From the Institute of Environmental Medicine

Karolinska Institutet, Stockholm, Sweden

Licentiate Thesis

Novel methods for the identification of cellular S-glutathionylated proteins and sites of glutathione-dependent modification using affinity chromatography and proteomic analyses

Ylva Hamnell-Pamment

Stockholm 2005

Abstract

Reactive oxygen species (ROS) are formed during normal respiration in the mitochondria through electron leakage to oxygen. During normal metabolism, several enzymes and low-molecular weight antioxidants work towards eliminating ROS in a well-coordinated manner. The tripeptide glutathione is an integral part of this antioxidant network, and functions both as a reducing factor and as a substrate by several enzymes. When the antioxidant defence of the cell is overwhelmed, "oxidative stress" develops, and cellular redox systems become oxidised. Oxidative stress can result through metabolism of foreign compounds, ischemia-reperfusion, or exposure to UV-radiation, and may also develop naturally during aging.

Glutathione (GSH) has long been known to reversibly bind cellular proteins, particularly during oxidative stress, in a reaction catalysed by glutaredoxin, previously known as thioltransferase. These modified protein substrates include several metabolic enzymes and cytoskeletal proteins, which may be thus functionally regulated as a cellular response to stress. However, a comparison of the constitutive and the stressed situations is difficult, since the current analysis protocols for high-throughput analysis are not sensitive enough to identify low level glutathionylation during constitutive metabolism. Hence, there is interest in developing more sensitive methods to accurately portray glutathionylation patterns in the cell.

A method was developed based on the specific reduction of glutathione-protein mixed disulphides by glutaredoxin, the reaction of liberated protein thiols with N-ethylmaleimide-biotin, affinity purification of tagged proteins, and identification by two-dimensional gel electrophoresis and mass spectrometry. The method unequivocally identified 43 mostly novel cellular protein substrates for S-glutathionylation. These included protein chaperones, cytoskeletal proteins, cell cycle regulators, and enzymes of intermediate metabolism. The method developed in this study is high-throughput, and more importantly, can specifically identify S-glutathionylated proteins from cells – both stressed cells and cells undergoing constitutive metabolism – with minimal disruption of cellular function. Therefore, the method can successively be used to study post-translational redox modification of cellular proteins – a potentially significant biochemical control mechanism in coordinating cellular function.

The protocol was further developed to allow for affinity purification and analysis of trypsinised S-glutathionylated proteins under non-reducing conditions, in an attempt to avoid the cumbersome 2-D gel electrophoresis step and at the same time identify the sites of S-glutathionylation by tandem mass spectrometry. The glutathionylation sites of gamma-actin, heat-shock protein 70 and elongation factor 1-α-1 were identified in this way, but the method was hampered by unspecific binding of peptides to the avidin-affinity column, a problem also encountered in other studies.

In this study, for the first time, a method has been developed for high-throughput identification of S-glutathionylated proteins without interference with cellular function. This protocol has also been modified to include identification of the S-glutathionylation sites of the proteins involved, although difficulties in peptide affinity capture interfere with specificity and high-throughput analysis.

To James, and to our unborn baby

CONTENTS

PAPERS I-II

LIST OF PUBLICATIONS

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

I.   Lind, C.; Gerdes, R.; Hamnell, Y.; Schuppe-Koistinen, I.; Brockenhuus von Löwenhielm, H.; Holmgren, A.; Cotgreave, I.A. Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch. Biochem. Biophys.* **406**(2): 229-40; 2002.

II.   Hamnell-Pamment, Y.; Lind, C.; Palmberg, C.; Bergman, T.; Cotgreave, I.A. Determination of site-specificity of S-glutathionylated cellular proteins by affinity isolation and liquid chromatography/tandem mass spectrometry. *Manuscript*. 2005.

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ABBREVIATIONS

ROS	Reactive oxygen species
$OH^{\bullet}$	Hydroxyl radical
$O_2^{\bullet-}$	Superoxide radical anion
H_2O_2	Hydrogen peroxide
GSH	Glutathione (reduced form)
GSSG	Glutathione disulphide
NADPH	Nicotinamide adenine di-nucleotide phosphate
$ROO^{\bullet}$	Peroxyl radical
SOD	Superoxide dismutase
$^{1}\Delta gO_2$	Singlet oxygen
ARE	Antioxidant response element
NFκB	Nuclear factor κ B
AP-1	Activator protein-1
Grx	Glutaredoxin
ABPs	Actin bundling proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
2-D	Two-dimensional
MALDI	Matrix-assisted laser desorption ionisation
ESI	Electrospray ionisation
TOF	Time-of-flight
MS/MS	Tandem mass spectrometry
NEM	N-ethyl-maleimide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
RP-HPLC	Reverse-phase high-performance liquid chromatography

Amino acids abbreviated in text:

Cys (C), Cysteine; Gly (G), Glycine; Glu (E), Glutamic acid; Pro (P), Proline; Ser (S), Serine; Tyr (Y), Tyrosine

INTRODUCTION

1. Oxidative stress

1.1 The aerobic living situation

During the Earth's development through billions of years, the appearance of oxygen in the atmosphere made possible an important evolutionary advantage: the use of oxygen as a terminal oxidant for the combustion of organic nutrients. At the same time, photosynthetic organisms could consume the carbon dioxide and water. This vital development in material and energy economy led to the evolution of aerobic organisms, including all animal species. However, the utilization of oxygen also presented a hazard; since aerobic systems utilize molecular oxygen for energy production, they are therefore subjected to the potential damaging effects of this molecule. Reactive oxygen species (ROS; see Table I) are transient derivatives of oxygen that are constantly produced as a by-product of aerobic respiration during normal cellular functions. Normally, ROS, some of which may cause harm, inflict little damage to the cell's normal processes, since antioxidant defences are been well developed to counteract any potential perturbations due to the redox processes. However, if the antioxidant capability of the cell is overwhelmed, this can lead to damage to cellular function [1-3].

Table I. Some reactive oxygen species. Adapted from [4].

Radicals	Non-radicals
Superoxide, $O_2^{\cdot-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\cdot}$	Ozone, O_2
Peroxyl, $RO_2^{\cdot}$	Singlet oxygen, $^1\Delta gO_2^*$
Alkoxyl, $RO^{\cdot}$	
Hydroperoxyl, $HO_2^{\cdot}$	

* Singlet oxygen is an energised, more reactive form of oxygen.

Mitochondria are the major endogenous source of ROS in the cell. In a cytochrome oxidase-catalysed reaction coupled to oxidative phosphorylation, oxygen undergoes stepwise and sequential reduction leading to the formation of water. An electron transport chain present in the inner mitochondrial membrane transports electrons

from NADH, via cytochrome and quinone sites, to cytochrome oxidase. Whereas cytochrome oxidase releases no detectable oxygen radicals into the cell, the electron transport chain involved in this process of four-electron donation to oxygen (outlined in Figure 1) can leak a few electrons directly onto oxygen molecules, leading to the formation of ROS [4, 5].

$$O_2 \xrightarrow{\;e^-\;} O_2^{\cdot-} \xrightarrow[2\,H^+]{\;e^-\;} H_2O_2 \xrightarrow[H^+]{\;e^-\;}{\searrow H_2O} OH^- \xrightarrow[H^+]{\;e^-\;} H_2O$$
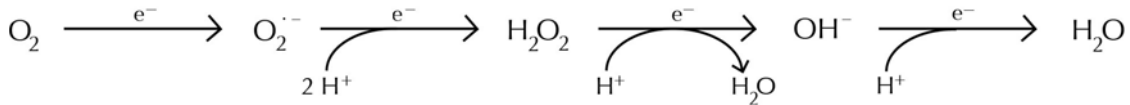
Figure 1. The four-electron reduction of oxygen. Adapted from [1].

During aging in mammals, mitochondrial damage is accumulated, especially in tissues that are particularly exposed to ROS, such as certain regions of the brain and muscle tissue. Mitochondrial proteins and mitochondrial DNA are damaged, leading to point mutations, and decreased mitochondrial protein synthesis [6].

Oxygen radicals are involved in the pathogenesis of a number of human diseases, including ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, and cancer. When the antioxidant systems of the cells are beleaguered, the immune system can also be thrown off balance, and conditions such as inflammation, hypersensitivity and autoimmunity may develop. Furthermore, ROS have been implicated in lung diseases, such as the Acute Respiratory Distress Symdromes (ARDS) associated with exposure to toxicants (for instance asbestos, paraquat or carbon tetrachloride). Several medical companies have shown an interest in developing ROS scavengers in order to treat, for instance, stroke, heart attacks, neurodegenerative diseases and symptoms of aging [3, 7].

