Mirva Peltoniemi

MECHANISM OF ACTION OF THE GLUTAREDOXINS AND THEIR ROLE IN HUMAN LUNG DISEASES
MIRVA PELTONIEMI

MECHANISM OF ACTION OF THE GLUTAREDOXINS AND THEIR ROLE IN HUMAN LUNG DISEASES

Academic dissertation to be presented, with the assent of the Faculty of Science of the University of Oulu, for public defence in Raahensali (Auditorium L10), Linnanmaa, on August 10th, 2007, at 12 noon

OULUN YLIOPISTO, OULU 2007
Abstract

Glutaredoxins (Grx) are small thiol disulphide oxidoreductases with a conserved active site sequence -CXXC/S- and a glutathione (GSH) binding site. They catalyze the reduction of protein disulphides, preferring protein-GSH mixed disulphides as substrates. The accumulation of protein-GSH mixed disulphides has been observed during oxidative stress, where they may serve both a regulatory and an antioxidant function by protecting the enzymes from irreversible oxidation. Once oxidative stress has been removed the GSH-protein mixed disulphides are reduced by GSH or, more efficiently, by Grx.

The present study showed for the first time that Grx1 and Grx2 can be detected in healthy human lung. Highly specific expression of Grx1 was observed in alveolar macrophages, but it could also be detected from sputum supernatant. Grx1 levels in alveolar macrophages were lower in selected inflammatory diseases than in control lung samples. Grx1 was also mainly negative in the fibrotic areas in usual interstitial pneumonia, an aggressive fibrotic lung disease. Overall, the present study suggests that Grx1 is a potential redox modulatory protein regulating the intracellular as well as extracellular homeostasis of glutathionylated proteins and GSH not only in healthy lung, but also in inflammatory and fibrotic lung diseases.

In order to study the mechanism of action of glutaredoxins in vitro, a new real-time fluorescence-based method for measuring the deglutathionylation activity of glutaredoxins using a glutathionylated peptide as a substrate was developed. The first reaction intermediate in the deglutathionylation reaction was shown to be exclusively Grx-GSH mixed disulphide and this specificity was solely dependent on the unusual γ-linkage present in glutathione. The study also demonstrated the role of conserved residues in the proximity of proposed GSH binding site to the GSH binding specificity of E. coli Grx1. Opening the binding groove and removing charged residues enabled Grx to form more readily mixed disulfides with other molecules besides GSH. Different members of the PDI family showed considerably lower activity levels compared to glutaredoxins and, in contrast to the glutaredoxin-GSH mixed disulphide, the only intermediate in the PDI catalysed reaction was PDI-peptide mixed disulphide.

Keywords: antioxidant, disulphide, glutaredoxin, glutathione, inflammation, lung, macrophage, oxidant
Acknowledgements

This work was carried out in the Department of Biochemistry and the Department of Internal Medicine of the University of Oulu and Oulu University Hospital. Significant portions of this study were performed in the Department of Pathology, Oulu University Hospital and the Department of Medicine, Division of Pulmonary Diseases, University of Helsinki.

I wish to express my gratitude to my supervisor Professor Vuokko Kinnula, who introduced me to the medical research world and the exciting field of oxidants, antioxidants and lung diseases. I thank her for her continuous support and encouragement.

I want to warmly thank my supervisor Professor Lloyd Ruddock, whose everlasting enthusiasm and bottomless well of knowledge drove me forward. I thank him for the opportunity to combine medical research and pure protein chemistry into one whole entity.

My sincere thanks go to Docent Anna-Liisa Levonen and Professor John Mieyal for the careful revision of this thesis and for the valuable comments.

I send my appreciation to Professor Arne Holmgren for generously providing the antibodies against human Grx1 and Grx2.

Warm thanks go to the pathologists, Riitta Kaarteenaho-Wiik, Kaisa Salmenkivi, Paavo Pääkkö and Ylermi Soini, without whom the analysis of immunohistochemical data would not have been possible. Especially I thank Riitta and Ylermi for their time and effort to teach me the basics of lung pathology.

I am grateful to Terttu Harju, Paula Rytilä, Raija Sormunen and Marjaana Säily for their contribution to this work. I thank them for their help with some practical parts as well as for valuable comments on manuscripts.

The skilful and valuable assistance of Heli Alanen, Jaana Jurvansuu, Satu Koljonen, Kirsi Kvist-Mäkelä, Maria Latva-Ranta, Tiina Marjomaa, Kirsi Salo, Raija Sirviö and Manu Tuovinen deserves my deepest thanks.

I wish to warmly thank all the present and former members of VK and LR groups. The friendly and helpful atmosphere has made working in the lab easy and enjoyable. Especially I thank my office- and labmates Anna-Riikka Karala and Anna-Kaisa Lappi for sharing my frustrations and occasional joys of success, both in life and in science.
I thank all the personnel in the departments of Biochemistry and Internal Medicine for creating scientifically inspiring atmosphere and for the excellent facilities to work with.

I am grateful to my mother Kerttu and my father Jorma for their love and support in all my studies and in life in general. I thank all my relatives and friends for the encouragement and the many joyful moments outside work.

My deepest love and thanks to Tuomo for believing in me. I thank him for his love and support, and for the opportunity to forget science and research at home and just to enjoy the small things.

This work was financially supported by the Academy of Finland, Biocenter Oulu, the Finnish Cultural Foundation, the Foundation of the Finnish Anti-Tuberculosis Association and Magnus Ehrnrooth Foundation, which all are gratefully acknowledged.

Oulu, May 31st, 2007 Mirva Peltoniemi
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper, zinc superoxide dismutase</td>
</tr>
<tr>
<td>DFA</td>
<td>Diffuse fibrosing alveolitis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECSOD</td>
<td>Extracellular superoxide dismutase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELF</td>
<td>Epithelial lining fluid</td>
</tr>
<tr>
<td>GCL</td>
<td>Glutamate-cysteine ligase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GS</td>
<td>Glutathione synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>γGT</td>
<td>γ-Glutamyltranspeptidase</td>
</tr>
<tr>
<td>HED</td>
<td>β-Hydroxyethyl disulphide</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PICOT</td>
<td>Protein kinase C-interacting cousin of thioredoxin</td>
</tr>
<tr>
<td>Prx</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed phase-high performance liquid chromatography</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-β₁</td>
<td>Transforming growth factor-β₁</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>UIP</td>
<td>Usual interstitial pneumonia</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


Contents

Abstract
Acknowledgements 5
Abbreviations 7
List of original articles 9
Contents 11
1 Introduction 15
2 Review of the literature 17
  2.1 Reactive oxygen species ................................................................. 17
    2.1.1 Intracellular sources of ROS ...................................................... 17
    2.1.2 Increased oxidative burden by exogenous oxidants .................... 19
    2.1.3 The effects of ROS ................................................................. 20
  2.2 Antioxidative mechanisms ............................................................... 21
    2.2.1 Glutathione .............................................................................. 22
    2.2.2 Antioxidative enzymes associated with GSH ......................... 23
    2.2.3 Other antioxidative enzymes .................................................... 24
  2.3 Glutaredoxins .................................................................................. 25
    2.3.1 Glutaredoxin isoforms .............................................................. 26
    2.3.2 Catalytic mechanism ................................................................ 28
    2.3.3 Measuring Grx activity ............................................................ 30
    2.3.4 Catalytically important structures ............................................ 30
    2.3.5 Glutaredoxins in cellular functions ......................................... 31
  2.4 Oxidative stress and glutathione associated antioxidants in human lung diseases ................................................. 33
    2.4.1 Inflammatory diseases ............................................................. 33
    2.4.2 Pulmonary fibrosis ................................................................. 34
    2.4.3 Glutathione homeostasis ......................................................... 35
3 Outlines of the present study 37
4 Materials and methods 39
  4.1 Cell cultures and exposures (I) ........................................................ 39
  4.2 Patient specimens (I, II) ................................................................. 39
  4.3 Qualitative RT-PCR (I) .................................................................. 40
  4.4 Immunohistochemistry (I, II) .......................................................... 40
  4.5 Immunoelectron microscopy (I) ....................................................... 41
  4.6 Protein expression and purification (III, IV) .................................... 41
  4.7 Western blot analysis (I, II, IV) ......................................................... 42
4.8 Assay for determining the deglutathionylation and glutathionylation activity (III, IV) .............................................................. 42
4.9 Analysis of fluorescence data (III, IV) ............................................. 43
4.10 Synthesis of alternative substrates (III) ............................................. 43
4.11 Analysis of reaction intermediates by mass spectrometry (III, IV) .......... 43
4.12 Stopped-flow measurements (III) .................................................... 44
4.13 Statistics (I, II) .............................................................................. 44
4.14 Ethical considerations (I, II, III, IV) ................................................. 44

5 Results

5.1 Expression of Grx1 and Grx2 in human lung cells (I) .......................... 45
5.2 Grx1 in healthy human lung and in different lung diseases (I, II) ........... 45
  5.2.1 Normal lung .............................................................................. 46
  5.2.2 Sarcoidosis and allergic alveolitis .............................................. 46
  5.2.3 Chronic obstructive pulmonary disease ...................................... 46
  5.2.4 Usual interstitial pneumonia ..................................................... 47
  5.2.5 Grx1 in BAL, plasma and induced sputum .................................. 47
5.3 Regulation of Grx1 in human lung cells in vitro (I) ............................... 47
5.4 Determination of the deglutathionylation and glutathionylation activities (III) ................................................................. 48
  5.4.1 Assaying deglutathionylation ..................................................... 48
  5.4.2 Assaying glutathionylation ....................................................... 48
  5.4.3 Analysis of the reaction kinetics ................................................ 49
  5.4.4 Analysis of C14S Grx1 catalysed deglutathionylation reaction .......... 49
  5.4.5 Effects of oxidised glutathione ................................................... 50
  5.4.6 pH-Dependence of the deglutathionylation reaction .................... 50
5.5 Deglutathionylation activity of different enzymes (III) .......................... 50
5.6 Intermediates in the deglutathionylation reaction (III) .......................... 50
5.7 Effect of conserved residues on the glutathione specificity of glutaredoxins (IV) ........................................................................ 51
5.8 Specificity of the second step in the deglutathionylation reaction (IV) ......... 53

6 Discussion

6.1 Grx1 and Grx2 in lung cell lines and in healthy human lung .................. 55
6.2 Possible export of Grx1 to extracellular space .................................... 55
6.3 Grx1 in different lung diseases ......................................................... 56
6.4 Mechanisms of action of glutaredoxins .......................................... 57
6.5 Deglutathionylation activity of different enzymes.......................... 58
6.6 Glutathione binding specificity of glutaredoxins.............................. 59

7 Conclusions 61
References 63
Original articles 75
1 Introduction

Most of the reduction of oxygen in aerobic organisms is carried out in pathways, which reduce oxygen to water. However, even under normal conditions, a small amount of the total oxygen consumed is reduced in a way that generates reactive oxygen species (ROS). When released at high quantities, ROS can cause severe damage to molecules, such as lipids, proteins, DNA and RNA, leading to the induction of apoptosis or even necrosis. Therefore, the production as well as removal of ROS must be strictly controlled in order to sustain favoured cellular functions and avoid harmful overproduction.

Cells have developed a variety of ways to protect themselves against ROS. Effective antioxidant defence mechanism is especially important in the lung since lung cells have to cope with the additional oxidative burden represented by inhaled oxidants. Glutathione (GSH) related pathways have been considered to be one of the major antioxidant defence mechanisms especially in human lung since GSH content in the epithelial lining fluid of human airways is over 100-fold higher than in the serum. The most important antioxidant enzymes associated with the GSH homeostasis include the rate-limiting enzyme in GSH synthesis, glutamate-cysteine ligase, and the enzymes participating in the degradation of hydrogen peroxide, glutathione peroxidases. In addition to these enzymes, the glutaredoxin (Grx) family of enzymes has possible antioxidative properties and close association with GSH homeostasis.

