the formation of superoxide and finally hydrogen peroxide, indicating a redox cycling for MGd. The reaction between TrxR and MGd required the fully active enzyme. Via the equilibration of a PD-10 Sephadex G-25 column with MGd in TE buffer and after loading MGd-incubated TrxR on the column, the binding between the enzyme and MGd was observed by studying the chromatogram. With rat and E. coli TrxR, approximately up to 1.4 and 6 molecules of MGd were eluted per enzyme dimer, respectively.

Following the incubation of the fully reduced human Trx1 with an equal concentration of MGd, no significant reaction was observed under different conditions, with and without EDTA and at different temperatures. In addition, there was no evidence of complex formation between MGd and reduced or oxidized Trx.

To study if MGd inhibited the protein disulfide reductase activity of the Trx system, a low rate limiting amount of rat TrxR (5 nM) was used to catalyze the reduction of 0.3 mM insulin in the presence of 0.1 mM NADPH and 1.8 µM Trx as well as 0-140 µM MGd. An inhibitory effect of MGd with the IC$_{50}$ of about 50 µM was observed. Using varying concentrations of Trx showed that MGd was a non-competitive inhibitor. The K$_i$ of MGd was negatively correlated to the insulin concentration, probably due to a binding interaction. We also used varying concentrations of NADPH via a method to continuously regenerate NADPH in the presence of glucose-6-phosphate dehydrogenase and glucose-6-phosphate, showing that MGd acted as a non-competitive inhibitor of NADPH in this assay system (a Km-value of 8.06 µM and a kcat of $26.9 \times s^{-1}$ without MGd, and a Km-value of 8.03 µM and a kcat of $24.0 \times s^{-1}$...
with MGd). The inhibition of TrxR by MGd independent of Trx was shown by using a saturating concentration of the artificial direct substrate DTNB. A relatively strong and direct inhibitory effect of MGd on TrxR with an IC₅₀ of 6 µM was observed.

MGd also inhibited the activity of ribonucleotide reductase. RNR activity was measured by using conversion of [³H] CDP into [³H] dCDP. Using the Trx system as an electron donor, MGd was a strong inhibitor of mouse RNR activity, with an IC₅₀ of 2 µM. Alternatively, 4 mM DTT was used as reducing substrate. Again, RNR activity was inhibited with an IC₅₀ of 6 µM.

2.2.2 Paper II

Oxidation and S-nitrosylation of cysteines in human cytosolic and mitochondrial glutaredoxins: effects on structure and activity

Oxidation of Grx1 by GSSG led to a complex mixture of monomers, dimers, and oligomers while only a small fraction of the protein kept free thiols. The physiological reductant GSH partially reduced Grx1; the number of free thiols increased to 2.5 per monomer. The amount of dimeric Grx1 was reduced and no oligomers could be detected. Incubation of oxidized Grx1 with TrxR, both in the absence and presence of Trx, decreased the amount of oligomeric protein, indicating the reduction of some intermolecular disulfides by TrxR. Reduction of the protein with DTT resulted in complete reduction and monomerization of the protein. The reaction between GSSG-treated Grx1 and mammalian TrxR showed an NADPH oxidation with a total oxidation of 1.6 mol of NADPH for each mol of Grx1. Since the disulfide bridge in Grx1 active site is not a substrate for mammalian TrxR, the reaction between GSSG-treated Grx1 and TrxR must be due to the reduction of intermolecular disulfides and/or glutathionylated Cys residues by TrxR. The activity of human Grx1 decreased after oxidation with GSSG of about 25 % and upon treatment with H₂O₂ by about 80 %. Full activity was regained by re-reduction with DTT, suggesting that the partial inactivation was caused by disulfide formation.

GSSG-treated Grx2 monomers did not form intermolecular disulfides upon oxidation. The number of free thiols was reduced to 0.1 per monomer, which could be explained by formation of an intramolecular disulfides. Although the active site in Grx2 was shown to be a substrate for both GSH and TrxR, neither of them nor Trx was able to fully reduce the protein. A partial reduction was observed which is most likely due to
the reduction of the active site. Incubation of the oxidized protein with DTT gave the most complete reduction, i.e. 3.6 free thiols per molecule. The activity of Grx2 monomer was not affected by treatment with oxidants or reductants.