1.2 Oxidative stress, reactive oxygen species and antioxidant defences of the cell

An 'oxidative stress' situation has been defined as 'an alteration of the steady-state concentrations of components of cellular redox systems in favour of the oxidized form' [8]. Causes of oxidative stress include metabolic activities of toxins, drugs or their formed metabolites (non-endogenous compounds are sometimes referred to as xenobiotics), ischemia-reperfusion, inflammatory processes and exposure to UV-radiation. Oxidative stress is a particularly common occurrence in the liver, since this

organ is exposed to chemicals in the diet, but oxidative stress can occur in all cell types, epithelial cells, erythrocytes and neurons which are all physiologically relevant examples [8]. The injury to the cell that this stress-situation causes is usually due to the formation of free radicals from oxygen, the most detrimental being the hydroxyl radical (OH·) [4, 8]. The formation of ROS in biological systems and the cellular defences that are utilized for protection against these radicals are discussed in detail below.

1.2.1. Production of reactive oxygen species
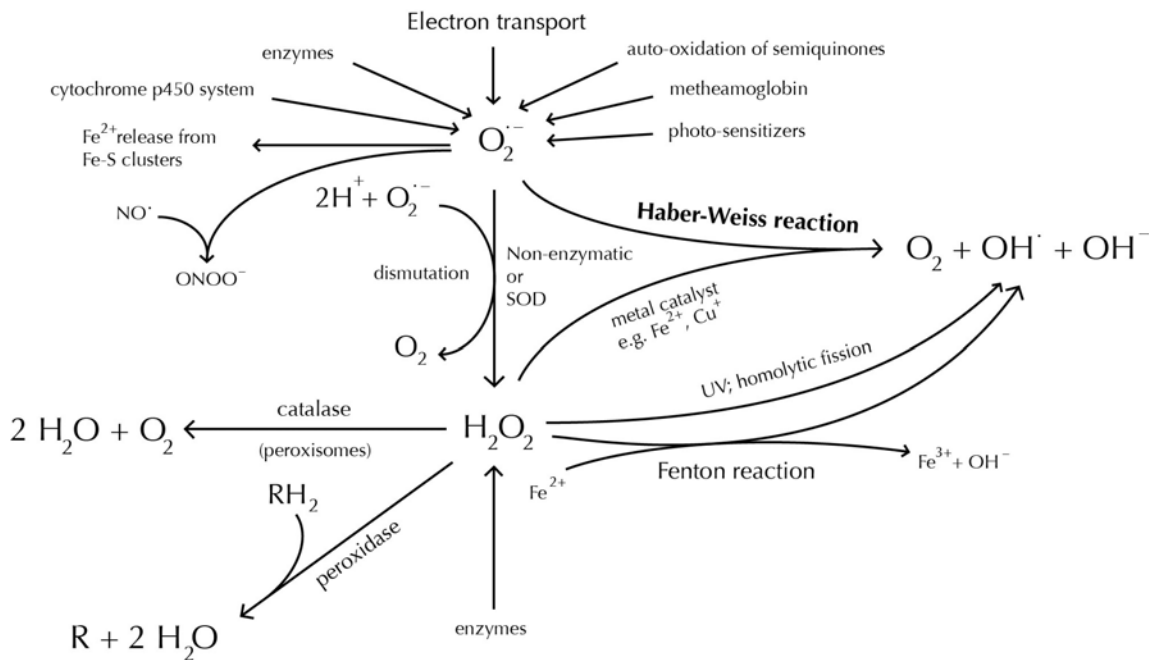
Figure 2. Production and elimination of reactive oxygen species in the cell.

The donations of electrons from various cellular sources to molecular oxygen (O_2) leads to the formation of the superoxide radical anion ($O_2^{·-}$) (see Figure 2). The electron transport chain (mainly semiquinone-sites in complexes I and III [5, 9]) is the major source of $O_2^{·-}$ in the cell, but $O_2^{·-}$ can also be produced through decomposition of haemoglobin into metheamoglobin, and via auto-oxidation of several compounds (for instance reduced flavins, thiols, noradrenaline and dopamine), in the presence of transition metals. Furthermore, $O_2^{·-}$ may be produced by enzymes such as xanthine oxidase, and due to uncoupling of the cytochrome P_{450} systems in the endoplasmic

reticulum during xenobiotic metabolism. Photosensitization (excitation of a photosenzitiser such as porphyrin, which then may transfer its excitation energy to a neighbouring molecule) has also been shown to produce $O_2^{\cdot-}$, although the major product of photosensitization is believed to be singlet oxygen [1, 2, 4].

The production of $O_2^{\cdot-}$ is often the first step in the generation of ROS in biological systems. Superoxide can either react with compounds in its vicinity (such as nitric oxide ($NO^{\cdot}$), or iron-sulphur clusters in enzymes), or quickly dismutate to form hydrogen peroxide (H_2O_2), which is membrane-permeable. Hydrogen peroxide is also produced through endogenous biological processes, mostly by various peroxisomal enzymes [2, 4].

If $O_2^{\cdot-}$, H_2O_2 and other ROS are not properly disposed of inside the different compartments of the cell, a number of reactions may take place, leading to the formation of various derivatives. Superoxide itself can release iron from storage sites by reducing Fe(III) to Fe(II). The very reactive and potent oxidant $OH^{\cdot}$ can be produced through metal ion catalysis in the cell, through the Haber-Weiss reaction. The production of $OH^{\cdot}$ via the reaction of metal ions with H_2O_2 is called the Fenton reaction (see Figures 2 and 3). Generation of $OH^{\cdot}$ is regarded as a particularly dangerous process, and the body's supply of iron (iron-sulphur clusters are used in many processes, for instance in the electron transport chain, or in active sites of enzymes) is therefore tightly regulated with respect to uptake, transport, storage and redox state [1, 2, 4].

a) $\quad Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$

b) $\quad H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$
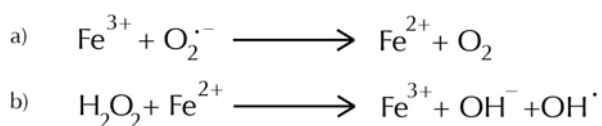
Figure 3. The Haber Weiss reaction is the sum of a) and b), whereas the Fenton Reaction is outlined in b). Both reactions can be catalysed by several metal ions, but most commonly iron, or copper ions.

The hydroxyl radical may also be produced from H_2O_2 by UV-radiation, by homolytic fission (for instance in sunlight-exposed skin) (see Figure 2) [4].

Reactive oxygen species are particularly destructive towards the cell in that they promote the formation of free radicals within biomolecules (e.g. lipids), which can escalate the cell damage through self-propagating radical chain reactions. Redox cycling agents, i.e. compounds that can become continuously reduced and oxidised to generate

superoxide in a redox cycle, can further exacerbate radical damage. Quinones are typical examples of redox cycling agents, and several xenobiotics are quinones (for instance some antitumour agents) [4]. A well-known quinone used to promote stress *in vitro* is menadione (vitamin K_3) [10].
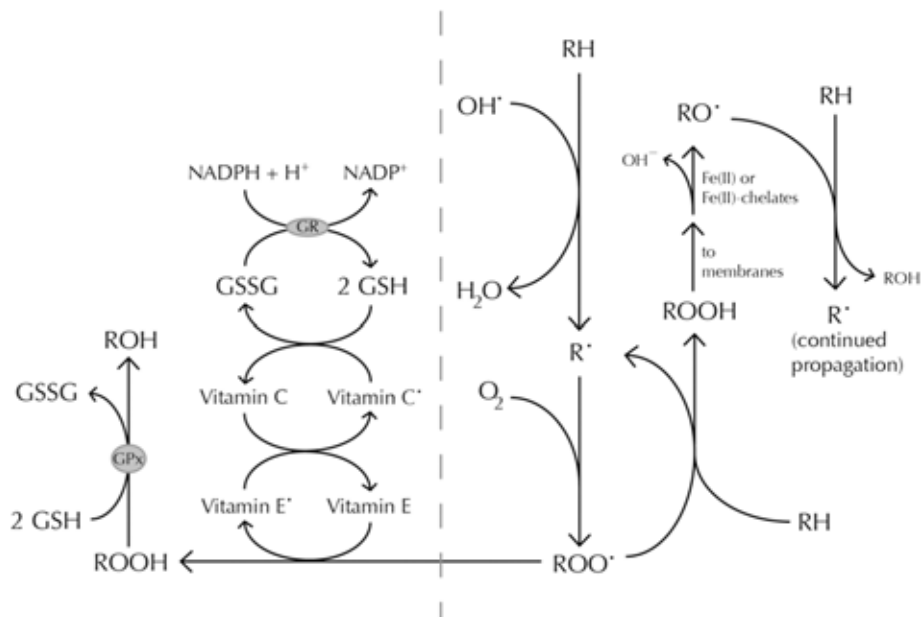
Figure 4. The process of lipid peroxidation. RH is a polyunsaturated lipid. Protective mechanisms of the cell are shown to the left of the dotted line. Vitamin E (α-tocopherol) is a free radical scavenger that ends the chain reaction (Vitamin E can be replaced by ubiquinone). Vitamin E is re-generated by for instance vitamin C (ascorbate), and the process is terminated by glutathione (GSH). Lipid peroxides have to be eliminated by glutathione peroxidase (GPx) not to cause damage to membranes: GSSG, oxidised glutathione; GR, glutathione reductase; NADPH, nicotinamide adenine di-nucleotide phosphate. For further information on these antioxidants, see Table II.