Classical glutaredoxins belong to the thioredoxin fold superfamily according to their three dimensional structures and have a disulphide/dithiol in the active site sequence (-CXXC-). Glutaredoxins catalyze general disulphide reductions of oxidized proteins and GSH-protein mixed disulphides, preferring the latter as substrates. It can be hypothesized that glutaredoxins due to their close functional association with GSH play a significant role in protecting human lung against endogenous and exogenous oxidants in vivo. However, with the exception of only two published microarray studies that mention Grx, glutaredoxins have not been studied in human lung. The present study characterized the localization and differences in Grx1 protein levels in healthy human lung and in different lung diseases. In addition possible regulatory mechanisms for Grx1 in human lung were investigated.

Since the activity and factors affecting the functions of glutaredoxins cannot be studied efficiently in vivo, the mechanism of action of glutaredoxins was assessed in vitro. Grx activity is often measured using an artificial non-specific
substrate in a spectrophotometric coupled assay. In the present study a new real-time method for measuring the deglutathionylation activity of glutaredoxins was developed. This method offers new insights into the mechanism of action of the glutaredoxins and allows the glutaredoxin-like activity of other thioredoxin superfamily members, such as protein disulphide isomerases, to be determined. The present study also demonstrated that the first reaction intermediate in the deglutathionylation reaction is exclusively Grx-GSH mixed disulphide and defined conserved residues affecting the specificity of GSH binding in the second step of the deglutathionylation reaction.
2 Review of the literature

2.1 Reactive oxygen species

A free radical can be defined as an atom or a group of atoms that have unpaired electron(s). Due to these unpaired electrons, free radicals are extremely reactive. There are many types of free radicals, but the most common forms important in biology and medicine are the ones that are derived from oxygen and are thus called reactive oxygen species (ROS). These species include the superoxide anion and hydroxyl radical which are formed during the partial reduction of molecular oxygen (for example see reactions 1-3 and Hassan 1997).

\[
\begin{align*}
O_2 + e^- & \rightarrow O_2^{•−} \quad (1) \\
O_2^{•−} + e^- + 2H^+ & \rightarrow H_2O_2 \quad (2) \\
H_2O_2 + e^- + H^+ & \rightarrow H_2O + HO^{•} \quad (3)
\end{align*}
\]

Hydrogen peroxide (reaction 2) is also considered to be a reactive oxygen species although it is not a radical.

2.1.1 Intracellular sources of ROS

Reactive oxygen species are formed in cells during normal cellular functions. Most of the oxygen reduction to water is accomplished through tetravalent reduction i.e. without releasing any partially reduced intermediates. However, even under normal conditions part of the molecular oxygen can be reduced via a univalent pathway resulting in the formation of reactive intermediates. Intracellularly ROS can be produced by the mitochondrial electron transport chain (Fig. 1), primarily due to electron leakage at complex I (also called NADH dehydrogenase or NADH Q reductase) and at complex III (also called ubiquinol-cytochrome c reductase or cytochrome bc₁ complex).
Fig. 1. The mitochondrial electron transport chain. Electrons are transferred from complex I or II dehydrogenases to coenzyme Q (Q, also called ubiquinone). The reduced form (QH₂) of coenzyme Q then undergoes two sequential one-electron reductions (the Q cycle) using oxidized and reduced forms of cytochrome b and cytochrome c (Cyt c). The unstable intermediate formed in the Q cycle (·Q⁻) can lead to superoxide formation by transferring electrons directly to molecular oxygen (modified from Finkel & Holbrook 2000).

Other cellular enzymes capable of producing ROS include NADPH oxidase, cytochrome P-450, xanthine oxidase, cyclooxygenase and lipoxygenase. The mechanisms of ROS production have been extensively reviewed (for example see reviews by Kinnula et al. 1995, Hassan 1997, Dalton et al. 1999, Finkel & Holbrook 2000, Sauer et al. 2001, Bayir 2005). Increased amounts of ROS have been detected in a variety of cells in response to cytokines and growth factors such as transforming growth factor-β₁ (TGF-β₁), interleukin-1 and tumour necrosis factor-α (TNF-α) (Meier et al. 1989, Ohba et al. 1994, Lo & Cruz 1995, Thannickal & Fanburg 1995, reviewed by Rhee 1999 and Thannickal & Fanburg 2000). Reactive oxygen species have also been detected in phagocytic cells (Weiss et al. 1977, Weiss et al. 1978), where they possibly play a role both in defence against pathogens and in redox signalling (reviewed by Forman & Torres 2001 and Forman & Torres 2002).
2.1.2 Increased oxidative burden by exogenous oxidants

In addition to cellular oxidants, cells and tissues have to cope with a variety of external oxidants from the environment (reviewed by Kelly 2003). For example, ozone, air pollution, such as nitrogen dioxide, sulphur dioxide and particulates, and cigarette smoke can cause additional oxidative stress especially in lungs. The architecture and large surface area of the lung (Fig. 2) is important for efficient gas exchange, but it also subjects lungs to higher molecular oxygen concentrations and makes them prone to additional oxidative stress. Epidemiological studies have demonstrated a clear association between decreased lung function, cardiovascular diseases, mortality, and airborne concentrations of pollutants (reviewed by Kelly 2003).

Fig. 2. Structure of human lung (modified from the Young Oxford Book of Science, OUP 2001, with permission of Oxford University Press). The large surface area of human lung is accomplished by 200-500 million alveoli, resulting in a combined internal surface area of approximately 75 m².
2.1.3 The effects of ROS

The effects of reactive oxygen species on cellular functions are diverse. On one hand they can damage cells, but on the other they can function as important molecules in cellular signalling events. ROS can harm many critical components in the cell as summarized in Table 1 and recently reviewed by Isabella Dalle-Donne and co-authors (Dalle-Donne et al. 2006).

Table 1. Effects of ROS on cellular components

<table>
<thead>
<tr>
<th>Target</th>
<th>Damage</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>modification of bases and sugars</td>
<td>mutations</td>
</tr>
<tr>
<td></td>
<td>strand breaks</td>
<td>DNA rearrangements</td>
</tr>
<tr>
<td></td>
<td>DNA-protein cross-links</td>
<td>induction of DNA repair systems</td>
</tr>
<tr>
<td>RNA</td>
<td>modification of bases and sugars</td>
<td>translational errors</td>
</tr>
<tr>
<td></td>
<td>strand breaks</td>
<td>inhibition of protein synthesis</td>
</tr>
<tr>
<td>Lipids</td>
<td>peroxidation of lipids</td>
<td>altered properties of membranes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inactivation of membrane-bound receptors or enzymes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>further generation of reactive oxidized products</td>
</tr>
<tr>
<td>Proteins</td>
<td>modification of amino acids</td>
<td>changes in protein structure</td>
</tr>
<tr>
<td></td>
<td>peptide backbone cleavage</td>
<td>inhibition of enzymatic and binding activities</td>
</tr>
<tr>
<td></td>
<td>cross-linking</td>
<td>increased susceptibility to aggregation and proteolysis</td>
</tr>
<tr>
<td></td>
<td>formation of carbonyl derivatives</td>
<td>increased or decreased uptake by cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>altered immunogenicity</td>
</tr>
</tbody>
</table>

These damaging effects of ROS are additive and can lead to chain reactions. Damage to DNA and/or RNA can cause mutations which in turn may affect the translated proteins including those involved in DNA repair. Peroxidation of lipids leads to formation of other reactive compounds, such as α,β-unsaturated aldehydes that can modify nucleophilic residues in proteins. In many cases the final outcome of oxidative stress is disturbance of normal cellular functions leading to apoptosis or even necrosis. When present at sublethal concentrations, ROS can play an important role in cellular signalling. The role of ROS in redox regulation has been implicated in inflammation, tissue injury, cell proliferation, cell differentiation and gene expression (for reviews see Thannickal & Fanburg 2000, Forman & Torres 2001, Sauer et al. 2001 and Forman & Torres 2002).
2.2 Antioxidative mechanisms

Oxidative stress can be defined as an imbalance between the production and detoxification of reactive oxygen species in cells. In order to maintain the balance cells have developed several ways to protect themselves against oxidative stress (see recent review by Rahman et al. 2006). These include many small non-enzymatic antioxidative compounds such as vitamins A, C and E and glutathione, and several enzymatic systems of which some examples are shown in Figure 3.

![Antioxidative mechanisms in mammalian cells and tissues. Superoxide radicals are produced in cells for example by xanthine oxidase (XAO), NADPH oxidase (NADPH-ox) and the mitochondrial electron transport chain (ETC). Superoxide can be converted to hydrogen peroxide by superoxide dismutases (SODs) and further to water and molecular oxygen by catalase (CAT), glutathione peroxidases (GPx), peroxiredoxins (Prx) and Thioredoxin (Trx) / Thioredoxin reductase (TrxR) system. Several enzymes are functioning in close association with glutathione (GSH), a major small molecular weight thiol compound in cells. GSH can be oxidised to GSSG, which is reduced back by glutathione reductase (GR). Other enzymes associated with GSH homeostasis include for example glutathione S-transferases (GSTs), glutamate-cysteine ligase (GCL), glutathione synthetase (GS) and γ-glutamyltranspeptidase (γGT).](image-url)
2.2.1 Glutathione

Glutathione (γ-glutamylcysteinylglycine, GSH) is a small tripeptide. It is synthesised from its constituent amino acids in sequential reactions catalysed by glutamate-cysteine ligase (GCL) (also known as γ-glutamylcysteine synthetase, γ-GCS) and GSH synthetase (GS), the reaction catalysed by GCL being the rate determining step (see reviews by Griffith 1999, Rahman & MacNee 2000 and Pastore et al. 2003). GSH has an unusual γ-linkage instead of normal peptide bond between the two amino acids glutamate and cysteine (Fig. 4).

![Fig. 4. Glutathione, γ-glutamylcysteinylglycine, has an unusual γ-peptide bond and a free sulphydryl group.](image)

The intracellular level of GSH ranges from 0.1 to 10 mM depending on the tissue (Tonkonogi et al. 2003, Satoh & Yoshioka 2006, reviewed by Pastore et al. 2003). It can also be found in micromolar concentrations in the extracellular space such as plasma (Wendel & Cikryt 1980, Jones et al. 2000, Ono et al. 2001, Sakhi et al. 2006, reviewed by Pastore et al. 2003) and lung epithelial lining fluid (Cantin et al. 1987, Baz et al. 1996, Behr et al. 2000). GSH reacts rapidly with hydroxyl radicals and peroxynitrite, and participates in the reduction of hydrogen peroxide and lipid peroxides in cooperation with different enzymes. These reactions lead to the oxidation of GSH to GSSG, which in turn is reduced back to GSH in an NADPH-dependent reaction catalysed by glutathione reductase (GR) (Fig. 3). Under normal conditions, the intracellular ratio of GSH/GSSG is around 100, but during oxidative stress it can transiently decrease significantly (see reviews by Griffith 1999, Rahman & MacNee 2000 and Pastore et al. 2003). Glutathione can
also be bound to proteins. Formation of these GSH-mixed disulphides can inactivate enzymatic processes, but it can also function as a protective mechanism to prevent irreversible modifications of thiols or be a part of redox signal transduction processes (reviewed by Klatt & Lamas 2000, Ghezzi 2005 and Shelton et al. 2005).

2.2.2 Antioxidative enzymes associated with GSH

Several antioxidative enzymes have their catalytic functions closely associated with GSH. These include enzyme families such as glutathione peroxidases (GPx), glutathione-S transferases (GST) and glutaredoxins (Grx).

Glutathione peroxidases are either selenium-dependent or independent enzymes found both intracellularly and in the extracellular space. GPxs can reduce hydrogen peroxide to water through oxidation of GSH. Total of seven potential GPxs have been found in humans (as listed in Swiss-Prot database and also reviewed by Imai & Nakagawa 2003, Rahman et al. 2006). GPx1 and GPx2 have the classical seleno-cysteine active site and are present in the cytosol, GPx2 being specific for gastrointestinal tract. Seleno-cysteine containing glycoprotein GPx3 is secreted to plasma. The fourth selenium-containing form of GPx is mitochondrial/ cytoplasmic GPx4, also called phospholipid hydroperoxide glutathione peroxidase, which is the only one capable of using phospholipid hydroperoxides as substrates. The less well characterized glutathione peroxidases include GPx5, which is selenium independent epididymal secretory glutathione peroxidase, as well as the possibly secreted, selenium-containing GPx6 and selenium independent GPx7.