Using MALDI-TOF and nESI mass spectrometry, digestion of reduced Grx1 generated three cysteine-containing peptides with all five Cys residues present in the reduced state. Digestion of oxidized Grx1 generated five cysteine containing peptides, corresponding to mixed disulfides between GSH and Cys 8, Cys79, and Cys83, respectively, as well as disulfide formation between Cys8 and Cys79 or Cys83, Cys79 and Cys83, and between the active site Cys residues. Trypsin digestion of reduced Grx2 resulted in two fragments that represented the reduced active site and reduced Cys113. Digestion of oxidized Grx2 resulted in only one Cys-containing peptide whose mass corresponded to the oxidized active site. The reduced and oxidized forms of purified Grx2 without a his-tag were also alkylated with iodoacetamide, and directly analyzed by mass spectrometry without trysinization. One mass corresponded to the mass of the protein without modification for the oxidized Grx1, and two masses in agreement with the addition of two and four acetamide moities for the partially reduced protein were recorded. The results can be explained by the formation of two intramolecular disulfides in Grx2, one in the active site, and the second disulfide between Cys28 and Cys113.

Treatment of Grx1 with GSNO under aerobic conditions irreversibly decreased its activity by almost 90 %; the number of free thiols decreased to 0.3, but no dimers or oligomers were formed. This inhibitory effect is due to the nitration of Grx1 since the treatment of Grx1 with SIN-1, which generates peroxynitrite in the solution, led to a dramatic decline in the activity of about 90 % which was not reversible. In the absence of oxygen, however, treatment of Grx1 with GSNO decreased its activity by 30 % and the enzyme regained its full activity after reduction with DTT. Furthermore, GSNO-treatment of Grx1 induced a new broad absorption band around 335 nm, characteristic for S-nitrosothiol groups and the recorded absorbance corresponded to three (2.8 ± 0.1) S-nitrosothiols. Because the active site Cys residues formed a disulfide, all three structural disulfides can be S-nitrosylated.

The activity of active Grx2 monomer was unaffected by pretreatment with GSSG or GSNO. Treatment of reduced Grx2 with GSNO reduced the number of free thiols to
0.3, it did not induce S-nitrosylation of the protein and had, as SIN-1 treatment, no effect on activity of the protein. On the other hand, when the enzymatically inactive, dimeric [2Fe2S]-complex was treated with GSNO or an alternative NO donor (SNAP), the holo complex dissociated resulting in enzymatically active Grx2.

2.2.3 Paper III

Regulation of the catalytic activity and structure of human thioredoxin 1 via oxidation and S-nitrosylation of cysteine residues

Chemically-reduced human Trx1 was incubated with different concentrations of diamide. A decrease in the number of free thiols was recorded by increasing the ratio between diamide and Trx1. With 10 molar equivalents of diamide, all Cys residues were oxidized and no free thiol was detected in the Trx1. Using non-reducing SDS-PAGE electrophoresis, oxidation by diamide led to a complex mixture of monomers, mainly dimers, but also oligomers. The effect of diamide on Cys62Ser/Cys73Ser Trx1 showed a progressive dimerization. Diamide inactivated the protein-disulfide reductase activity of Trx1 in a dose-dependent fashion. The inhibition of Trx catalysis was transient and activity was regained after a lag phase. The duration of the lag phase was a function of diamide concentration. For Cys62Ser/Cys73Ser mutant protein, the activity was not affected by diamide, indicating that the dimers generated by Cys 69 oxidation were active.

Similar to the effect of diamide, the oxidation of human Trx1 by H2O2 revealed a progressive decrease in the number of –SH groups. However, the number of free thiols did not reach zero. Even with a 100-fold molar excess of H2O2, one free thiol was left in the structure of human Trx1. Using non-reducing SDS-PAGE electrophoresis, H2O2- treated Trx1 was still in the monomeric form even after treatment with 100 molar equivalents of H2O2. After treating human Trx1 with H2O2, a progressive inhibition of Trx1 activity was recorded by increasing the concentration of H2O2. As with diamide, the inhibitory effect of H2O2 on Trx1 activity was reversible, and Trx1 regained its activity after a lag phase.

Fully-reduced wt Trx1 was treated with GSNO for 60 min at 37 °C. After a chromatographic step on a NAP-5 column, the spectrum of GSNO-treated Trx1 showed an extra peak of absorbance at 335 nm, indicating the S-nitrosylation of the protein. The number of nitrosothiols was calculated using an extinction coefficient of
920 M$^{-1}$ cm$^{-1}$ at 335 nm, showing two nitrosothiols in nitrosylated hTrx1. In order to find the localization of these nitrosothiols, different mutants of hTrx1 as well as the wt protein were utilized. After treatment with GSNO, cysteines 69 and 73 became nitrosylated, and cysteines 32 and 35 in the active site were oxidized to a disulfide. Furthermore, *E. coli* Trx was treated by GSNO to investigate if cysteines in the active site can be nitrosylated. Pro34 was shown to have a protective effect against the S-nitrosylation of the Cys residues in the active site, a result consistent with active site nitrosylation of PDI.