Lipid peroxidation is one particularly detrimental ROS-induced process, in which, for instance, OH˙ abstracts a hydrogen from a polyunsaturated lipid, which forms a conjugated diene radical. This radical can form a peroxyl radical (ROO˙) in the presence of oxygen, and this radical can, in turn, abstract a hydrogen from a neighbouring lipid, starting a chain reaction (see Figure 4). In the presence of Fe(II) irons or certain Fe(II) chelates, the peroxyl radical can decompose into an alkoxyl radical (RO˙), which can abstract hydrogens from lipids and other peroxides, which then continue the propagation of lipid peroxidation. Lipid peroxidation damages the cell membrane and leads to the production of reactive adduct-forming epoxides and several lipid-derived toxins [4].

DNA strand breakage, altered DNA bases and DNA-protein cross-links are other outcomes of ROS in the cell [2, 4]. DNA scission is particularly common with mitochondrial DNA, which is not protected by histone proteins, in a manner similar to nuclear DNA, and is, therefore, not as well protected from insult. Mitochondrial DNA damage increases with age, which results in impaired functionality of the mitochondria, mostly in tissues with low mitochondrial DNA turnover, such as the brain [6].

Proteins can also be affected by ROS, through oxidation of amino acids. When cellular proteins are damaged, they usually misfold, the protein protection machinery of the cell is activated, and heat-shock proteins try to refold misfolded proteins. If the damaged proteins cannot be repaired, they are rapidly degraded. However, if abnormal proteins are allowed to accumulate, protein aggregates will form, such as in cataracts [11]. Oxidative injury to proteins can lead to aggregate formation, either by induction of protein-protein cross-links, or by damage to the protein degradation machinery itself. If not properly taken care of by the cell, formation of protein aggregates may lead to cell death [12, 13].

1.2.2. Elimination of reactive oxygen species

Several enzymes co-ordinately eliminate ROS from the cell (see Figure 2), the most important being:

Catalase: One of the most efficient enzymes known, with a turnover of 300000 sec^{-1} at 37°C; reacts with H_2O_2 to form water and O_2. It can also react with other peroxides, but does this at slower rates. Hydrogen peroxide is catabolised in areobic systems by both catalases and peroxidases [2, 3, 14].

Superoxide dismutase (SOD): Converts $O_2^{\cdot-}$ into H_2O_2 and O_2, through successive oxidation and reduction of a transition metal in its active site. The SOD enzymes have very high reaction rates [3, 15].

Peroxidases: The glutathione peroxidases are divided into selenium-containing and non selenium-containing peroxidases, where the selenium-containing peroxidases are quantitatively the most important for hydrogen peroxide detoxification. They catalyse the reduction of a variety of hydroperoxides, including H_2O_2, and the levels of the different

isoforms vary in tissues and in cell compartments. The seleno-cysteine amino acid residue is essential for enzyme activity [3, 14, 16, 17].

Table II. Summary of some non-enzymatic antioxidant defences by low-molecular mass agents. Adapted from [4].

Endogenous low-molecular mass antioxidants	
Glutathione	Scavenger of reactive oxygen species (ROS) and peroxyl radicals (ROO$^{\bullet}$). Involved in numerous antioxidant enzymatic reactions and protection mechanisms of the cell, and plays a pivotal role in the defence against oxidants. Discussed separately in sections *1.2.3 The role of glutathione* and *2. The mechanisms of protein S-glutathionylation*.
Bilirubin	Scavenger of peroxyl radicals and singlet oxygen ($^{1}\Delta gO_2$)
α-Keto acids	Scavenge hydrogen peroxide (H_2O_2).
Coenzyme Q (called ubiquinol in its reduced form)	Scavenges peroxyl radicals, thereby preventing lipid peroxidation. Can regenerate Vitamin E (α-tocopherol) from its radical in lipoproteins and membranes. Generates radicals when part of the electron transport chain.
Urate	Scavenges ROS in blood. Chelates metal ions.
Low molecular mass antioxidants derived from the diet	
Vitamin C (ascorbic acid)	Can reduce more reactive ROS, such as hydroxyl radical (OH$^{\bullet}$), superoxide ($O_2^{\bullet-}$) and urate radical. However, ascorbate acts as an oxidant in the presence of metal ions. Can regenerate α-tocopherol from its radical.
Vitamin E (α-tocopherol)	The most important ROO$^{\bullet}$ scavenger (reacts faster with ROO$^{\bullet}$ than do neighbouring lipids). Can quench $^{1}\Delta gO_2$.
Caroteniods	Low oxygen concentration dependent scavengers of ROS. Antioxidant importance uncertain; also have oxidant properties.
Plant phenols (many variants, some of which exist in green tea, red wine and cocoa)	Inhibitors of lipid peroxidation, prevent low-density lipoprotein oxidation, scavenge ROS. Physiological importance and impact still uncertain.

Apart from the enzymatic antioxidant system, several low-molecular weight compounds (a couple already shown in Figure 4) function as scavengers of ROS and

peroxyl radicals, thereby acting as a second-line of defence during oxidant insult. Some of these antioxidants are summarised in Table II.

Some other adaptive defence mechanisms of the cell include:

Gene transcription: the most studied relevant gene transcription element is the antioxidant response element (ARE). This regulates the induction of several antioxidant/detoxifying enzyme genes. The transcription factors nuclear factor κ B (NFκB) and activator protein-1 (AP-1) are also induced by oxidative stress, and AP-1 binds to ARE (NFκB and AP-1 are further discussed in section 2.3.2.) [4].

Metal sequestration: Iron ions are bound to transferrin in plasma, and copper ions are sequestered in plasma by albumin (although 95% of copper found in plasma is contained by the abundant ferroxidase ceruloplasmin, this is not a copper transport protein [18]). Both metals are stored inside cells within specialised, high-capacity storage proteins. Cellular iron levels and iron metabolism are controlled by iron regulatory proteins IRP1 and IRP2 [4].

DNA repair systems: These may be chemical or enzymatic, and include DNA glycosylases, endo- or exonucleases and ligases [2, 4].

1.2.3 The role of glutathione

Glutathione (GSH) is the most abundant non-protein thiol in the cell [19]. Despite the fact that glutathione is particularly abundant the mitochondria, glutathione appears to be synthesised primarily in the cytoplasmic compartment of cells [20, 21]. A fraction of the GSH cellular pool also exists in the nucleus, where it acts as a protector for DNA. Intracellular pools typically contain 1-10 mM concentrations of GSH, whereas the extracellular pools usually are in the 1-10 µM range [20]. The concentration of glutathione disulphide (GSSG) is generally very low in comparison, and the cellular ratio of GSSG/GSH lies around ≥1:100 [22, 23].

As mentioned previously, oxidative stress is involved in the pathogenesis of a number of human diseases, including neurodegenerative diseases and cancer. Glutathione has been shown to play an important role in several diseases, for instance HIV, Parkinsons disease and cystic fibrosis [24]. The brain is particularly sensitive to oxidative

stress as it has a relatively low antioxidant defence, and the levels of glutathione play a major role in how the brain cells manage to combat stress situations [3]. Other than protecting cells from stress, glutathione is also involved in a number of metabolic processes [24].

During oxidative stress, in addition to its radical scavenging properties, GSH can convert hydroperoxides into their corresponding alcohols through the action of glutathione peroxidase (see Figure 5a). This reaction can also be catalysed by peroxiredoxins, although their rate constants for the reaction with hydroperoxides in mammals is believed to be comparatively slow [25]. If not taken removed, the peroxides could decompose into radicals in the presence of reduced metal ions such as Fe^{2+} and Cu^+ [4].

a)

$$2\ GSH \qquad\qquad GSSG$$
$$\text{glutathione peroxidase}$$
$$ROOH \longrightarrow 2\ ROH$$

b)

$$GSH \qquad\qquad XH$$
$$\text{glutathione-S-transferase}$$
$$RX \longrightarrow RSG$$
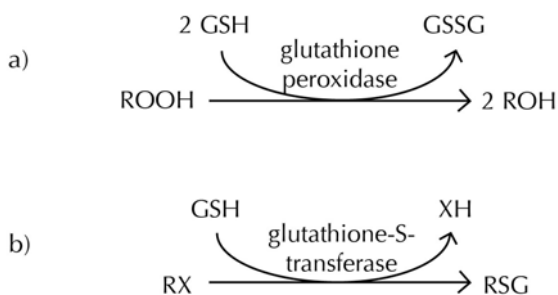
Figure 5. The actions of glutathione peroxidases and glutathione S-transferases. ROOX is a peroxide converted enzymatically into an alcohol with the help of glutathione (GSH), which is oxidised to glutathione disulphide (GSSG). RX is a foreign compound that is enzymatically conjugated with GSH, forming RSG.