Glutathione S-transferases are enzymes, which catalyse GSH transfer reactions. There are two GST families: the family of soluble enzymes and the family of microsomal enzymes (MAPEG; membrane-associated proteins in eicosanoid and glutathione metabolism). In humans the soluble GST-family is encoded by 16 genes that are divided into eight different gene classes named alpha, mu, theta, pi, zeta, sigma, kappa and omega (reviewed by Hayes & Strange 2000 and Strange et al. 2001). GSTs are able to detoxify a variety of electrophilic compounds such as oxidised lipids, DNA and catecholamines, acrolein present in tobacco smoke and carcinogenic epoxides. Some members of the GST family also have selenium independent glutathione peroxidase activity toward organic hydroperoxides. GST omega also has thioltransferase activity (Board et al. 2000) similar to that of glutaredoxins (glutaredoxins are described in more detail in
section 2.3). The MAPEG GST-family contains six human proteins (reviewed by Jakobsson et al. 1999), namely MGST (microsomal glutathione S-transferase) 1, 2 and 3 with GST and GPx activity, MGST1-L1 (microsomal glutathione S-transferase 1-like 1), which is inducible by p53, and FLAP (5-lipoxygenase activating protein) and LTC4S (leukotriene C₄ synthase), which are crucial for leukotriene biosynthesis.

### 2.2.3 Other antioxidative enzymes

The harmful free radical, superoxide anion can be reduced either non-enzymatically or by superoxide dismutases (SOD) to less damaging hydrogen peroxide which in turn is reduced to water for example by GPx, peroxiredoxins (Prx) and catalase (CAT) (Fig. 3). Three different forms of SODs with different structure, localization, inducibility and metal ion requirement have been described (reviewed by Kinnula & Crapo 2003, Kinnula & Crapo 2004 and Rahman et al. 2006). These include cytosolic copper, zinc SOD (CuZnSOD), mitochondrial manganese SOD (MnSOD) and extracellular SOD (ECSOD) which requires copper and zinc for its activity. CuZnSOD is a homodimer whereas MnSOD and ECSOD are homotetramers. SODs are the only enzymes capable of dismutating superoxide radicals. Studies on knock-out mice have shown that MnSOD is essential, whereas CuZnSOD knock-out mice are viable for two weeks and ECSOD knock-out mice develop normally and remain healthy until at least 14 months of age (reviewed by Kinnula & Crapo 2003).

Catalase is a homotetrameric enzyme that is ubiquitous to most aerobic cells in animals where it can be found mainly in peroxisomes, but also in the cytoplasm. It has a heme, ferriprotoporphyrin IX, at the active site in each subunit. In the basic reaction in scavenging hydrogen peroxide this heme iron is oxidized by H₂O₂ to form an oxyferryl group which is further oxidised by another molecule of H₂O₂ thus resulting in the release of water, oxygen and catalase in an active form (reviewed by Kirkman & Gaetani 2007).

Peroxiredoxins are selenium independent peroxidases (see reviews by Rhee et al. 2001, Kang et al. 2005 and Manevich & Fisher 2005). In mammals, total of six Prxs have been characterized. They contain either one or two conserved cysteines and can thus be divided to 1-Cys (PrxVI) or 2-Cys peroxiredoxins (PrxI-IV). PrxV has one conserved and two additional cysteines and it shares only ~10% sequence homology with other 2-Cys Prxs. Although PrxV has only one conserved cysteine, it is classified as an atypical 2-Cys Prx since its function is
dependent on two cysteine residues. All 2-Cys Prxs use thioredoxin (Trx) as a hydrogen donor when scavenging hydrogen peroxide. PrxVI has both GSH peroxidase and phospholipase A₂ activities. It uses GSH as an electron donor to reduce H₂O₂ or other hydroperoxides. PrxI, PrxII and PrxVI have similar subcellular localization mainly in cytoplasm, but at least PrxI has been suggested to also be located in the nucleus, mitochondria and peroxisomes. PrxV is also widely distributed in cells and it has been found in cytoplasm, nucleus and in different cell organelles. PrxIII has a cleavable mitochondrial targeting signal, whereas PrxIV has been suggested to exist in either an intracellular form or as a membrane bound inactive precursor which can be cleaved and released to the extracellular space in some cell types.

Thioredoxins have a dithiol/disulphide active site -CGPC-. Together with thioredoxin reductase (TrxR) and NADPH they form the thioredoxin system, a robust cellular protein disulphide reductase system. One cytoplasmic (Trx1) and one mitochondrial (Trx2) thioredoxin have been found in humans, whereas bacteria, yeast and plants have multiple thioredoxin isoforms. Thioredoxin reductase is a selenoprotein with one cytosolic and one mitochondrial isoform, and one additional cytosolic isoform expressed mainly in testis (reviewed by Arnér & Holmgren 2000). Trx1 and TrxR have also been detected in the extracellular space such as plasma (Nakamura et al. 1998, Söderberg et al. 2000). The glutaredoxin system has overlapping functions with the thioredoxin system, but the two systems display different disulphide substrate preferences as glutaredoxins are considered to be more specialized in reducing glutathione-mixed disulphides.

2.3 Glutaredoxins

Glutaredoxin was probably first found in 1955 by Racker who discovered an enzyme in liver, which catalysed a hydrogen transfer from glutathione to homocystine which he called glutathione-homocystine transhydrogenase (Racker 1955). Twenty years later a cytosolic protein capable of catalyzing the reduction of protein disulphides as well as GSH-mixed disulphides was characterized from rat liver and named thioltransferase (Mannervik & Axelsson 1975). Around same time an alternative hydrogen donor for ribonucleotide reductase from a thioredoxin 1 knockout Escherichia coli strain was found and this enzyme was named glutaredoxin (Holmgren 1976). A couple of years later this glutaredoxin was shown to also have GSH-disulphide transhydrogenase activity thus
functioning as general thiol disulphide oxidoreductase (Holmgren 1979a, 1979b). Eventually it became evident that thioltransferases and glutaredoxins purified from different organisms were highly similar and were actually the same enzymes just named differently (Gan & Wells 1988, Yang & Wells 1990).

2.3.1 Glutaredoxin isoforms

Classical glutaredoxins are small proteins with a conserved active site -CPYC- and a glutathione recognition site (Höög et al. 1983, Klintrot et al. 1984, Bushweller et al. 1992, Bushweller et al. 1994, Luikenhuis et al. 1998). They belong to the thioredoxin fold superfamily (reviewed by Martin 1995, Carvalho et al. 2006 and Pan & Bardwell 2006) according to their three dimensional structures (Fig. 5) (Sodano et al. 1991, Xia et al. 1992, Katti et al. 1995, Sun et al. 1998).

Fig. 5. The thioredoxin fold superfamily members have common structural features. Human Trx1 (PDB code 1ERV, Weichsel et al. 1996) and human Grx1 (PDB code 1JHB, Sun et al. 1998) represent the classical forms in their protein family, whereas E. coli Grx2 (PDB code 1G7O, Xia et al. 2001) has also GST-like elements.

Besides the classical dithiol active site glutaredoxins, two other classes of glutaredoxins can be identified. These can be defined as glutaredoxins structurally related to glutathione-S-transferases and as monothiol glutaredoxins with the active site -CGFS- and thus lacking the C-terminal cysteine (reviewed by Fernandes & Holmgren 2004 and Herrero & Torre-Ruiz 2007).
Multiple glutaredoxins have been found in different organisms as summarized in Table 2. In humans two dithiol glutaredoxins have been characterized so far, namely the classical cytosolic Grx1 (Mieyal et al. 1991, Padilla et al. 1995) and mitochondrial/nuclear Grx2 with an unusual -CSYC- active site (Gladyshev et al. 2001, Lundberg et al. 2001). Also two possible monothiol glutaredoxins have been found in humans, namely Grx5, which is a single domain protein with mitochondrial targeting sequence (Molina-Navarro et al. 2006), and PICOT (protein kinase C-interacting cousin of thioredoxin), which has Trx-Grx structure (Isakov et al. 2000, Witte et al. 2000). A total of five glutaredoxins have been found from Saccharomyces cerevisiae (reviewed by Grant 2001). These include two classical dithiol glutaredoxins (cytosolic Grx1 and cytosolic/mitochondrial Grx2) (Luikenhuis et al. 1998, Pedrajas et al. 2002) and three monothiol glutaredoxins (Rodriguez-Manzaneque et al. 1999) of which single domain Grx5 localizes to mitochondria (Rodriguez-Manzaneque et al. 2002). Two other S. cerevisiae monothiol glutaredoxins have Trx-Grx structure and localize predominantly to the nucleus (Molina et al. 2004). E. coli has one single domain monothiol glutaredoxin (Fernandes et al. 2005) and three dithiol glutaredoxins (Åslund et al. 1994) of which Grx2 belongs to the category structurally related to GSTs (Vlamis-Gardikas et al. 1997, Xia et al. 2001). The largest numbers of glutaredoxins are found among plants. For example Populus trichocarpa has 36, Arabidopsis thaliana has 31 and Oryza sativa has 27 genes coding for glutaredoxins that can be divided into three subgroups according to their active site structures (reviewed by Rouhier et al. 2006). Subgroup I contains classical glutaredoxins with single -CPYC-, -CGYC-, -CPFC- or -CSY[C/S]- active sites whereas subgroup II enzymes possess a -CGFS- active site, but the number of repeated modules varies. Subgroup III is more complex with a wide range of active site sequences -C[C/F/P/G/Y][M/L][C/S/G/A/I]- with species specific combinations (reviewed by Rouhier et al. 2006).
Table 2. Glutaredoxin isoforms

<table>
<thead>
<tr>
<th>Species</th>
<th>Isoform</th>
<th>Classification</th>
<th>Active site</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Grx1</td>
<td>classical</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>Grx2</td>
<td>classical</td>
<td>-CSYC-</td>
<td>nuclear/mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Grx5</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>mitochondrial</td>
</tr>
<tr>
<td>PICOT</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>cytosolic</td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Grx1</td>
<td>classical</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>Grx2</td>
<td>classical</td>
<td>-CPYC-</td>
<td>cytosolic/mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Grx3</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>nuclear</td>
</tr>
<tr>
<td></td>
<td>Grx4</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>nuclear</td>
</tr>
<tr>
<td></td>
<td>Grx5</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>mitochondrial</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Grx1</td>
<td>classical</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>Grx2</td>
<td>GST-like</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>Grx3</td>
<td>classical</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>Grx4</td>
<td>monothiol</td>
<td>-CGFS-</td>
<td>cytosolic</td>
</tr>
<tr>
<td>Populus trichocarpa*</td>
<td>GrxC1,1</td>
<td>subgroup I</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>GrxC1,2</td>
<td>subgroup I</td>
<td>-CPYC-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>GrxC2</td>
<td>subgroup I</td>
<td>-CPFC-</td>
<td>ER/cytosolic</td>
</tr>
<tr>
<td></td>
<td>GrxC3</td>
<td>subgroup I</td>
<td>-CPYC-</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>GrxC4</td>
<td>subgroup I</td>
<td>-CPYC-</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>GrxS12</td>
<td>subgroup I</td>
<td>-CSYS-</td>
<td>plastid</td>
</tr>
<tr>
<td></td>
<td>GrxS14</td>
<td>subgroup II</td>
<td>-CGFS-</td>
<td>plastid</td>
</tr>
<tr>
<td></td>
<td>GrxS15</td>
<td>subgroup II</td>
<td>-CGFS-</td>
<td>mitochondrial</td>
</tr>
<tr>
<td></td>
<td>GrxS16</td>
<td>subgroup II</td>
<td>-CGFS-</td>
<td>plastid</td>
</tr>
<tr>
<td></td>
<td>GrxS17,1</td>
<td>subgroup II</td>
<td>-CGFS-</td>
<td>cytosolic</td>
</tr>
<tr>
<td></td>
<td>GrxS17,2</td>
<td>subgroup II</td>
<td>-CGFS-</td>
<td>cytosolic</td>
</tr>
</tbody>
</table>

* Only subgroups I and II are presented here.