Oxidation of NADPH by 20 µM of either oxidized or nitrosylated Trx1 in the presence of 10 nM rat TrxR revealed a lower rate of NADPH oxidation with nitrosylated hTrx1 than that of the oxidized protein. However, this rate was increased after a lag phase. It shows that active Trx-(SH)$_2$ molecules can reduce the nitrosothiol groups of Trx1 through an autocatalytic reaction. Fully nitrosylated Trx was almost inactive as an insulin-disulfide reductase, although it restarted to regain activity after a lag phase. A progressive inhibition of Trx activity was observed following the preincubation of reduced hTrx1 with increasing concentrations of GSNO, and under no conditions an increase in activity was detected. Rat TrxR was not affected by either H$_2$O$_2$ or GSNO.
3 CONCLUSIONS

The main conclusions from each individual paper were as follows:

Paper I:

- MGd acts as a substrate of the mammalian cytosolic TrxR, generating ROS from NADPH in the presence of oxygen, with hydrogen peroxide as the reaction end product. *E. coli* TrxR showed a higher reactivity with MGd.
- MGd formed a complex with TrxR; a higher number of MGd molecules bound to *E. coli* TrxR compared to the mammalian enzyme.
- MGd did not react with *E. coli* or human Trx under any assay conditions.
- MGd was a powerful inhibitor of both the mammalian and *E. coli* Trx system. The effect on the mammalian Trx system was much more moderate than for the *E. coli* TrxR.
- The other major effect of MGd is the direct inhibition of the activity of RNR, an essential enzyme required for DNA synthesis and repair. It occurred both with Trx and TrxR as well as with the artificial electron donor DTT, as electron donors, clearly demonstrating a direct effect on RNR. The low IC$_{50}$ of 2 µM of MGd for RNR makes it a strong inhibitor of the enzyme. RNR activity could also be indirectly affected as a downstream consequence of inhibition of the Trx system.

Paper II:

- Human cytosolic Grx1 was inhibited by oxidation and nitrosylation of structural Cys residues.
- S-Nitrosylation induced by GSNO under anaerobic conditions was reversible. However, in the presence of oxygen, inhibition was stronger and irreversible which was most likely caused by formation of peroxynitrite and subsequent nitration of the enzyme.
- Human Grx1 was inactivated by oxidation of protein thiols to disulfides, with a complex pattern of homodimers and oligomers on an SDS-PAGE formed by intermolecular disulfide bonds between Cys8 and either Cys79 or Cys83.
- In addition, an intramolecular disulfide between Cys79 and Cys83 was formed; Cys8 as well as Cys79 and Cys83 formed mixed disulfides with GSH.
- Reactive nitrogen species effectively induce dissociation of the holo-complex.
of Grx2 as well as reactive oxygen species. Because apo-Grx2 is not inactivated by nitrosylation or nitration, reactive nitrogen species represent a second mechanism of Grx2 activation.

- The two additional Cys residues in Grx2 form a structural disulfide. This disulfide appears to be important for the structure of Grx2 under oxidative conditions ensuring a high pool of active protein.

- There are large differences between human cytosolic Grx1 and mitochondrial Grx2 upon oxidation. The three additional cysteinyl residues of Grx1 provide mechanisms for inactivation during oxidative and nitrosative stress perhaps for secretion and avoiding planting destructive disulfides in other proteins. Iron sulfur cluster coordination involving the N-terminal active site cysteine offers a mechanism for activation of Grx2 under these conditions. These astonishing differences most likely reflect adaptions to their different subcellular localization and also to different regulatory functions *in vivo*.

**Paper III:**

- Both diamide and H₂O₂ induced a second disulfide Cys 62-Cys 69 in Trx1 with profound effects on the structure.

- Only H₂O₂ gave rise to a monomeric two disulfide form of Trx1 which was inactive as a substrate for TrxR and NADPH. Diamide also inactivated Trx1 but in contrast gave rise to covalent dimers and multimers.

- H₂O₂-dependent oxidation will block the catalytic activity of Trx and it may be a signal for secretion of Trx and potentially generation of truncated thioredoxin (Trx80).

- Regarding S-nitrosylation of human Trx1, the fully reduced protein was nitrosylated by GSNO on both Cys 69 and Cys 73 *in vitro*.

- The different results published on the subject of Trx1 and NO may be derived by the kinetics of nitrosylation and by the different conditions including the redox state of the starting material.