Glutathione can also form conjugates with electrophilic compounds through the action of glutathione S-transferases (see Figure 5b), and thus protect the cell against the redox cycling of quinones and oxidative stress that otherwise might result from accumulation of these metabolites [3, 26]. The glutathione conjugates are excreted in the urine in the form of processed mercapturates, and the mechanism is a part of the coordinated detoxification machinery of the cell. The liver, which is particularly rich in glutathione S-transferases, often excretes glutathione conjugates into bile [4]. Reduced glutathione is regenerated from glutathione disulphide by glutathione reductase, in a nicotinamide adenine di-nucleotide phosphate (NADPH) dependent reaction (see Figure

6). The reducing equivalents of NADPH are supplied predominantly by the pentose phosphate metabolism [27, 28].

NADPH + H$^+$ NADP$^+$
              glutathione
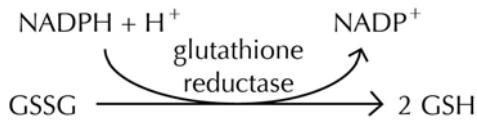   reductase
GSSG ─────────────────→ 2 GSH

Figure 6. The regeneration of reduced glutathione (GSH) from glutathione disulphide (GSSG).

The levels of GSH may vary diurnally in some cells, especially in liver cells, where the levels also are affected by detoxification processes and during oxidative stress. In these situations glutathione levels are decreased *reversibly* by formation of GSSG (via radical scavenging or oxidation via glutathione peroxidases) and formation of protein-mixed disulfides, and *irreversibly* by formation and efflux of glutathione-S-conjugates and direct efflux of GSSG. When GSSG levels rise, GSSG is exported from the liver into bile, in order to avoid oxidative effects (i.e. oxidative stress), and the efflux is directly related to the hepatic GSSG levels [4, 29, 30]. In cells other than liver cells, such as erythrocytes and heart cells, transport of GSSG across the plasma membrane into the extracellular space is used both to alleviate stress and recycle glutathione [4, 28].

Depletion of glutathione compromises the glutathione peroxidase detoxification system. This leads to accumulation of H_2O_2 and lipid hydroperoxides, and subsequent oxidative damage that may overpower the cell's defence systems and affect mitochondrial membrane permeability and enzyme function [31]. Redox imbalance has long been associated with cell death. In fact, glutathione efflux plays an integral part in the damage-induced apoptotic pathway, and GSH depletion causes cytochrome C release from mitochondria. Depending on the strength of the oxidative stimulus, this may lead to activation of the apoptotic pathway via caspase 3 [32].
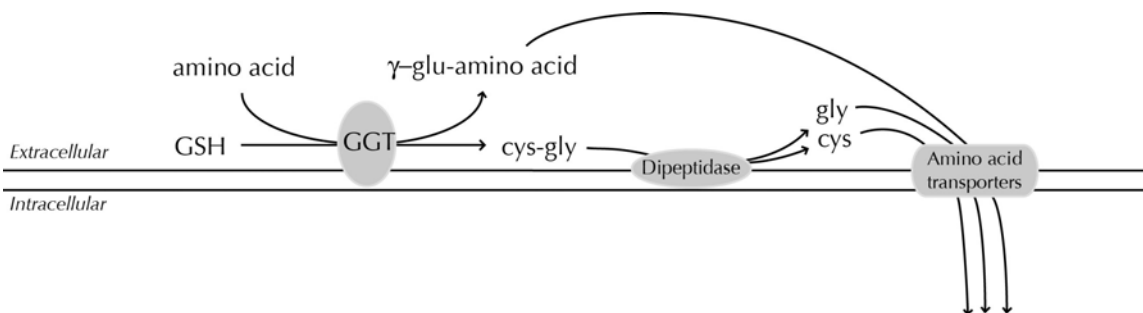
Figure 7. Recycling of glutathione (GSH), and import of its amino acid constituents into the cell through the action of γ-glutamyl-transpeptidase (GGT), dipeptidase and amino acid transporters. Adapted from [30].

Depletion of glutathione can either be counteracted by the cell through GSSG reduction by glutathione reductase (see Figure 6), release of GSH from cellular proteins by the action of glutaredoxin or thioredoxin (for further discussion on protein thiol-disulphide exchange reactions, see section *2. Protein S-glutathionylation*), or through glutathione recycling and *de novo* synthesis. Glutathione recycling involves importing GSH from the extracelullar space, where it is degraded, firstly by the membrane-bound enzyme γ-glutamyl-transpeptidase (GGT) (see Figure 7), the only enzyme capable of breaking the γ-glutamyl bond in GSH and GSH conjugates. The cys-glu dipeptide is then cleaved by dipeptidase, and the constituents can be imported as amino acids, which are used in glutathione synthesis. This recycling of glutathione preserves the amino acid cysteine, which is often limiting in *de novo* synthesis [28, 33]. Blood GSH can also, in this way, be used as a source for cysteine, which easily auto-oxidises and could be potentially toxic in high amounts. The liver actively exports GSH and is the main source of circulatory GSH [30, 34]. Circulatory GSH may also play a role in the extracellular antioxidant defence [20].

Glutathione is synthesised through the action of two enzymes: Glutamate cysteine ligase (γ-glutamyl cysteine synthetase, GCS) and glutathione synthase (see Figure 8). Glutamate cysteine ligase is the rate-limiting enzyme and its activity is influenced by feedback inhibition by GSH, through availability of substrate, through cell signalling pathways, or at the gene transcription level. In this way, several cytotoxic compounds have the ability to influence glutathione synthesis [30, 33].
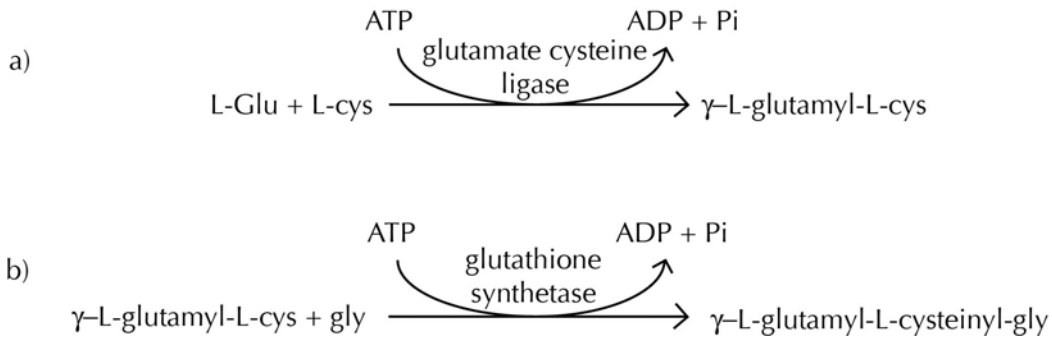
Figure 8. The two enzymatic steps of glutathione *de novo* synthesis.

2. Protein S-glutathionylation

2.1 Reversible S-glutathionylation

Reversible S-glutathionylation (sometimes referred to as glutathiolation, or 'thiolation') of protein thiols has long been known as an enzyme-catalysed reaction. The enzyme, glutaredoxin (Grx; sometimes called thioltransferase), is capable of efficiently catalysing the reaction in both directions (see Figure 9) [35-38].

Figure 9. Reversible S-glutathionylation of S-glutathionylated proteins, catalysed by glutaredoxin. Adapted from [35].

Glutaredoxin is an enzyme seemingly ubiquitous to mammalian cells, and present in most organisms [39-41]. Its structural relative, thioredoxin [41], may have a small capacity to aid Grx in the reduction of mixed disulphides *in vitro* [42]. Glutaredoxin has an *in vitro* efficiency of about 5000 times that of thioredoxin in de-glutathionylation reactions [43], and a broad specificity for various S-glutathionylated proteins. Hence, glutaredoxin appears to be the most physiologically relevant candidate for de-glutathionylation reactions *in vivo* [37, 41]. Glutaredoxin also has the capacity to reduce intramolecular disulphides, and can therefore function as backup for thioredoxin in some situations [41, 44, 45].