2.3.2 Catalytic mechanism

Glutaredoxins catalyse the GSH-dependent reduction of protein disulphides or protein-GSH mixed disulphides, preferring the latter as substrates (Gravina & Mieyal 1993). The reduction of protein disulphides is thought to proceed via the dithiol pathway (Fig. 6A) whereas the reduction of GSH-mixed disulphides i.e. deglutathionylation can be accomplished by the monothiol pathway (Fig. 6B) (for proposed reaction mechanisms see Yang & Wells 1991b, Bushweller et al. 1992, Gravina & Mieyal 1993, Srinivasan et al. 1997, Yang et al. 1998 and review by Fernandes & Holmgren 2004).
Fig. 6. Reaction mechanisms of glutaredoxins. A. The dithiol pathway is used in reducing protein disulphides. The intermediate in the reaction is a mixed disulphide between Grx and the substrate protein. Finally the substrate is released in reduced form and Grx in oxidised form, which can be reduced back to active form by GSH. B. The monothiol pathway is utilized in the reduction of GSH-mixed disulphides. The first intermediate in the reaction is Grx-GSH mixed disulphide. Glutathionylated Grx can then be either reduced directly by another molecule of GSH or oxidised and then re-reduced by GSH.

Both pathways use GSH as reducing power and concomitantly produce GSSG which in turn is reduced back to GSH by GR and NADPH (Fig. 6A-B). The first step in the monothiol mechanism is a nucleophilic attack initiated by the N-terminal active site thiol of Grx on the mixed disulphide between protein thiol and GSH. This leads to a formation of a new disulphide between Grx and GSH and
release of a protein substrate in the reduced form (Fig. 6B, reaction 1). The mixed disulphide between Grx and GSH is in turn reduced by another molecule of GSH through a nucleophilic attack on the mixed disulphide to form GSSG and reduced Grx as the final products (Fig. 6B, reaction 2). In the dithiol pathway, the first step is a nucleophilic attack from glutaredoxin on a sulphur atom of the disulphide in the target protein (Fig. 6A, reaction 1). Through series of nucleophilic attacks, Grx is finally released in oxidised form and the target protein in the reduced form (Fig. 6A, reaction 2). Grx can then be reduced back to its active form by GSH (Fig. 6A, reaction 3). A partition reaction to the oxidised form of Grx is also possible in the monothiol pathway of dithiol glutaredoxins (Fig. 6B, reaction 3).

2.3.3 Measuring Grx activity

The deglutathionylation activity of Grx is often measured using an artificial non-specific substrate HED (β-hydroxyethyl disulphide) in a spectrophotometric coupled assay measuring the consumption of NADPH by GR as described by Holmgren (Holmgren 1979a). Less frequently more specific substrates such as cysteine-glutathione mixed disulphides or (radiolabelled) glutathionylated proteins are used for measuring the activity either as coupled to NADPH or by radioactive determinations (Mannervik & Axelsson 1975, Mannervik & Axelsson 1980, Gravina & Mieyal 1993, Srinivasan et al. 1997, Yang et al. 1998, Lundström-Ljung et al. 1999). To measure the disulphide reduction activity the ribonucleotide reductase assay is usually used (Holmgren 1979a, Bushweller et al. 1992). The assay measures the formation of [3H]dCDP from [3H]CDP with ribonucleotide reductase in the presence of GSH, GR and NADPH or with all three replaced with DTT.

2.3.4 Catalytically important structures

Residues which affect Grx activity have been studied with structural and site directed mutagenesis studies. It has been demonstrated that the N-terminal cysteine in the active site is essential for Grx activity, but that mutating the C-terminal cysteine to serine in human Grx or pig liver Grx can even increase the deglutathionylation activity of the enzyme (Yang & Wells 1991a, Yang & Wells 1991b, Yang et al. 1998). In contrast, in E. coli Grx1 or Grx3 this mutation seems to decrease the deglutathionylation activity (Bushweller et al. 1992, Nordstrand et
al. 1999) and furthermore, the monothiol glutaredoxins, *E. coli* Grx4 and *S. cerevisiae* Grx5, seem to be totally inactive in the HED assay (Tamarit *et al.* 2003, Fernandes *et al.* 2005).

The N-terminal cysteine residue in the -CXXC- active site has an unusually low pKₐ (3.5-5.0; Mieyal *et al.* 1991, Yang & Wells 1991a, Sun *et al.* 1998, Foloppe *et al.* 2001, Foloppe & Nilsson 2004). This low value has been attributed to the influence from peripheral residues such as Arg8 in *E. coli* Grx1 (Foloppe & Nilsson 2004) or Lys19 in the corresponding position in human Grx1 (Jao *et al.* 2006). Mutating Lys19 to glutamine or leucine has also been shown to decrease the deglutathionylation activity down to approximately 20% of wild type activity (Jao *et al.* 2006). In one study Arg26 in pig liver Grx1 was shown to be partly responsible for the low pKₐ of the N-terminal cysteine and mutating this amino acid resulted also in lower deglutathionylation activity of the enzyme (Yang & Wells 1991a).

Besides direct binding to the N-terminal active site cysteine in *E. coli* Grx1 C14S mutant, there are other residues, namely Tyr13, Thr58, Val59, Tyr72, Thr73 and Asp74, which have been described to be involved in the interactions between the –SG moiety and the protein (Bushweller *et al.* 1994). A similar study with human Grx1 found residues in analogous positions which also affect GSH binding (Yang *et al.* 1998). Grx has also a cis-proline, which is found in all thiol-oxidoreductases at a structurally analogous place (Katti *et al.* 1995, reviewed by Martin 1995), and which is thought to be involved in interactions with protein substrates.

### 2.3.5 Glutaredoxins in cellular functions

Glutaredoxins have been suggested to be involved in a variety of cellular functions. These include defence against oxidative stress, as demonstrated by the ability of yeast glutaredoxins to directly reduce hydroperoxides with catalytic rates comparable to glutathione peroxidases (Collinson *et al.* 2002), and the sensitivity of the yeast mutants lacking Grx to oxidative stress (Luikenhuis *et al.* 1998). Mammalian Grx2 is also able to catalyse the reduction of hydroperoxides *in vitro*, although reasonably high concentration of enzyme is needed (Fernando *et al.* 2006). Furthermore, human Grx1 has been shown to be able to scavenge glutathione-thiol radicals (Starke *et al.* 2003). Grx has also been shown to mediate the recovery from oxidative damage by reducing protein-GSH mixed disulphides.
and restoring the protein thiol homeostasis in brain mitochondria (Kenchappa et al. 2002) or human lens epithelial cells (Xing & Lou 2003).

In addition to being a defence/recovery mechanism against oxidative stress, glutaredoxins have been demonstrated to play a role in many redox signal transduction events (reviewed by Shelton et al. 2005) including restoring the DNA-binding activity of nuclear factor I (NFI)-C by reducing the single cysteine residue of NFI-C which is probably glutathionylated in oxidative environment (Bandyopadhyay et al. 1998). Grx can also deactivate the OxyR transcription factor which is active in the oxidised form and thus activates the expression of antioxidant genes under oxidative stress conditions in \textit{E. coli} (Zheng et al. 1998). Since OxyR also activates Grx itself, it can thus provide a mechanism of autoregulation (Zheng et al. 1998).

Grx can protect cells against apoptosis as shown by a study demonstrating the critical role of glutaredoxin system in protecting macrophages from cell death induced by oxidised low density lipoprotein (Wang et al. 2006). In addition, Grx functions as a negative regulator of ASK1 (apoptosis signal-regulating kinase 1) and dissociation of Grx from ASK1 activates JNK (Jun N-terminal kinase) pathway (Song et al. 2002, Song & Lee 2003). Grx has also been shown to protect cerebellar granule neurons from dopamine-induced apoptosis by activating the nuclear factor-\(\kappa\)B (Daily et al. 2001a, Daily et al. 2001b). Grx can regulate apoptosis through the redox state of the serine/threonine kinase Akt (Murata et al. 2003). Human Grx2 seems to prevent cardiolipin oxidation and cytochrome \(c\) release from mitochondria thus protecting cells from mitochondrially mediated apoptosis (Enoksson et al. 2005). On the other hand Grx activity might be also proapoptotic as demonstrated in a study where small interference RNA knock down of Grx in endothelial cells significantly inhibited TNF-\(\alpha\) -induced cell death through attenuated caspase-3 cleavage with a concomitant increase in caspase-3 glutathionylation (Pan & Berk 2007).

In addition to above mentioned functions, glutaredoxin has been detected within HIV-1 and has been shown to regulate the activity of glutathionylated HIV-1 protease \textit{in vitro} (Davis et al. 1997). Mammalian Grx1 can also reduce dehydroascorbic acid to ascorbic acid which in turn can reduce oxidants in cells (Wells et al. 1990, Park & Levine 1996, Washburn & Wells 1999). Grx has also been proposed to have a role during cell differentiation in differentiating macrophages (Takashima et al. 1999). Human Grx1 has been found to be present in the extracellular space such as plasma (Nakamura et al. 1998, Lundberg et al. 2004) and to have a role in exocytosis (Padilla et al. 1992, Ivarsson et al. 2005).
E. coli Grx1 has been shown to serve as electron donor for PAPS (3’-phosphoadenylylsulfate) reductase, an enzyme which reduces inorganic sulphate to sulphite making reduced sulphur available for biosynthetic reactions (Tsang 1981, Lillig et al. 1999). All three dithiol E. coli glutaredoxins can reactivate the PAPS reductase by reducing the inhibitory GSH-mixed disulphide formed between GSH and the PAPS reductase active site thiol (Lillig et al. 2003). They are also able to serve as hydrogen donors for the reduction of arsenate by ArsC by reducing the ArsC GSH-mixed disulphide formed in the reaction (Shi et al. 1999).

2.4 Oxidative stress and glutathione associated antioxidants in human lung diseases

Oxidative stress and the impairment of antioxidative mechanisms have been implicated in the pathogenesis of many lung diseases (for example see reviews by Rahman & MacNee 2000, Kinnula 2005, Kinnula et al. 2005 and Rahman et al. 2006). Cigarette smoke is a well known external contributor to oxidative stress and pathogenesis of several lung diseases. Many of the antioxidative functions in lungs are related to the small antioxidative thiol, glutathione. The glutathione content in the epithelial lining fluid (ELF) of human airways is over 100-fold higher than in the plasma (Cantin et al. 1987), and together with reasonably high concentrations of intracellular glutathione, GSH-related pathways can be considered as one of the major antioxidant defence mechanisms of human lung. Alterations in GSH homeostasis have been found in smokers and in many lung diseases including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome, idiopathic pulmonary fibrosis (IPF), neonatal lung damage and asthma (reviewed by Rahman & MacNee 2000, Comhair & Erzurum 2002 and Rahman et al. 2006).

2.4.1 Inflammatory diseases

The accumulation of inflammatory cells such as macrophages and neutrophils in the respiratory tract is a general phenomenon both in inflammatory airway diseases such as asthma and COPD, and also in several interstitial lung diseases including sarcoidosis and allergic alveolitis. In all of these diseases inflammatory cells show increased generation of reactive oxygen species (Fig. 7). In addition, lung epithelial cells can release ROS thus directly stimulating inflammatory cells and further promoting inflammation.
Immunopathogenesis of sarcoidosis and allergic alveolitis includes the accumulation and activation of inflammatory cells as well as formation of pulmonary granulomas originating from monocytic cells (reviewed by Müller-Quernheim 1998 and Bourke et al. 2001). In COPD, besides the increased number of inflammatory cells in the airways and airway obstruction, other characteristic features involve tissue injury due to emphysema and mucus hypersecretion (reviewed by Barnes 2004 and MacNee 2005). According to the severity of symptoms COPD patients can be classified into different GOLD stages (Pauwels et al. 2001).