- In this study, Cys 32 and Cys 35 which are in the active site of Trx1 were not nitrosylated by GSNO, but became oxidized to a disulfide bond. In addition, Pro 34 was involved in protection of the active site against S-nitrosylation since the *E. coli* P34H Trx1 was sensitive to S-nitrosylation.
o When reduced Trx1 was treated with different ratios of GSNO, the disulfide redox activity was progressively lost without evidence of activation. In our studies, we could not detect any increase in redox regulatory activity of human Trx1 after nitrosylation under any conditions.

o The lag phase in the redox activity of H$_2$O$_2$-treated and S-nitrosylated Trx1 could be a mechanism by which transient inhibition of Trx1 activity provides more time for the transmission of oxidative and/or nitrosative signals.

o Nitrosylation of Trx1 may provide a protective mechanism against irreversible oxidation of thiol groups or inactivation of the protein via disulfide formation between structural cysteines during oxidative and nitrosative stress.

o Nitrosylation of hTrx1 might be a protective effect against nitrative stress by removing NO, leading to the inhibition of ONOO$^-$ formation.
4 FUTURE PERSPECTIVES

In our first paper, we showed that MGd binds to TrxR, leading to the induction of its NADPH-oxidase activity as well as the inhibition of the protein-disulfide reductase activity of the enzyme. The details of the binding between MGd and TrxR are not known yet, and it would be interesting to know which domains of TrxR are targeted by the drug. Since a larger number of MGd molecules bind to *E. coli* TrxR compared to the mammalian enzyme, and the inhibition of the bacterial enzyme is stronger than the mammalian TrxR, it would be possible to study MGd as a new potential antibacterial drug. In order to approach this goal, it is necessary to study the inhibitory effects of MGd on the activity of the Trx system as well as the growth and survival of pathogenic bacteria.

Moreover, we showed a direct and strong inhibitory effect of MGd on the activity of RNR, an essential enzyme for DNA synthesis and repair. Further studies are required to assess if MGd can bind to RNR. Besides, since RNR is composed of two non-identical R1 and R2 subunits, we should analyze which of the two RNR subunits is targeted by MGd.

With respect to the subcellular localization of MGd which mainly accumulates in lysosomes and endoplasmic reticulum, and to a lesser extent in the Golgi apparatus and mitochondria, further studies are required to investigate the *in vivo* interaction between MGd and TrxR1 or RNR which are located mainly in cytosol and nucleus. It is also necessary to study diverse inhibitory effects of MGd on cytosolic and mitochondrial TrxRs concerning their different subcellular localization.

In the second and third papers, the modifications of the structure and activity of human glutaredoxins and thioredoxin by oxidation and S-nitrosylation were investigated. We showed that human Grx1 can be oxidized through the formation of different kinds of inter- and intracellular disulfide bonds, leading to the inactivation of the protein. However, under oxidizing conditions, human Grx2 worked as a redox sensor since the holoenzyme was dissociated to the active monomers which were not affected by oxidants. The treatment of human Trx1 with H$_2$O$_2$ may lead to the formation of a second disulfide bond between Cys 62 and Cys 69, besides the disulfide bridge in the active site. The physiological and pathophysiological
implications of these oxidative modifications are not understood well. Further studies are needed to understand under which conditions and at what extent these modifications happen and how they are regulated by cells. The secretion of human Trx1 and Grx1 to the extracellular space is another fact which has not still explained how to happen. We think that the oxidized forms of these proteins with two disulfide bonds in the structure are the forms which can be excreted from the cells. However, this is not proven yet and needs more investigations.

Regarding to the S-nitrosylation of these proteins, we showed that human Grx1 which has three structural cysteine residues can be nitrosylated on three of them. Human Trx1 which also has three structural cysteines, became nitrosylated on two of them (Cys 69 and Cys 73) and the third one (Cys 62) was not affected by GSNO. In both cases, the active site cysteines became oxidized through the formation of a disulfide bridge. Apo-Grx1 was not nitrosylated and inactivated by GSNO, while the holo-enzyme with the iron-sulfur cluster became dissociated and activated. The kinetics of the S-nitrosylation of human Trx1 and Grx1 are unknown. The S-nitrosylation of human Grx1 was reported for the first time in our paper; however, S-nitrosylation of human Trx1 has been extensively studied by different groups. The outcomes are very controversial and complicated even concerning which Cys residues that can be nitrosylated and it shows the necessity of further investigations in this field. It is required to study which Cys residues react and in which order these can be nitrosylated under nitrosative stress. Since the transnitrosation process is one of the mechanisms for nitrosylation/denitrosylation of proteins, it would be interesting to know the target proteins which can be nitrosylated/denitrosylated by human Trx1 or Grx1. Moreover, appropriate methods such as site-directed mutagenesis can be applied to investigate in which transnitrosation reactions, each individual structural cysteine is involved. The effects of this modification on other activities of these proteins such as the binding to other proteins are the other appealing questions.

Moreover, the irreversible inhibition of human Grx1 by GSNO which was observed under aerobic conditions suggested the nitration of this protein that was confirmed by the treatment of human Grx1 with peroxynitrite. Further studies are required to confirm the nitration of human Grx1. For example, anti-nitrotyrosine antibody can be utilized to detect if the protein is modified via nitration. Since this modification is irreversible and covalent, mass spectrometry is another option to study this post-
translational modification. In addition, the physiological importance of this modification is not understood and it needs more studies to show under which \textit{in vivo} conditions the nitration of human Grx1 happens.
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