The catalytic site of glutaredoxin typically contains a CXXC motif (usually CPYC), a solvent-accessible hydrophobic area, and a binding site for GSH. To reduce a mixed disulphide, the enzyme recognises and binds to the GSH moiety of the mixed disulphide via its N-terminal cysteine thiol, forming a mixed intermediate. The protein is reduced and released, and the second intermediate glutaredoxin-SG is then in turn reduced by glutathione (see Figure 10). This mechanism is referred to as a "monothiol mechanism", and is functionally separate from its (less effective [42]) "dithiol mechanism", in which the enzyme utilises both its catalytic site cysteines to reduce intramolecular disulphides [41, 46].
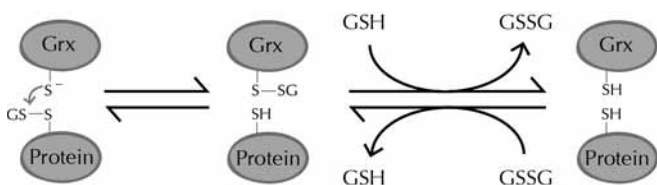
<u>Figure 10</u>. The glutaredoxin monothiol mechanism. The grey arrow shows the initiation of de-glutathionylation (reaction progressing from left to right). The S-glutathionylation reaction progresses from right to left. Adapted from [41].

Glutaredoxin has been shown to be sensitive to the redox status of the cell. Wild-type *Escherichia coli* Grx3 has a tendency to form mixed disulfides with GSH [38], with accompanying decreased catalytic activity [47]. A mutant construct of *E.coli* Grx3, with Tyr replacing Cys^{65} (a conserved cysteine also present in human Grx [48]) does not, like the wild-type enzyme, have this tendency, suggesting that S-glutathionylation of Cys^{65} regulates the wild-type enzyme's activity [49, 50]. This particular cysteine is S-glutathionylated *in vitro* during H_2O_2-induced stress, and it is possible that S-glutathionylation of residue Cys^{65} regulates Grx activity towards S-glutathionylation of protein thiols in oxidative stress situations [38].

Many factors may influence the process of S-glutathionylation *in vivo*. For a full appreciation on how different factors may influence glutaredoxin during oxidative stress, a thorough discussion concerning the properties of thiol-disulphide exchange is needed.

Several mechanisms have been proposed for S-glutathionylation, i.e. the formation of mixed disulphides between GSH and proteins. A decrease in the cellular

GSH/GSSG ratio due to the actions of oxidants such as diamide, H_2O_2, *t*-butyl-hydroperoxide and redox-cycling of quinones such as menadione, leads to the formation of mixed disulphides [51-57]. The properties and thermodynamic possibilities of the reversible glutathione-disulphide exchange reaction in the absence of enzymatic catalysis (see Figure 11) have been thoroughly discussed [22, 23, 58].

Thiol-disulphide exchange reactions

$$PrS^- + R\text{-}S\text{-}S\text{-}R \rightleftharpoons Pr\text{-}S\text{-}S\text{-}R + RS^-$$

$$PrS^- + Pr\text{-}S\text{-}S\text{-}R \rightleftharpoons Pr\text{-}S\text{-}S\text{-}Pr + RS^-$$

$$RS^- + R'\text{-}S\text{-}S\text{-}R' \rightleftharpoons R\text{-}S\text{-}S\text{-}R' + R'S^-$$

$$PrS^- + Pr'\text{-}S\text{-}S\text{-}Pr' \rightleftharpoons Pr\text{-}S\text{-}S\text{-}Pr' + Pr'S^-$$

Disulphide exchange reactions

$$PrS\text{-}SPr + R\text{-}S\text{-}S\text{-}R \xrightarrow{R'S^-} 2\ PrS\text{-}SR$$
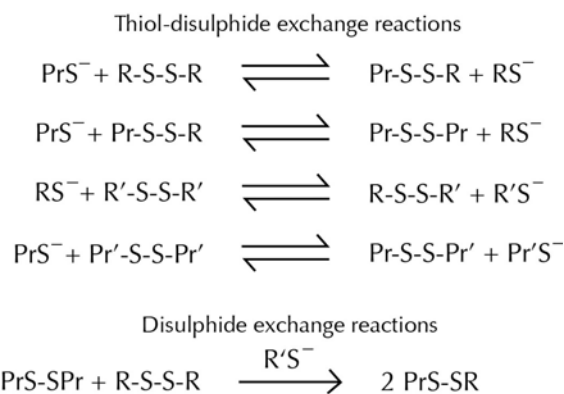
Figure 11. Proposed possible non-enzymatic thiol-disulphide exchange (four different kinds) and disulphide exchange reactions: Pr, protein; R, low molecular weight thiols (Cotgreave, Atzori and Moldéus, 1989).

Generally, for thiol-disulphide and disulphide-disulphide exchange reactions to take place under physiological conditions, the formation of a thiolate anion is necessary. Furthermore, the thiol-disulphide oxidation potentials of proteins are hard to predict. Thus, a large variation between those potentials, due to steric factors and amino-acid interactions within the proteins, is to be expected, and hence, one may presume that many proteins are not functionally affected simply by a physiologically plausible change in the GSH/GSSG ratio [23, 27, 58]. There are, of course, proteins whose functions can be non-enzymatically modified *in vitro* by a change in GSH redox status, one such protein being the transcription factor c-Jun [59] (for further reading on this transcription factor, see *2.2.2. Transcription factors and gene regulation*).

The larger complexity of the mechanisms of S-glutathionylation are made apparent by the observations that oxidative stress-induced formation of GSH-mixed-disulphides in cells is not necessarily dependant on perturbation of the cellular GSH/GSSG redox status [60, 61]. Would it be possible for oxygen radicals to interact

non-enzymatically with glutathione, or protein thiols, and form glutathione-mixed-disulphides?

Enzyme-catalysed formation of GSH-mixed-disulphides possibly becomes more rapid in the presence of free radicals [62], and the thiyl-radical intermediate mechanism suggested by Thomas and co-workers [63, 64] may explain this. In this mechanism, free radicals in the cell, like for instance OH$^{\cdot}$, induce the formation of reactive protein sulfhydryl intermediates, such as thiyl radicals, which then react rapidly with reduced GSH to form mixed disulphides (see Figure 12). A theory is that Grx can stabilise a GS$^{\cdot}$-radical in its active site and that the transfer of this radical to protein sulfhydryls is particularly rapid [62]. Thus, it does seem possible that the local redox environment in the cell will influence the enzymatic catalysis of S-glutathionylation and de-glutathionylation, and that the rate and direction of Grx-catalysed reversible S-glutathionylation is an event influenced by several factors.
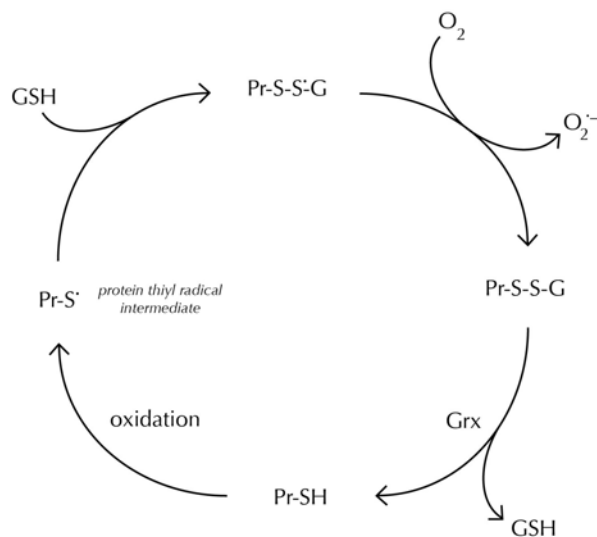
Figure 12. Proposed mechanism of reversible S-glutathionylation via a thiyl-radical intermediate: Pr, protein; GSH, glutathione. Adapted from [64].

2.2 Does S-glutathionylation have a purpose? Three functional examples:

There is no denying the importance of the cellular stress response, as it has the possibility to affect such diverse functions as apoptosis and proliferation, ion channel function, the cytoskeletal apparatus, signal transduction pathways, gene regulation and expression,

carbohydrate metabolism, neurotransmitter release and proteolysis [22, 65-67]. The repeated observations of S-glutathionylation of especially GAPDH, actin, and certain heat shock proteins and peroxiredoxins, in various cell types in response to various types of stress [61, 68-71], indicates that the binding of glutathione to these proteins is no random process. To shed further light upon the relationship between glutathione-mediated regulation and oxidative stress, some important examples are discussed below, including the complex regulation of actin polymerisation involving glutaredoxin [72-74], the influence of GSH/GSSG perturbations on transcription factors NFκB and AP-1 [59, 75], which has been used as an argument for the possibility of non-enzymatic redox regulation [67], and finally, the control of NADPH-production by stress-induced regulation of GAPDH and other glycolytic enzymes [27, 38].