Fig. 7. Mechanisms of oxidant-mediated lung inflammation. In addition to inhaled oxidative compounds, oxidants can be released by activated neutrophils, alveolar macrophages (AMs) and epithelial cells. This can lead to an imbalance in glutathione homeostasis and activate inflammatory responses. GSH, reduced glutathione; GSSG, oxidised glutathione; GSX, GSH-mixed disulphides and other glutathionyl adducts (modified from Rahman & MacNee 2000).

2.4.2 Pulmonary fibrosis

In addition to inflammatory diseases, other lung diseases are also associated with impaired antioxidative defence. One of these is idiopathic pulmonary fibrosis (IPF), the histopathological findings of this clinical entity being usual interstitial
pneumonia (UIP), which is an aggressive and progressive disease with poor prognosis (classification according to ATS/ERS 2002). Typical features in IPF include dyspnea, diffuse interstitial infiltrates, progressive lung fibrosis appearing as patchy fibrotic lesions and only weak inflammation (reviewed by Kinnula et al. 2005).

### 2.4.3 Glutathione homeostasis

GSH is present in increased concentrations in the ELF and BALF (bronchoalveolar lavage fluid) of chronic smokers (Morrison et al. 1999, Nagai et al. 2006), and both acute and chronic exposure of experimental animals to cigarette smoke causes depletion in the intracellular GSH concentration (Rahman et al. 1995, reviewed by Rahman & MacNee 2000). The GSH content in the ELF of human airways has been shown to be decreased in different lung diseases such as IPF (Cantin et al. 1989), allergic alveolitis (Behr et al. 2000), diffuse fibrosing alveolitis (DFA) (Behr et al. 1995) and during COPD exacerbations (Drost et al. 2005), as well as in HIV-seropositive subjects (Pacht et al. 1997).

The rate limiting enzyme in GSH synthesis, GCL, is induced by acute oxidant exposure and cytokines (reviewed by Rahman & MacNee 1999, Rahman & MacNee 2000 and Kinnula 2005) and downregulated by TGF-β1 in vitro (Arsalane et al. 1997). Also uptake of cystine into cells regulates the intracellular glutathione homeostasis as demonstrated by exposing rats or cultured cells to hyperoxia and oxidative stress (Deneke & Fanburg 1989, Miura et al. 2000). The level of glutathione peroxidase (classical intracellular form) in human lung is constant (Asikainen et al. 1998), whereas extracellular glutathione peroxidase (GPx3) is induced in the ELF of asthma patients (Comhair et al. 2001) and during exposure to cigarette smoke (Comhair et al. 2000). One microarray study showed increased GPx2, GPx3 and GCL mRNA levels in airway epithelial cells in smokers compared to non-smokers (Hackett et al. 2003). Polymorphism of GSTs is usually associated with different cancers (for example see Gaspar et al. 2004, Sørensen et al. 2004, Srivastava et al. 2005, Agalliu et al. 2006, Gonlugur et al. 2006, Marahatta et al. 2006, Nagle et al. 2007, Saadat 2006 and review by Parl 2005) but there are some studies showing association with COPD (Ishii et al. 1999, Cheng et al. 2004), asthma (Fryer et al. 2000) and with the decline in lung function parameters in smokers (He et al. 2002, He et al. 2004).

One of the enzymes with close association with GSH and potential antioxidative properties is glutaredoxin, but the data on glutaredoxins and their
role in lung diseases is very limited. One microarray study found no change in Grx1 mRNA levels in the bronchial epithelium of healthy smokers when compared to non-smokers (Hackett et al. 2003). In another study on cultured bronchial epithelial cells Grx was found to be elevated by 10 fold during the first 10 hours of exposure to cigarette smoke (Yoneda et al. 2003). One recent study in mouse model of allergic airway disease reported Grx1 levels to be increased in the lungs of these mice and showed decreased Grx1 expression in primary tracheal epithelial cells exposed to TGF-β1 (Reynaert et al. 2007).
3 Outlines of the present study

The purpose of this study was to determine possible differences in the localization and protein expression levels of glutaredoxins in healthy and diseased human lung and to assess the mechanism of action of glutaredoxin family members in vitro. More specifically the aims were:

1. To characterise the localization of Grx1 in healthy human lung and in different lung diseases
2. To compare Grx1 protein levels in different lung diseases and find out possible regulatory mechanisms for Grx1 in human lung
3. To develop a new method for measuring the deglutathionylation activity of glutaredoxins in vitro
4. To assess the mechanism of action of glutaredoxins
5. To elucidate the role of conserved residues in the specificity of GSH binding by *E. coli* Grx1
4 Materials and methods

4.1 Cell cultures and exposures (I)

Human primary bronchial epithelial (PBE), alveolar epithelial (A549) and nonmalignant simian virus 40 transformed mesothelial (MeT5A) cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). SV40 transformed bronchial epithelial (BEAS 2B) cells were obtained from the National Cancer Institute, Laboratory of Human Carcinogenesis (Dr Harris, Bethesda, MD, USA). A549 cells were grown in Ham’s Nutrient mixture F-12 with L-glutamine (Gibco Life Technologies, Gaithersburg, MD, USA) supplied with foetal bovine serum (FBS), penicillin and streptomycin (all from Gibco). MeT5A cells were cultured in monolayer in RPMI 1640 medium with 25 mM HEPES buffer and L-glutamine (Gibco) and supplied with FBS and antibiotics as above. PBE and BEAS 2B cells were cultured in BEBM supplemented with non-essential amino acids (Clonetics, San Diego, CA, USA). All cells were grown at 37 °C in 5 % CO₂ atmosphere. A549 cells were exposed to 2 ng/ml of TGF-β₁ or 50 ng/ml of TNF-α (both from Sigma, St. Louis, MO, USA) for varying times with corresponding unexposed control at each time point.

4.2 Patient specimens (I, II)

The tissue material included uninvolved peripheral lung tissue from lung surgery (hamartomas, carcinoid tumours and lung carcinomas) representing healthy lung from non-smokers and smokers, and histopathologically typical cases of sarcoidosis, extrinsic allergic alveolitis, UIP (ATS/ERS 2002) and different stages (Pauwels et al. 2001) of COPD (all from Department of Pathology, Oulu University Hospital). The specimens of the patients with very severe COPD or emphysema from α-1-antitrypsin deficiency (stage IV) were retrieved from lung transplantations (Department of Pathology, Helsinki University Hospital). Bronchoalveolar lavage (BAL) samples (normally representing > 90% alveolar macrophages) included healthy controls, sarcoidosis, allergic alveolitis and UIP. Plasma and induced sputum samples were collected from healthy non-smokers and smokers and from COPD patients with a stable disease state as well as during exacerbation. The biopsy material was fixed in 10 % neutral formalin, dehydrated
and embedded in paraffin. The material for Western analysis was immediately frozen and stored at -80 °C or in liquid nitrogen.

4.3 Qualitative RT-PCR (I)

Total cellular RNA was extracted from A549, MeT5A, BEAS 2B and PBE cell lines with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Samples containing 0.5 µg or 1.0 µg of total cellular RNA were reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (Gibco) and appropriate downstream primer for Grx1 or Grx2. The resulting cDNA fragments were PCR amplified using Dynazyme II DNA polymerase (Finnzymes Oy, Espoo, Finland) and specific upstream primers for Grx1 or Grx2. The PCR products were electrophoresed in an ethidium bromide stained 1.5 % SeaKem Le agarose (FMC BioProducts, Rockland, ME, USA) gel in 1xTBE (Tris-Borate-EDTA), and visualized under ultraviolet light with Kodak 1D Image Analysis Software (Eastman Kodak Companies, Rochester, NY, USA).

4.4 Immunohistochemistry (I, II)

Four micrometer thick paraffin sections were cut from the representative paraffin blocks and collected onto 3’-aminopropyltriethylsilane coated glass slides. The sections were deparaffinized in xylene, rehydrated through a series of ethanol solutions, and washed with phosphate buffered saline (PBS). Frozen sections were collected on untreated glass slides and fixed in acetone. The following staining protocol was used for both paraffin and frozen sections: Endogenous peroxidase activity was eliminated by incubation in 1 % H2O2 in absolute methanol and nonspecific antibody binding sites were blocked using normal rabbit serum (Vector Laboratories Inc, Burlingame, CA, USA). The sections were incubated with goat anti-human Grx1 antibody followed by incubation with biotinylated rabbit anti-goat antibody (Zymed Laboratories Inc, San Francisco, CA, USA). The avidin-biotin complex and aminoethylcarbazole (AEC) colour reactions were made according to the instructions of the Histostain™ Plus –kit (Zymed). The sections were counterstained with Mayer’s hematoxylin (OY Reagenia LTD, Kuopio, Finland) and mounted with ImmuMount (Shandon, Pittsburg, PA, USA). As a negative control, the primary antibody was replaced by PBS or non-immune goat serum (Zymed). The paraffin embedded sections from normal lung, sarcoidosis, extrinsic allergic alveolitis, UIP and COPD were
evaluated independently by at least two investigators by semiquantitatively grading the staining intensity separately in alveolar macrophages, bronchial epithelial cells and alveolar cells.

4.5 Immunoelectron microscopy (I)

Macrophages from BAL of sarcoidosis were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer with 2.5 % sucrose, pH 7.4 for 1 hour. Cells were embedded in 4 % gelatin, immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome (Leica, Vienna, Austria) and the immunolabeling was performed as described previously (Harju et al. 2002) using a polyclonal antibody to Grx1.

4.6 Protein expression and purification (III, IV)

All constructs used in the experiments were cloned into an expression vector generated previously, which incorporates an N-terminal His-tag (Alanen et al. 2003) or Myc-tag to the cloned gene. Site directed mutagenesis was performed according to instructions of the Pfu™Turbo DNA polymerase kit (Stratagene, La Jolla, CA, USA). All plasmids were checked for correctness by sequencing. Proteins were expressed in E. coli strains BL21 (DE3) pLysS or rosetta gami. Strains were grown in LB medium supplemented with appropriate antibiotics at 37 °C and induced at an A600 of 0.3 for four hours with 1 mM isopropyl β-D-thiogalactoside (Fermentas, Burlington, ON, Canada). Cells were harvested by centrifugation and the pellet was resuspended in 20 mM sodium phosphate pH 7.3 (Buffer A) with 1 μg/ml DNase (Roche Diagnostics GmbH, Mannheim, Germany). The cells were lysed by freezing and thawing twice and centrifuged. Myc-tagged constructs were used as cell lysates in Western blotting. His-tagged constructs were purified by loading the filtered supernatant on immobilized metal affinity chromatography column (HiTrap™ Chelating HP, Amersham Pharmacia Biotech AB, Uppsala, Sweden) precharged with Ni²⁺ and equilibrated in buffer A. The column was washed with 50 mM imidazole, 0.5 M sodium chloride in buffer A and bound proteins were eluted with three column volumes of 50 mM EDTA in buffer A. Depending on the properties of each protein of interest, they were further subjected to either anion (Resource Q, Amersham) or cation (Resource S, Amersham) exchange chromatography. The proteins were eluted with linear gradient from buffer A to 0.5 M sodium chloride in buffer A. The purity of eluted
fractions was tested on Coomassie Brilliant Blue stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and pure fractions were combined and buffer exchanged into buffer A. The purity and size of proteins was also tested by mass spectrometry.

4.7 Western blot analysis (I,II, IV)

The cell pellets or tissue specimens were homogenized and a known amount of total cell protein was boiled for 5 min at 95 °C with reducing electrophoresis sample buffer. *E. coli* lysates expressing Myc-tagged constructs were mixed with either reducing or non-reducing sample buffer. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose or polyvinylidene fluoride membrane. The blotted membranes were incubated with antibodies to Grx1, Grx2 (both a gift from Arne Holmgren, Karolinska Institutet, Stockholm, Sweden), β-actin (Sigma) or Myc-tag (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), followed by treatment with appropriate horseradish peroxidase-conjugated secondary antibodies. The proteins were detected using the enhanced chemiluminescence (ECL) system (Amersham), and the luminol excitation was imaged on X-ray film.

4.8 Assay for determining the deglutathionylation and glutathionylation activity (III, IV)

The glutathionylated substrate peptide SQLWC(glutathione)LSN was designed and ordered, also in reduced form, from University of Kent, Department of Biosciences (Canterbury, Kent, UK). Fluorescence measurements were performed with Perkin-Elmer LS50B spectrometer using a 315 μl cuvette. Deglutathionylation assays were carried out in McIlvain buffer (0.2 M disodium hydrogen phosphate/0.1 M citric acid) at desired pH, including appropriate amounts of GSH, NADPH, glutathione reductase, bovine serum albumin (BSA) (all from Sigma), EDTA, substrate peptide and enzyme of interest. In some measurements GSH was replaced with corresponding reduced tripeptide ECG, which has normal peptide bond instead of the γ-linkage present in GSH. Glutathionylation measurements were performed in McIlvain buffer at pH 7.0, including GSSG, BSA, EDTA, reduced substrate peptide and the enzyme of interest. All measurements were done at 25 °C, excitation 280 nm, emission 356 nm and slit widths 5 nm.
4.9 Analysis of fluorescence data (III, IV)

The fluorescence data was analysed with Igor Pro 3.14 (Wavemetrics Inc., Lake Oswego, OR, USA). Initial rates of reaction were determined from linear fits over 20 units of change in fluorescence signal or other applicable linear part of the curve, and correlating it to the total change in fluorescence observed during the reaction. The corresponding non-catalysed background reaction rates were subtracted from the catalysed rates.

4.10 Synthesis of alternative substrates (III)

The reduced substrate peptide was incubated with 10 fold excess of ECG, QCG or pECG in 0.1 M sodium phosphate buffer, at pH 8 for one hour. Peptide-ECG, -QCG and -pECG were purified in RP-HPLC using SOURCE 5RPC ST 4.6/150 column (Amersham). The peptides were eluted from the column with a linear gradient from buffer A (0.1 % TFA) to 100 % buffer B (90 % ACN, 0.1 % TFA) over 10 column volumes. The peptides were dried in speed vacuum and resuspended into 20 mM phosphate buffer, pH 7.3.

4.11 Analysis of reaction intermediates by mass spectrometry (III, IV)

The protein of interest was reduced with DTT, gel filtrated (NAP™ 10 columns, Amersham) and further concentrated with Biomax Ultrafree centrifugal filter device (Millipore, Bedford, MA, USA). The reduced protein (40 μM) was reacted with either substrate peptide (50 μM) or buffer alone in McIlvaine buffer at pH 7.0. The reaction was quenched with 50 mM NEM (N-ethylmaleimide) or 1.1 M IAA (iodoacetamide) (both from Sigma). The excess of NEM/IAA was removed with pepClean™ C-18 spin columns (Pierce, Rockford, IL, USA) according to manufacturer’s instructions. Proteins were eluted with 50 % ACN and CH₃COOH to final concentration of 0.1 % was added to samples. Molecular masses were measured with electrospray ionisation mass spectrometer (Micromass LCT, Manchester, UK) using positive ionisation. Additional time-dependent trapping experiments were carried out using a RQF3 quenched-flow apparatus (KinTek, Austin, TX, USA). The reduced protein was reacted with either substrate peptide or buffer alone in McIlvaine buffer at pH 7.0 and the reaction was quenched with HCl.
4.12 Stopped-flow measurements (III)

Stopped-flow experiments were performed with a SF2004 stopped-flow apparatus (KinTek, Austin, TX, USA) with 20 μM *E. coli* C14S Grx1 and 0.14 mg/ml (357 μM) Ellman’s reagent in McIlvaine buffer at the desired pH (4.5 - 7.5). The absorbance at 412 nm was measured for 0.2 seconds after mixing the two reagents and the pseudo first-order rate constants calculated using KinTek StopFlow v9.06 software.

4.13 Statistics (I, II)

Statistical analyses were performed with the Statistical Package for Social Studies (SPSS) version 11.5 (Chicago, IL, USA). Differences between controls and selected diseases were compared using analysis of variance and post hoc comparisons were performed using two-tailed *t* tests. Categorical data were compared using Kruskal-Wallis or Fisher’s exact test. *P*-values less than 0.05 were considered statistically significant. Correlations to lung functions were analyzed with the Spearman rank correlation test. The two independent evaluations of immunohistochemical samples were compared using Cohen’s kappa statistics.

4.14 Ethical considerations (I, II, III, IV)

The study protocol was accepted by the ethical committees of the University of Oulu, Oulu University Hospital and Helsinki University Hospital and it is in accordance with the ethical standards of the Helsinki declaration of 1975.
5 Results

5.1 Expression of Grx1 and Grx2 in human lung cells (I)

All lung cell lines studied here showed detectable amounts of Grx1 and Grx2 mRNA as well as protein. In comparison with cultured cells, Grx1 was most prominently expressed in the cells of BAL (i.e. alveolar macrophages) from healthy lung, whereas the level of Grx2 in these cells was nearly undetectable. Homogenized lung specimens from healthy lung showed positive and constant expression of Grx1 whereas again Grx2 was hardly detectable.

5.2 Grx1 in healthy human lung and in different lung diseases (I, II)

The results of cell specific distribution of Grx1 in healthy lung and in different lung diseases have been summarized in Table 3.

Table 3. Distribution of Grx1 in healthy human lung and in different lung diseases.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>AM</th>
<th>BE</th>
<th>MBAL</th>
<th>P</th>
<th>SS</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>No statistically significant differences in Grx1 levels between non-smokers and smokers</td>
</tr>
<tr>
<td></td>
<td>(healthy non-smokers and smokers)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>Granulomas + Decreased total Grx1 levels when compared to controls</td>
</tr>
<tr>
<td>Allergic alveolitis</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>Granulomas + Decreased total Grx1 levels when compared to controls</td>
</tr>
<tr>
<td>COPD</td>
<td>+</td>
<td>–</td>
<td>/</td>
<td>NA</td>
<td>+</td>
<td>Decreased total Grx1 levels when compared to controls</td>
</tr>
<tr>
<td>UIP</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>Low or absent Grx1 immunoreactivity in the fibrotic areas and fibroblast foci</td>
</tr>
</tbody>
</table>

AM = alveolar macrophages; BE = bronchial epithelium; MBAL = macrophages in bronchoalveolar lavage; P = plasma; SS = sputum supernatant; + = positive; - = negative; NA = not analysed
5.2.1 Normal lung

In immunohistochemical samples of normal human lung, Grx1 was expressed mainly in the alveolar macrophages. Bronchial epithelium showed weak Grx1 positivity while endothelial cells, fibroblasts and type II pneumocytes were mainly negative. Since Grx1 is highly expressed in alveolar macrophages, a detailed analysis was conducted where the number of alveolar macrophages per square millimetre of tissue specimen was analyzed from healthy non-smokers and smokers. These analyses showed an increased number of macrophages in the lung tissue of smokers (p = 0.020) compared to non-smokers. No statistically significant differences were observed in Grx levels between non-smokers and smokers.

5.2.2 Sarcoidosis and allergic alveolitis

Both granulomatous lung diseases showed Grx1 expression mainly in alveolar macrophages, the intensity being significantly lower in these diseases than in the control lung (p < 0.001, Kruskall-Wallis). Granulomas observed in sarcoid lung and in allergic alveolitis showed from very weak to moderate positivity. In contrast to the Grx1 positivity in the bronchial epithelium of control lung, bronchial epithelium was negative in all cases of sarcoidosis and allergic alveolitis. As with the controls, occasional weak positivity could be seen in type II pneumocytes.

5.2.3 Chronic obstructive pulmonary disease

In different stages (Pauwels et al. 2001) of COPD Grx1 was mainly expressed in alveolar macrophages. Occasional faint positivity could be also detected in the bronchial epithelium in some cases. There were no statistically significant differences in the semi-quantitative analysis of the immunohistochemical staining intensities between healthy lung and different stages of COPD.

When the cells were divided into two groups, either Grx1 positive or negative, the percentage of Grx1 positive macrophages from the total macrophage population showed a tendency to decrease during disease progression, being lowest in stage IV COPD. This reduction was statistically significant when compared to smokers (p = 0.021). Furthermore, there was a significant correlation between Grx1 positive macrophages in the lung tissue specimens (containing lung
samples from all smokers and different stages of COPD) and lung function parameters (FEV1, $r = 0.45$, $p = 0.008$; FEV1%, $r = 0.46$, $p = 0.007$, FEV/FVC%, $r = 0.55$, $p = 0.001$).

In agreement with this result the Western blot analysis of lung homogenates showed that Grx1 levels were decreased in COPD when compared to healthy smokers (stage I-II $p = 0.045$ and stage IV $p = 0.022$).

### 5.2.4 Usual interstitial pneumonia

Fibrotic lesions of UIP patients investigated showed negative or very weak Grx1 positivity in the regenerative epithelium overlying the focal zones of fibroblast proliferation i.e. fibroblast foci, in the areas of active ongoing fibrosis as well as in old fibrotic lesions. Alveolar macrophages were positive when present.

### 5.2.5 Grx1 in BAL, plasma and induced sputum

The immunoreactivity for Grx1 in BAL could be detected only in macrophages, whereas lymphocytes (present especially in the BAL of sarcoidosis and allergic alveolitis) were negative. By immunoelectron microscopy Grx1 could be detected in the plasma membrane, in the nucleus and in the intra-cytoplasmic vacuoles of macrophages from BAL. Grx1 could be detected by Western blot analysis in the plasma samples from non-smokers, smokers and COPD patients, but with high individual variability. There was a prominent Grx1 immunoreactivity in the cells of induced sputum as analyzed by immunocytochemistry and Western blot. Furthermore, Grx1 could also be detected from the induced sputum supernatants of healthy controls, smokers and COPD patients.

### 5.3 Regulation of Grx1 in human lung cells in vitro (I)

Human alveolar epithelial (A549) cells were exposed to TNF-$\alpha$ or TGF-$\beta_1$ for up to 72 hours with a corresponding unexposed control at each time point of the exposure. TNF-$\alpha$ exposure had no significant effect on Grx1 levels in A549 cells whereas TGF-$\beta_1$ caused a 32% decrease after 72 hours of exposure.
5.4 Determination of the de-glutathionylation and glutathionylation activities (III)

5.4.1 Assaying de-glutathionylation

The glutathionylated substrate peptide SQLWC(glutathione)LSN had an emission maximum at 356 nm and upon removal of the glutathione i.e. the reduction of the cysteine moiety, a $38 \pm 2\%$ ($70 \pm 2$ arbitrary units) increase in total fluorescence was observed with no shift in the emission maximum (Fig. 8A). Hence de-glutathionylation of the peptide could be measured directly in real time by monitoring the change in fluorescence at 356 nm in the presence of reducing agents. At pH 7.0 in McIlvaine buffer, the non-catalysed reaction was very slow, just 3 % of the enzyme catalysed reaction with 20 nM *E. coli* Grx1 and 1 mM GSH (Fig. 8B). To remove the GSSG formed during the de-glutathionylation reaction, GR and NADPH were added to the reaction.

![Fig. 8. Fluorescence analysis of peptide de-glutathionylation. A. Typical emission spectra for glutathionylated (---) and de-glutathionylated (→) peptide (5 μM) at pH 7.0; B. Representative time-dependent fluorescence profiles during the de-glutathionylation of the substrate peptide catalysed by 20 nM *E. coli* Grx1 (black) and the non-catalysed reaction (grey); McIlvaine buffer pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM.]

5.4.2 Assaying glutathionylation

In order to monitor the glutathionylation activity of Grx, reduced substrate peptide was added to GSSG in the presence and absence of enzyme. Consistent with the de-glutathionylation results, a decrease of $69 \pm 2$ arbitrary units in total
fluorescence was observed upon addition of GSH moiety to reduced peptide. The rate constant for this pseudo first order reaction was 0.002 s\(^{-1}\) at pH 7.0 ([peptide = 5 \(\mu\)M], [GSSG] = 5 mM). The addition of \textit{E. coli} Grx1 at levels comparable to those used in the deglutathionylation assay (20 nM) or at ten fold higher levels showed no activity, and the addition of the C14S Grx1 mutant at 200 nM resulted in only very minor changes in rate compared with the non-catalysed rate.