2.2.1. Actin and the effect on cell morphology.

Actin is a cytoskeletal protein that is used by the cell for maintaining its structure and producing movement. It exists in two forms: Monomeric G-actin, and polymeric F-actin (which forms large fibres), and the polymerisation into F-actin is an ATP-dependent process. Actin fibres can, in turn, be organised into two distinct structures in the cell: Parallel, unbranched bundles of filaments and highly branched, interlaced filaments networks. The structures are formed with the aid of actin-bundling proteins (ABPs) such as villin and fimbrin, and filamin and Arp2/3, respectively. The combinations of the different ABPs give specific characteristics to the polymerisation of actin, and ABPs can thereby control actin morphology and polymerisation/depolymerisation rates. Several other proteins also influence the actin structure, most notably the actin-membrane-protein linkers (the ezrin/radixin/moesin family), which maintain directionality of protrusions, aid remodelling of focal adhesions and bind several membrane-associated proteins. The different actin structures are modified through a tightly regulated, continuous polymerisation and depolymerisation of the actin fibres. In this way, the cell remains flexible and can adapt structurally to its environment both by sending out spike-like protrusions of unbranched actin and by subtly changing the meshlike actin network which supports the membrane and the overall cell shape [76].

Oxidant-stressed mammalian cells undergo morphological changes, and their appearance is often characterised by round membrane protrusions, so-called 'blebs'. The formation of these is associated with an oxidative environment (membrane 'blebbing' can be blocked via inhibition of GSH oxidation) and the depolymerisation of actin [72, 77, 78].

Actin is a common substrate for S-glutathionylation, and its C-terminal cysteine (Cys^{374}) is a target for glutathione *in vitro*. When glutathione forms a reversible disulfide bond to the actin C-terminal cysteine *in vitro*, the actin polymerisation rate shows a 33% inhibition compared to native actin. This is believed to be at least partly due to a less efficient assembly of monomers, as the binding of glutathione induces a conformational change in a C-terminal loop surface involved in intermonomer interactions [74]. S-glutathionylation of actin and the effect on its polymerisation rate is not, however, affected by high concentrations of GSSG *in vitro*, as actin needs an activated thiol group to react with glutathione. Therefore, it was suggested that actin is probably not subjected to non-enzymatic thiol-disulphide exchange *in vivo*, and that the regulation of actin polymerisation is likely to be catalysed by Grx [79]. Interestingly, epidermal growth factor (EGF)-treated epidermal cells increase their F-actin content by 12% in a de-glutathionylation process dependent on the presence of glutaredoxin, which leads to polymerisation of F-actin in the periphery of the cell, and transient membrane ruffling. Treatment of the same cells with H_2O_2 leads to S-glutathionylation of actin, pointing towards an integrated Grx-modulated process in epidermal cells in response to stress or growth [73]. In conclusion, the binding of GSH specifically modifies a functional site on actin and affects its polymerisation rate, and this environment-sensitive (stress versus growth), reversible process can be regulated by Grx, but *not* via non-enzymatic GSSG-exchange.

Sulfhydryl groups on actin activated by reactive oxygen species readily react non-enzymatically with glutathione [62], and therefore one cannot simply assume that the S-glutathionylation processes are mediated by Grx only. However, a regulatory process of some kind seems to exist with regard to actin polymerisation, and it is even possible that the injury-associated bleb-formation during oxidative stress is a direct result of enzymatic redox-regulation. Glutathione-depleted cells respond severely to stress by irreversibly

contracting and detaching from culture plates, and hence, it has been suggested, that by inhibiting actin polymerisation, the cell finds a way to preserve its microfilament dynamics, by hindering polymerisation reactions that may perturb its focal adhesions [72].

Cell-cell contacts and cellular adhesion commonly involve linker molecules that attach the actin cytoskeleton to the cell membrane. Actin polymerisation is essential both to the formation of cell-cell contacts (adherens junctions) and in the cell's adhesion to a surface or the extracellular matrix (fibrillar adhesion and also possibly focal adhesions), and inhibition of actin polymerisation leads to inhibited cell movement [80, 81]. A decreased polymerisation rate could hence function as a stabilising factor for a stressed cell (prolonged attachment to a surface or extracellular matrix), and decreased movement seems a logical response to a stressful environment.

Bleb formation on the plasma membrane is a common occurrence in both stressed and non-stressed cells, and is directly related to impaired polymerisation of F-actin [82, 83]. The formation of cell membrane blebs has been shown to be a result of cell membrane expansion outpacing the local rate of actin polymerisation [82]. Also, many cellular blebs feature detachment of the cell membrane from the actin cytoskeleton during their formation [83]. Hence, the occurrence of blebs in stressed cells may well be a symptom of decreased actin polymerisation in combination with stress-induced disruption of cell-cell contacts and cellular adhesion mechanisms.

2.2.2 Transcription factors and gene regulation

Several transcription factors are modified by oxidative stress, which affects the transcription of proteins involved in many important response pathways of the cell, such as immune responses, protective heat shock responses, apoptosis and cellular proliferation [84-87]. Two transcription factors that especially have received a lot of attention lately are NFκB and AP-1. Both NFκB (including related proteins of the v-Rel and c-Rel oncoprotein family) and AP-1 carry a DNA-binding site containing a well-conserved cysteine, important for binding [59, 88], flanked by basic amino acids [89, 90]. Furthermore, a cell study has shown that both transcription factors can have their activity increased by increased expression of Grx [91].

The transcription factor NFκB is very much involved in inflammatory responses [92] but it is also, among its other functions, activated by a number of conditions that disrupt the intracellular redox status [84, 85, 93]. It is possible that the difference between the redox status in the cytoplasm and the nucleus may play a part in this regulation, since the release of NFκB from its inhibitory protein in the cytoplasm (induced by oxidative events) and the binding of NFκB to DNA in the nucleus (dependent on a reducing environment) appear to be two separately regulated, redox-sensitive events [84]. It has been proposed that since NFκB is activated by a physiologically relevant increase in GSSG but inhibited by higher GSSG levels, this reflects its ability to react optimally to oxidative bursts during an immune response [90, 94]. Impaired DNA-binding of NFκB due to high levels of GSSG is restored by induced expression of thioredoxin in several cell studies [88, 90, 95], however, indicating that the reduction of internal disulphide bonds is involved in this redox regulatory event.

The p50 subunit of NFκB can undergo non-enzymatic S-glutathionylation *in vitro*, preferentially on the Cys^{62} residue of its DNA-binding domain, when exposed to a radical decrease in the GSH/GSSG ratio, reversibly inhibiting NFκB's DNA-binding capacity [75].

The transcription factor AP-1 is involved in the execution of proliferative and differentiating responses in the cell, and binds to growth-factor inducible genes implicated in tumour promotion. The transcription factor is a usually a dimer of the proteins c-Jun and c-Fos, although c-Jun has the possibility to form homodimers. The activity of AP-1 has both been found to be regulated by several antioxidants and to be related to the overall cell redox status [84, 85]. Transcription events dependent on AP-1 were activated by a cytosolic increase in GSSG (at a ratio of GSH/GSSG of 10:1 and below) in a cell study, although the DNA binding of the transcription factor was slightly inhibited at the lowest ratios [94]. It has been suggested that AP-1's relative unresponsiveness to inhibition due to H_2O_2-induced decreases in the GSH/GSSG ratio in cell studies could reflect its primarily nuclear localisation, as GSSG levels are normally low in the nucleus [90].

The c-Jun protein has, as NFκB, a free cysteine in its DNA-binding site, which can be reversibly (non-enzymatically) S-glutathionylated *in vitro* following a radical

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decrease in the GSH/GSSG ratio, thereby disrupting DNA binding of the homodimer. The DNA binding capacity could not be restored with the aid of thioredoxin [59].

It is to be noted that both of the studies done on S-glutathionylation of transcription factors (by Pineda-Molina et al [75] and by Klatt et al [59]) were *in vitro* studies performed on cloned and purified protein domains using direct manipulations of the GSH/GSSG ratios. Physiologically, the ratio of GSH/GSSG rarely drops as low as 10, and non-enzymatic glutathione-mediated regulation of these transcription factors could only really be argued to be a response to severe oxidative stress. The influence of Grx upon these seemingly slow S-glutathionylation reactions in intact cell systems remains to be examined. Since Grx isoforms exist in the nucleus [96, 97], Grx could well be involved in this type of regulation.

As has been found in the present study [70], and in others [69], proteins involved in translation can be substrates for S-glutathionylation. Therefore, it seems likely that transcription factors also can be modified. Certainly, the connection between stress, inflammatory responses, heat-shock responses, apoptosis and cell proliferation are important from a physiological viewpoint, and the redox-mediated regulation of gene expression provide evidence for a complex regulatory response process in the cell during oxidant exposure. The structural motif suggested for redox-regulation is an interesting hypothesis, but its potential role needs further investigation.