### 5.4.3 Analysis of the reaction kinetics

In order to analyse the kinetics as a function of enzyme, substrate and GSH concentrations, the initial rates of deglutathionylation reaction were determined from each time-course and plotted against the concentration of the varying substance. \textit{E. coli} Grx1 showed a linear dependence between the initial rate of reaction and the enzyme concentration. The initial rate of reaction increased with increasing substrate concentrations and fitted well to the Michaelis-Menten equation with \(K_m = 7.8 \ \mu\text{M}\) and \(k_{cat} = 4.4 \ \text{s}^{-1}\) ([GSH] = 1 mM). Also an increase in the initial rate with increasing GSH concentration was observed, with a plateau at the highest GSH concentrations measured, however, the plot could not be fitted to the Michaelis-Menten equation and instead showed a sigmoidal shape ([substrate peptide] = 5 \(\mu\)M).

### 5.4.4 Analysis of C14S Grx1 catalysed deglutathionylation reaction

The sigmoidal shape for the GSH dependence of \textit{E. coli} Grx1 activity is probably due to the net effect of the partitioning of the Grx-GSH mixed disulphide intermediate between the formation of reduced or oxidised Grx1 and the re-reduction of oxidised Grx1 by GSH. Mutating the C-terminal active site cysteine to serine should prevent this partitioning. Under standard conditions the initial rate of the C14S mutant catalysed reaction was only 23 \% of that catalysed by the wild-type enzyme. As with wild-type, a linear dependence of the initial rate of reaction on the enzyme concentration was observed with the C14S mutant and the increase in the initial rate with increasing substrate concentrations fitted well to the Michaelis-Menten equation with \(K_m = 44 \ \mu\text{M}\) and \(k_{cat} = 4.0 \ \text{s}^{-1}\) ([GSH] = 1 mM). In contrast to wild-type, the data with increasing GSH concentrations could be fitted to the Michaelis-Menten equation giving \(K_m = 48 \ \mu\text{M}\) and \(k_{cat} = 0.36 \ \text{s}^{-1}\) ([substrate peptide] = 5 \(\mu\)M).
5.4.5 Effects of oxidised glutathione

The initial rate of both the wild-type Grx1 and C14S mutant catalysed peptide deglutathionylation reaction decreased with increasing concentrations of GSSG. Using the $K_m$ values for the peptide substrate obtained earlier, the competitive inhibition constant for GSSG was calculated to be 5.6 $\mu$M for wild-type and 50 $\mu$M for C14S Grx1.

5.4.6 pH-Dependence of the deglutathionylation reaction

Over the pH range 4.5 to 7.5 the initial rate of reaction increased with increasing pH and showed a single $pK_a$-dependent event with a $pK_a = 6.47 \pm 0.04$ for the wild-type enzyme catalysed reaction and a $pK_a = 5.90 \pm 0.07$ for C14S catalysed reaction. Since these values were significantly higher than the $pK_a$ values previously reported for the N-terminal active site cysteine (3.8 to 5.0) (Sun et al. 1998, Foloppe et al. 2001, Foloppe & Nilsson 2004), measurements for the pH-dependence of the reaction of the C14S Grx1 with Ellman’s reagent were done by stopped-flow. This data fitted to a single- $pK_a$ dependent event with a $pK_a = 4.86 \pm 0.07$ for the N-terminal cysteine.

5.5 Deglutathionylation activity of different enzymes (III)

The deglutathionylation activity of different members of the thioredoxin superfamily was measured under standard conditions (pH 7.0; [GSH] = 1 mM; [substrate] = 5 $\mu$M). While yeast Grx1 and *E. coli* Grx1 had comparable activities, members of the human protein disulphide isomerase (PDI) family showed considerably lower deglutathionylation activity than glutaredoxins ranging from approximately 1 % (PDIp) to 22 % (ERp46) of the *E. coli* Grx1 wild-type catalysed reaction.

5.6 Intermediates in the deglutathionylation reaction (III)

Only the oxidised Grx1 form was detected when wild type enzyme was reacted with glutathionylated peptide hence studies on the reaction intermediates used C-terminal active site cysteine mutant. In the reaction between the C14S Grx1 mutant and glutathionylated peptide, only the Grx-GSH mixed disulphide was observed as a first intermediate in the reaction. Under the test conditions used
([Grx1] = 80 μM, [substrate] = 100 μM) in quench-flow experiments, the half time for this reaction was around 100 ms.

The mass spectrometric analysis of reaction intermediate in the C39S PDI a - catalysed deglutathionylation reaction revealed only the PDI a domain-peptide mixed disulphide, no PDI a domain-GSH mixed disulphide intermediate was detected at any time point. When using a mixed disulphide between glutamylcysteinylglycine and the peptide as substrate, only C14S Grx-peptide or C39S PDI a domain-peptide intermediates were seen in the enzyme catalysed reduction of this substrate. No Grx-glutamylcysteinylglycine intermediates were seen. The same result was obtained when using the peptide-QCG or -pECG mixed disulphides.

5.7 Effect of conserved residues on the glutathione specificity of glutaredoxins (IV)

In order to investigate the role of selected conserved residues on the GSH specificity of glutaredoxin, the following mutations either singly or in combination were made in the C14S background of *E. coli* Grx1: R8A, Y13A, T58A and D74A. After one minute incubation of *E. coli* Grx1 mutants with slight excess of glutathionylated peptide, a heterogeneous population of products was observed, including a small proportion of reduced Grx and a significant population of Grx-GSH mixed disulphide. For Y13A, T58A and D74A a significant amount of Grx-peptide was also observed, with the largest amount being observed for the Y13A mutant (Fig. 9A). The effects of Y13A, T58A and D74A were additive, resulting in more Grx-peptide being formed when combined. The R8A mutation had no significant effect on the specificity of the intermediate formed.
Fig. 9. Mass spectrometric analysis of reaction intermediates. The amount of reduced Grx1, Grx-GSH mixed disulphide and Grx-peptide mixed disulphide A. after one minute incubation and B. after five minutes incubation. Concentrations were as follows: McIlvaine buffer, pH 7.0, [substrate peptide] = 50 μM, [enzyme] = 40 μM. The reaction was quenched with 50 mM NEM or 1.1 M IAA.

After one minute incubation, some Grx-peptide mixed disulphide was observed also with C14S mutant alone. Since this finding was inconsistent with previous results showing that the first intermediate in the deglutathionylation reaction is exclusively Grx-GSH mixed disulphide, this effect was studied in more detail. When the incubation time was extended to allow the system to come to equilibrium, the amount of Grx-peptide mixed disulphide observed increased with all constructs tested, though mutations in Y13, T58 and D74 still showed a clear effect on specificity compared with the C14S control (Fig. 9B). Quenched-flow reactions at 50 ms, 0.1 s, 0.5 s and 1.0 s, revealed that for all constructs tested the first step in the reaction was the formation of the Grx-GSH mixed disulphide.

When lysates of \textit{E. coli} expressing Myc-tagged Grx were analysed by non-reducing western blot, many additional bands were observed in the lysates expressing \textit{E. coli} Grx1 Y13A C14S, C14S D74A and Y13A C14S D74A mutants compared with the C14S control. This indicates that these mutants are able to form stable Grx-protein mixed disulphides \textit{in vivo}. 

52
5.8 Specificity of the second step in the deglutathionylation reaction (IV)

All of the mutants showed very significantly lower activity than wild type *E. coli* Grx1 (pH 7.0, [GSH] = 1 mM, [substrate] = 5 μM). The Y13A mutation had the greatest effect, and when combined with other mutations the effect on activity was additive leading to almost inactive enzyme. To examine the glutathione specificity of glutaredoxins in the second step of deglutathionylation reaction, the peptide deglutathionylation reaction was performed using either GSH or the corresponding tripeptide ECG, which has a normal peptide bond instead of the γ-linkage. When GSH was replaced with ECG the activity of the wild type Grx decreased by over 99%. Similarly the activity of the R8A, Y13A, T58A and D74A mutations showed a strong dependence on GSH vs. ECG, implying that none of these residues in isolation is responsible for the specificity of the second reaction. In contrast to the specificity of the wild type enzyme, the C14S mutant showed no difference in the catalytic efficiency when GSH or ECG was used. Furthermore the R8A, Y13A, T58A and D74A mutants in the C14S background also showed no specificity for GSH or ECG in the second reaction. This effect was not dependent on the specific mutation made since the C14A mutant also showed no specificity for GSH or ECG, though it should be noted that this mutant is prone to forming aggregates making quantification more difficult.
6 Discussion

Glutaredoxins constitute a family of small thiol disulphide oxidoreductases with possible antioxidant capacity and an intimate connection to GSH, one of the major antioxidants of human lung. They catalyse the reduction of protein disulphides, preferring GSH-protein mixed disulphides as substrates. Accumulation of such GSH-protein mixed disulphides can be caused by oxidative stress (Cotgreave & Gerdes 1998) and their formation may serve both a regulatory and an antioxidant function by protecting the enzymes from irreversible oxidation that might lead to enzyme inactivation. Once oxidative stress has been alleviated the protein-GSH mixed disulphides are efficiently reduced by glutaredoxins liberating free protein and glutathione.

6.1 Grx1 and Grx2 in lung cell lines and in healthy human lung

The present study showed for the first time that Grx1 and Grx2 can be detected in healthy human lung. Grx1 expression was highly cell-specific, being localized prominently to alveolar macrophages. Alveolar macrophages are oxidant resistant cells that also contain other antioxidant enzymes (Cantlay et al. 1994, Lakari et al. 1998, Harju et al. 2002, Kinnula et al. 2002, Kaarteenaho-Wiik & Kinnula 2005). The high Grx1 expression in macrophages and specialized function in reducing GSH-mixed disulphides can be hypothesized to be directed towards the primary oxidant defence of human lung and the role of macrophages in lung protection.

Human airway epithelial and pleural mesothelial cell lines were capable of synthesizing Grx1 and Grx2 mRNA and protein in vitro. Interestingly, Grx2 protein could hardly be detected in alveolar macrophages or lung homogenates. Grx2 may, however, have significance in lung protection, since it is located in the mitochondria (Gladyshev et al. 2001, Lundberg et al. 2001), a cell organelle which maintains a separate GSH pool and has the capability to produce ROS (Meredith & Reed 1982, reviewed by Fernández-Checa et al. 1998).

6.2 Possible export of Grx1 to extracellular space

Highly specific expression of Grx1 was observed in alveolar macrophages, specifically in the nucleus, plasma membrane and in intra-cytoplasmic vacuoles, which may be associated with the export of Grx1 out of cells. This observation is
in line with a study suggesting a role for Grx in exocytosis in pancreatic islet β-cells where it was shown to localize to distinct microdomains (Ivarsson et al. 2005). The present study also confirmed that Grx1 can be detected from plasma as shown earlier (Nakamura et al. 1998, Lundberg et al. 2004) and more importantly demonstrated that Grx1 could be detected from sputum, both from the cells and the supernatant, the latter further supporting the possibility that Grx1 may be exported from alveolar macrophages to the extracellular space. Epithelial lining fluid also contains other enzymes (e.g. GPx3, ECSOD and Trx1) with antioxidative properties as well as glutathione, thus Grx1 may well play a role in this first line of defence system in lungs.

6.3 Grx1 in different lung diseases

Sarcoidosis and extrinsic allergic alveolitis were selected to represent well-characterized in vivo models for lung inflammation. The immunopathogenesis of sarcoidosis and extrinsic allergic alveolitis include accumulation and activation of inflammatory cells, mainly macrophages, and the formation of pulmonary granulomas originating from monocytic cells (see review by Müller-Quernheim 1998). COPD was chosen to represent an in vivo model of cigarette smoke related disease. Accumulation and activation of neutrophils and macrophages is a common feature also in this disease, but in addition tissue injury due to emphysema, airway obstruction and mucus hypersecretion are characteristic for COPD (reviewed by Barnes 2004). Our results indicated that Grx1 levels in alveolar macrophages were lower in these three diseases than in control lung samples. Previous studies suggest the importance of GSH-associated antioxidant mechanisms in the primary antioxidant defence of human lung and their impairment in parenchymal lung diseases (Cantin et al. 1989, Behr et al. 2000, Harju et al. 2002), and our results are in line with these findings.

Further studies were conducted with specimens obtained from the fibrotic lung of UIP, where Grx1 was mainly negative or very weak in the fibroblast foci, fibroblasts and myofibroblasts. UIP is an aggressive fibrosing disease and the amount of fibrosis correlates with the prognosis of the disease (Titto et al. 2006). Low or absent expression of several antioxidant enzymes in the fibrotic areas of UIP, as has been reported (Lakari et al. 2000, Lakari et al. 2001, reviewed by Kinnula et al. 2005), and low expression of Grx1 in these same locations may contribute to the progression of lung injury in fibrotic lung diseases. Downregulation of Grx1 in cultured airway epithelial cells by TGF-β1, which is a
major cytokine involved in the pathogenesis of fibrotic lung disorders, further supports the role of impaired antioxidative defence mechanisms, including Grx1, in the pathogenesis of fibrogenic lung diseases.

6.4 Mechanisms of action of glutaredoxins

The activity and factors affecting the functions and specificity of glutaredoxins cannot be studied efficiently in vivo, thus we assessed these questions by in vitro applications. Human Grx1 and E. coli Grx1 are structurally highly homologous in their catalytically important features (Bushweller et al. 1994, Yang et al. 1998) and they are capable of catalysing same reactions. Human Grx1 wild type enzyme has one unpaired cysteine making it prone to aggregation hence the E. coli Grx1 was chosen for in vitro studies.

The fluorescence-based method developed here measures in real-time the deglutathionylation activity of glutaredoxins using a glutathionylated peptide as a substrate. Most of the previous assays developed for studying this reaction are based on indirect measurements in coupled reactions using nonphysiological substrates, such as HED (Holmgren 1979a), or interval measurements of radiolabelled substrates such as BSA-, papain- or haemoglobin-SSG mixed disulphides (Gravina & Mieyal 1993, Srinivasan et al. 1997). The new method developed here allowed the comparison and determination of apparent $K_m$ and $k_{cat}$ values for wild-type E. coli Grx1 and the C14S mutant. The activity of C14S mutant was only 23 % of the wild-type enzyme, which was found to represent a decrease in $K_m$ for the substrate rather than a decrease in $k_{cat}$. Hence consistent with previous results (Yang & Wells 1991b, Bushweller et al. 1992, Yang et al. 1998), only the N-terminal active site thiol is required for the deglutathionylation of the glutathionylated substrate by glutaredoxins.

The deglutathionylation activity of both wild-type E. coli glutaredoxin and the C14S mutant was competitively inhibited by oxidised glutathione. Comparing the $K_m$ values for substrate peptide and GSH, and $K_i$ value for GSSG revealed that the $K_m$ values for the C14S mutant for the substrate and for GSH and the $K_i$ value for GSSG were effectively equivalent, as were the $K_m$ value for substrate and $K_i$ value for GSSG for the wild-type enzyme. This implies that Grx has the same affinity for all glutathione moieties thus explaining its specificity towards all glutathionylated substrates. The GSH-dependence of wild-type Grx1 was sigmoidal probably due to partitioning of Grx-GSH mixed disulphide to oxidised Grx1 and re-reduction by GSH. Since the activity of Grx1 wild-type is
significantly decreased at low GSH concentrations and potentially inhibited by GSSG, it is likely that Grx1 cannot function under conditions mimicking oxidative stress (i.e. where concentration of GSH significantly decreased or concentration of GSSG increased). Instead, it will only be able to have significant deglutathionylation activity once the oxidative stress has been removed. In fact, dithiol glutaredoxins are probably present in the oxidised state under oxidative conditions, hence the C-terminal active site cysteine residue may protect the reactive N-terminal active site cysteine residue by forming a less reactive disulphide. This reaction in turn is readily reversed once GSH levels rise, generating an active enzyme.

The pH-dependence of the assay developed here was shown to have significantly higher pKₐ values for wild-type and C14S mutant catalysed reactions than those previously reported for the N-terminal active site cysteine. When measuring the pH-dependence of the reaction of the C14S Grx1 with Ellman’s reagent, a single- pKₐ dependent event for the N-terminal cysteine was observed, with a similar pKₐ value to that reported in earlier studies (Sun et al. 1998, Foloppe et al. 2001, Foloppe & Nilsson 2004). Hence, the pH-dependence of activity in our assay most likely does not simply represent the nucleophilicity of the N-terminal active site cysteine.

6.5 Deglutathionylation activity of different enzymes

The deglutathionylation activities of other thioredoxin superfamily members were also tested in the new assay. Although the deglutathionylation activities of the PDI-family members tested here were low compared to that of glutaredoxin (ranging from 1-22 %), they are probably physiologically relevant since they are in line with their catalysed rates of peptide oxidation under standard conditions (Ruddock et al. 1996, Alanen et al. 2006). Only a few direct in vitro studies have shown PDI-family members to have deglutathionylation activity (Lundström-Ljung et al. 1999, Xiao et al. 2005), but additional evidence comes from in vitro refolding studies where PDI is able to form native disulphide bonds in fully glutathionylated protein substrates (for example see Ruoppolo & Freedman 1995). Furthermore, the catalysed folding pathway for reduced protein substrates in a glutathione buffer never results in the significant accumulation of glutathionylated protein (for example see Ruoppolo et al. 1996).
6.6 Glutathione binding specificity of glutaredoxins

The reaction pathways for Grx and PDI catalysed deglutathionylation are significantly different. Mass spectrometric studies on reaction intermediates demonstrated only the presence of Grx-GSH mixed disulphide in the first step of deglutathionylation reaction, whereas the PDI-peptide mixed disulphide was shown to be the exclusive intermediate in the PDI-catalysed reaction. The intermediates observed in the Grx catalysed reduction of GSH-peptide and glutamylcysteinylglycine-peptide mixed disulphides demonstrate the specificity of Grx substrate binding to the $\gamma$-linkage. Grx has previously been reported to show specificity for GSH using cysteine-glutathione and cysteinylglycine-glutathione mixed disulphides (Gravina & Mieyal 1993, Srinivasan et al. 1997, Yang et al. 1998), but our results provide the first demonstration of the importance of the highly unusual $\gamma$-linkage found in GSH on Grx-specificity.

Previous structural studies using C14S mutants have implicated several conserved residues in the potential GSH binding site of glutaredoxins (Bushweller et al. 1994, Foloppe & Nilsson 2004). These include Arg8, Tyr13, Thr58 and Asp74 in E. coli Grx1 that were assessed in this study. Asp74 and Arg8 are thought to form electrostatic attractive forces with the N- and C-terminii of GSH respectively and the side chains of Tyr13 and Thr58 help form the binding groove for GSH in the mixed disulphide structures of E. coli and human Grx (Bushweller et al. 1994, Yang et al. 1998). The mutation D74A results in a shift in the equilibrium of mixed disulphides formed by Grx, while R8A does not. However, both mutations significantly decrease the activity of Grx, consistent with a role for Asp74 in substrate binding (Bushweller et al. 1994) and a role for Arg8 in modulating the pKa of the active site Cys of Grx (Foloppe & Nilsson 2004). Our observations on the capability of Y13A and T58A mutants to form mixed disulphides with peptide and proteins are in line with their role in defining the GSH binding site and hence specificity of Grx. Furthermore both mutations significantly reduce the activity of Grx, consistent with a reduction in affinity for glutathionylated substrates.

The effects of the mutations in Tyr13, Thr58 and Asp74 were additive, suggesting all contribute to the GSH binding site. Surprisingly while the equilibrium positions of the multiple mutants were very significantly towards the Grx-peptide mixed disulphide, the initial product in all cases was exclusively the Grx-GSH mixed disulphide.
Since another molecule of GSH is needed for the reduction of Grx-GSH mixed disulphide, the specificity of this second reaction of the catalytic cycle was examined by determining the initial rates of reaction of peptide deglutathionylation when using GSH or ECG. The wild type enzyme showed very significant specificity for GSH, with the activity in the ECG-based assay being less than 1% of that in the GSH-based assay. Similar effects were seen for the R8A, Y13A, T58A and D74A mutants suggesting that none of them significantly changed the specificity of the second reaction. In contrast to the wild-type enzyme, the C14S or C14A mutants showed no specificity for GSH over ECG in the second reaction i.e. while the wild-type enzyme requires the $\gamma$-linkage of GSH for it to act as a substrate in the second reaction, the Cys14 mutants do not. This finding is significant since C-terminal active site mutants of Grx have been used in structural studies on Grx-GSH mixed disulphides to define the glutathione binding site and as the starting point for defining the determinates of the specificity of the second reaction.

Overall these results demonstrate the role of conserved residues in the proximity of proposed GSH binding site to the GSH binding specificity of E. coli Grx1. Opening the groove and removing charged residues enabled Grx to form more readily mixed disulphides with other molecules besides GSH.
7 Conclusions

1. Both Grx1 and Grx2 could be detected in healthy human lung. Grx1 was most prominently expressed in alveolar macrophages, where it was ultrastructurally localized to plasma membrane, nucleus and cytoplasmic vacuoles. Grx1 could be also detected in plasma and in induced sputum supernatant.

2. The levels of Grx1 were lower in inflammatory diseases including sarcoidosis, allergic alveolitis and COPD compared to healthy controls, and the levels correlated with lung function parameters. Grx1 was mainly negative in fibrotic areas in UIP. Furthermore Grx1 was downregulated by TGF-β1, a major cytokine involved in the pathogenesis of fibrotic lung diseases. These results suggest that Grx1 is a potential redox modulatory protein regulating the intracellular as well as extracellular homeostasis of glutathionylated proteins and GSH not only in healthy lung, but also in cigarette smokers and in inflammatory as well as fibrotic lung diseases.

3. A new real-time fluorescence-based method for measuring the deglutathionylation activity of glutaredoxins using a glutathionylated peptide as a substrate was developed.

4. Kinetic analysis revealed that E. coli Grx1 seems to act equally well on all glutathionylated moieties. Consequently, Grx catalysis of deglutathionylation is inhibited by oxidised glutathione and cannot function under conditions mimicking oxidative stress. The first reaction intermediate in the deglutathionylation reaction is exclusively Grx-GSH mixed disulphide and this specificity is solely dependent on the unusual γ-linkage present in glutathione. Different members of the PDI family showed considerably lower activity levels compared to glutaredoxins and, in contrast to Grx-GSH mixed disulphide, the only intermediate in the PDI catalysed reaction was PDI-peptide mixed disulphide thus demonstrating different reaction pathways.

5. The glutathione binding specificity of glutaredoxin could be modified by mutating selected conserved residues in E. coli Grx1 C14S background. Opening the binding groove and removing charged residues enabled Grx to more readily form mixed disulfides with other molecules besides GSH, the first step still being the formation of Grx-GSH mixed disulfide. The specificity for the second step in the deglutathionylation reaction was altered in Cys14 mutants.
References


Original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


Permission to reprint the original articles has been obtained from the following copyright owners:

I  Elsevier Inc.

III  The American Society for Biochemistry and Molecular Biology

Original publications are not included in the electronic version of the dissertation.
475. Klaavuniemi, Tuula (2006) PDZ-LIM domain proteins and α-actinin at the muscle Z-disk
478. Hillukkala, Tomi (2006) Roles of DNA polymerase epsilon and TopBP1 in DNA replication and damage response
482. Sipilä, Laura (2007) Expression of lysyl hydroxylases and functions of lysyl hydroxylase 3 in mice
487. Välimäki, Panu (2007) Reproductive tactics in butterflies – the adaptive significance of monandry versus polyandry in Pieris napi
488. Oinas, Janne (2007) The degree theory and the index of a critical point for mappings of the type (S)
Mirva Peltoniemi

MECHANISM OF ACTION OF THE GLUTAREDOXINS AND
THEIR ROLE IN HUMAN LUNG DISEASES