2.2.3. Regulation of GAPDH activity, glycolysis regulation and the generation of NADPH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that catalyses the oxidative phosphorylation of glycealdehyde-3-phosphate into 1,3-bisphosphoglycerate, in a NAD^+-dependent reaction. The enzyme has long been known to be inactivated in the presence of H_2O_2. This is a fully reversible process if glutathione is supplied immediately after peroxide treatment. The inactivation was proposed to most likely be due to the formation of sulphenic acids on critical cysteine residues, and did not lead to intermolecular disulphide bond formation [98]. Glyceraldehyde-3-phosphate dehydrogenase was later discovered to be strongly S-glutathionylated in endothelial cells exposed to H_2O_2, and during the respiratory burst of monocytes. Hydrogen peroxide

treatment was coupled to reduced enzyme activity, with stronger inhibition in endothelial cells than in monocytes, and the removal of oxidants led to full recovery of function [61, 68]. Lind and colleagues [38] discovered that S-glutathionylation and de-glutathionylation of GADPH could be regulated by the redox-responsive enzyme Grx, but that this did not affect the enzyme's catalytic ability. Thereby, it was confirmed that it was the oxidation reaction with H_2O_2 that inactivated the enzyme, not the binding of GSH. A functional role for enzymatic S-glutathionylation of GAPDH, therefore, remains to be discovered. It was suggested that the S-glutathionylation could perhaps "switch" the enzyme towards its other reported functions [38], although this has not been confirmed.

Glyceraldehyde-3-phosphate dehydrogenase is not the first enzyme involved in carbohydrate metabolism discovered to be modified by glutaredoxin. Pyruvate kinase, which catalyses the last step in glucolysis (see Figure 13), has been shown to be reversibly inhibited by Grx-catalysed S-glutathionylation [36]. A number of other enzymes involved in glucose metabolism can have their activities modified by glutathione, as outlined in Table III.

Redox regulation appears to influence several aspects of glucose metabolism, including enzyme activities and protein turnover. It has been proposed, from observing the pattern of S-glutathionylation, protein activation and protein inhibition, that cells may rapidly respond to oxidative stress by switching glucose equivalents away from glycolysis and into the production of NADPH-reducing equivalents, via pentose phosphate metabolism [99, 27]. The committed step of the pentose phosphate pathway, catalysed by glucose-6- phosphate dehydrogenase, is activated by GSSG, although this effect could be indirect through generation of $NADP^+$ via reduction of GSSG and not a result of direct interaction between glutathione and the enzyme itself. It is noteworthy that, however, for activation of the pentose phosphate pathway, the NADPH/NADP+ ratio needs to be reduced to one tenth of its normal level [65]. An increase of the GSH/GSSG ratio, or sustained levels of H_2O_2, both stimulate the pentose phosphate pathway in red blood cells, an effect inversely proportional to the cellular level of GSH [99]. Further, in a yeast study, the exposure to H_2O_2 lead to reversible S-glutathionylation and inactivation of several glycolytic enzymes, but H_2O_2 did not adversely affect NADPH-production via the pentose phosphate pathway [100]. The production of
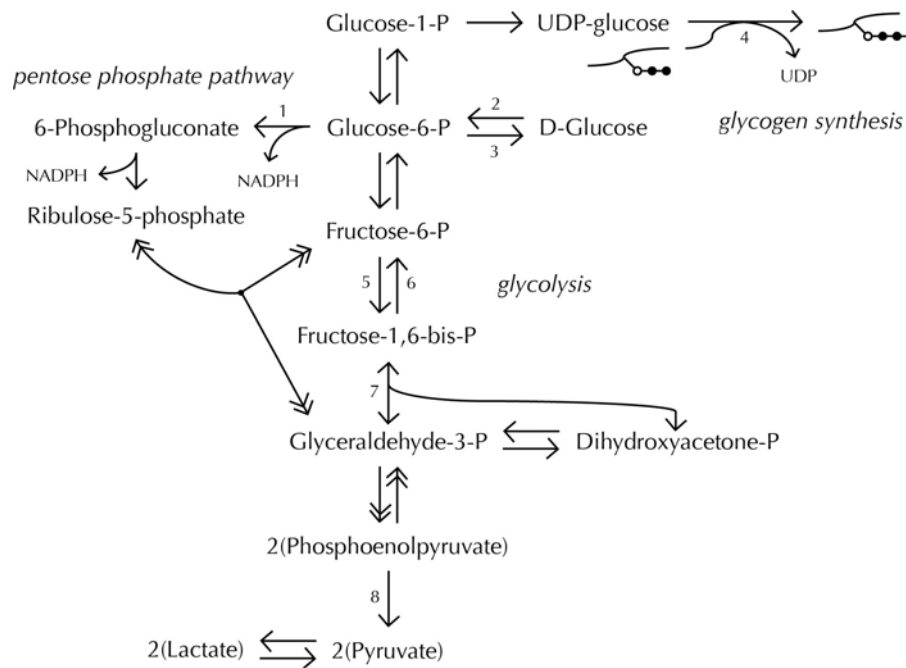
Figure 13. Scheme of carbohydrate metabolism outlining the reactions influenced by a change in reduced glutathione to glutathione disulphide ratio. Double arrows indicate several reactions that are not shown. Enzymes that are affected are represented by numbers: 1. Glucose-6-phosphate dehydrogenase, 2. Hexokinase, 3. Glucose-6-phosphatase, 4. Glycogen synthase, 5. Phosphofruktokinase, 6. Fructose-1,6-bis-phosphatase, 7. Fructose-1,6-bis-phosphate-aldolase, 8. Pyruvate kinase.

Table III. Enzymes of carbohydrate metabolism known to be reversibly affected by treatment with glutathione disulphide (or, in the case of Fructose-1,6-bis-phosphatase, Coenzyme A-SSG). Adapted from [27] and [65].

Enzyme	Regulation	Comment
Glucose-6-phosphate dehydrogenase	↑	Committed step in the pentose phosphate pathway; controlled by the ratio of NADPH/NADP$^+$. Is generally inhibited.
Hexokinase	↑	Effect: Prevents inactivation
Glucose-6-phosphatase	↑	
Glycogen synthase I	↓	Effect: Loss of activity *and* dissociation of the enzyme from glycogen granules
Glycogen synthase D	↓	
Phosphofruktokinase	↓	Presence of ATP makes the enzyme less sensitive to inactivation
Fructose-1,6-bis-phosphatase	↑	Effector: CoASSG
Fructose-1,6-bis-phosphate-aldolase	↓	
Pyruvate kinase	↓	Discussed in text

NADPH equivalents would be beneficial to the cell during stress, considering the fact that NADPH is used by glutathione reductase to reduce glutathione.

3. Methods for studying protein S-glutathionylation

3.1. The cell line ECV304, and its use as an oxidative stress model

The cell line ECV304 has long been used as a model cell line for human endothelium, as it was originally reported to be a spontaneously transformed human umbilical vein endothelial line [101], but its true origin has recently been questioned. ECV304 cells express many specific endothelial cell markers, such as vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM-1), thrombomodulin and the vitronectin receptor CD51, and they also exhibit typical endothelial features such as uptake of acetylated low-density protein LDL and expression of angiotensin-converting enzyme (ACE) [101, 102]. However, they do lack expression of the common endothelial marker von Willebrand factor [101], and possess many typical epithelial-like features [102-104]. A karyotypic (DNA-fingerprint) analysis performed on the ECV304 cell line showed it to be identical to the human bladder cancer derived cell line T24/83, and the cell line is therefore not recommended to be used as an endothelial model [104, 105].

Previous use of ECV304 shows its responsiveness towards oxidative stress, and capacity to form large amounts of mixed disulphides when exposed to the oxidant diamide [87, 70]. However, the plasticity and functional heterogeneity of endothelial cells is well-documented [105-107], and it is possible that the properties of ECV304 will change during culturing. Hence, although the cell line ECV304 cannot be used as an endothelial model in this case, it is very useful for the development of new methods for isolating mixed disulphides from cells, as long as efforts are made to ensure the cell line retains its stress-response properties.

3.2 Commonly used oxidising agents

Several agents are available for inducing oxidative stress and perturbing the redox status in the cell, such as redox-cycling quinones (e.g. menadione), *tert*-butyl hydroperoxide (an

organic peroxide), hydrogen peroxide and diamide. All of these increase GSSG and protein-glutathione mixed disulphides in cells [51-57]. As mentioned in section *1. Oxidative stress*, both the redox cycling compounds and the peroxides have the ability to either produce or aggravate free radical damage to the cell.

Diamide is a small oxidant molecule that has been commonly used in laboratories for about thirty years to modulate the cell redox status. It rapidly oxidises glutathione (GSH) to glutathione disulphide, and does this without producing free radicals or causing irreversible damage to biological systems [108-110]. Furthermore, it hydrolyses very slowly in aqueous solution ($t_{1/2}$ at pH 7.4, 25°C is approximately 3000 hrs) [110], which makes it a stable and experimentally reliable compound to use in biological systems.

Diamide reacts with GSH in a two-step reaction, in which a thiolate anion, GS$^-$, first attacks the diazene double bond to form a sulfenylhydrazine. This reacts with a second thiolate anion, which leads to the formation of a disulphide and a hydrazine (see Figure 14). In theory, Diamide can undergo the same reaction with any thiol [108].

$$GS^- + H^+ \qquad\qquad\qquad GS \qquad\qquad GS^- + H^+$$

$$(CH_3)_2NCON=NCON(CH_3)_2 \longrightarrow (CH_3)_2NCON-NHCON(CH_3)_2 \longrightarrow (CH_3)_2NCONHNHCON(CH_3)_2$$

$$GSSG$$

Figure 14. The two-step reaction between diamide and glutathione thiolate anions, leading to the formation of glutathione disulphide. Adapted from [108].

The preference for glutathione exhibited by diamide is due to the small disulfide's relative acidity and small size compared to the more sterically hindered protein thiols [108]. Diamide will also react easily with other small thiols in the cell such as cysteine and coenzyme A, as well as some other compounds (lipoic acid, ferredoxin and NADH), but the comparatively high concentration of GSH in the cell cytosol will lead to GSH being the primary target for oxidation by diamide. Additionally, GSH reacts with diamide much faster than the other reaction candidates in the cell. However, there is always a possibility in an experimental setting that diamide may react with other compounds to a small extent [108, 111, 112]. These reactions are of no relevance to the present study.

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3.3. [^{35}S] -labelling, biotin-labelled thiols and glutathione affinity columns

One of the most established methods for tracing S-glutathionylated poteins is by introducing cysteine labelled with radioactive sulphur (^{35}S) into the cell under conditions of protein synthesis inhibition. This cysteine is incorporated into GSH, cells are exposed to oxidative stress, and proteins are then extracted from the cell, separated by gel electrophoresis, and detected by autoradiography and sequence analysis. This method has been developed for proteomics analysis by Fratelli et al [69], and has led to the discovery of several substrates for S-thiolation in T-lymphocytes during stress induced by diamide or H_2O_2. A few problems arise when using radiometric analysis in this way, however. Firstly, the proteins identified cannot be identified exclusively as 'S-glutathionylated', since proteins may form mixed disulphides with small sulphur compounds other than glutathione, even though this occurs to a smaller extent intracellularly [113]. Secondly, the perturbation of protein synthesis imposes a large impact on the cell, and hence, the protein profile extracted is also dependent on this treatment. Thirdly, the method has not been shown to be very sensitive towards S-glutathionylated proteins present in low quantities, rendering the study of constitutively modified proteins difficult [70, 113].

Another approach to identify S-glutathionylated proteins is by incorporating membrane-permeant biotin-labelled thiols into the cell media. This has been tested with biotinylated cysteine [114] and biotinylated glutathione ethyl ester [115], where the thiols cross the cell membrane and become coupled to cellular proteins during pro-oxidizing conditions. Biotin-labelled proteins in isolated cell fractions can then be purified with the aid of affinity chromatography, separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and identification by either immunoblotting, mass spectrometry or automated Edman degradation [114, 115]. Although these approaches interfere less with cellular processes than the radiolabelling procedures, the problem with adding biotinylating thiols to cells is the competition of protein-binding between the existing cellular glutathione and the biotinylated thiols used, which means that not all proteins modified by S-glutathionylation will be identified.

The susceptibility of proteins towards S-glutathionylation can also be tested through the use of various types of glutathione affinity columns. These columns can absorb proteins susceptible to non-enzymatic S-glutathionylation, which can later be

eluted with the help of a sulphur reducing agent, for instance dithiothreotol or β-mercaptoethanol. The problem with these types of methods are that the proteins identified have not actually been labelled by the cellular glutathione pool. Additionally, the methods are not able to take into account either the effects of enzymatic S-glutathionylation or the effects of stress-induced radical-exchange reactions in the cell [67, 114].

3.4 Proteomics, mass spectrometry and their uses in protein identification

Proteomics is the study of total protein content (proteome) from a particular cell line, tissue or organism. This field can further be roughly divided into three categories: *Expression proteomics*, which deals with finding disease markers and elucidating biological pathways, *cell-map proteomics*, which is the systematic study of protein-protein interactions, and *structural proteomics*, which involves the determination of three-dimensional protein structures on a genome-wide scale with the aid of crystallography, nuclear magnetic resonance and computational studies. The most commonly used experimental techniques in proteomics are two-dimensional (2-D) gel electrophoresis for separation of proteins and mass spectrometry (MS) for identification of proteins [116-118].

Two-dimensional gel electrophoresis can separate a large number of proteins, since it separates them in two ways: First by charge using isoelectric focusing and then by size using SDS-PAGE. The proteins are visualised after separation by staining the gel with dyes like Coomassie Brilliant Blue, silver ions or fluorophores, allowing approximate determination of pI and molecular weight. Two-dimensional gel electrophoresis is very effective at separating charge and size isoforms of the same protein [119].

Excising protein from a gel manually is very time-consuming, which has led to the development of robotic systems. There are other drawbacks that are harder to deal with, however: Most 2-D gels can only separate proteins between pI 4 and 10 (excluding very acidic and basic proteins), and proteins with molecular weights below 15kDa or above 200kDa require special gels. Also, low abundance proteins and membrane proteins are often not observed in the gel [119, 120].

Mass spectrometers consist of three essential components: An ionisation source that converts molecules into gas-phase ions, a mass analyser that separates ions by their mass-to-charge (m/z) ratios and an ion detector that determines the m/z ratio of individual ions. The development of mass spectrometry for protein identification has been essential for the fast processing of proteomics data. During the 1990s, changes in MS instrumentation and associated techniques hence revolutionised the analysis of proteins, beginning with the development of two ionisation techniques for biomolecules: Matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). These techniques solved the difficult problem of generating ions from large, volatile analytes without significant sample fragmentation, and gave rise to two new kinds of information generated by the mass spectrometer: Firstly, an accurate measurement of weight of biomolecules and secondly, an amino acid sequence [121, 122].

Matrix-assorted laser desorption ionisation creates ions by laser-excitation of an energy-absorbing matrix. The matrix consists of small, UV-absorbing molecules, which co-crystallise with peptides or proteins on a sample plate. Energy absorption leads to rapid thermal expansion of both matrix and analyte into the gas phase. The most common mass analysers used with MALDI are time-of-flight (TOF) mass spectrometers, which generate mass-to-charge ratios from the time it takes a packet of ions with a set of electric potentials to traverse a flight tube [119, 121].

Electrospray ionisation creates ions by application of a potential to a flowing liquid, causing the liquid to charge and subsequently spray. The electrospray creates very small droplets of solvent-containing analyte, and the solvent is removed by, for instance, heat or energetic collision with a gas (a "drying gas"). As ESI produces a continuous stream of ions, it can easily be coupled to a high-performance liquid chromatography instrument, which can pre-separate the proteins or peptides in the sample prior to analysis. The generation of a range of multiply charged ions by ESI allows for analysis of higher molecular weight compounds by mass spectrometers with limited m/z ranges [119, 121].

Mass analysers commonly used in proteomics are quadropole mass analysers and TOF analysers, usually coupled in various combinations. Quadropole analysers use a radiofrequency voltage applied to four metal rods with radiofrequency voltage of

alternate polarity placed on opposite rods. A direct current is overlayed on the rods, and the combinations of the currents can be set to stabilise the trajectory of ions of a particular m/z value as they pass through the rods, and the currents are recorded for each ion. Quadropole ion trap (IT) analysers create three-dimensional radiofrequency fields to trap ions in the centre of a ring electrode. The field can then be manipulated to eject the ion for further analysis, or induce collisions with the carrier gas, generating fragments for analysis. Collisions with a carrier-gas can also be used in tandem mass spectrometers (MS/MS): In TOF-TOF, ions of a particular m/z value are selected, and then pass into a collision cell to be fragmented. Quadropole-TOF instruments combine quadropole selection with time-of-flight identification. Ion trap and TOF analysers are compatible with MALDI [119].

Tandem mass spectrometers and, to a more limited extent, single-stage mass spectrometers, can fragment peptide ions and record the resulting ion spectra. Generally, the low-collision energy spectra generated by ESI-MS/MS are of high quality and are sequence specific. Low-collision energy spectra generated from these mass spectrometers are dominated by fragment ions resulting from cleavage at amino bonds, but internal fragments also arise, particularly if proline or aspartic acid residues are present. The ions are differentiated by which end of the peptide that retains a charge after fragmentation: If the positive charge associated with the parent peptide ion remains on the amino-terminal side of the fragmented ion, this fragment is referred to as a *b* ion, but if the charge remains on the carboxy-terminal side of the bond the fragment ion is referred to as a *y* ion. All ions generated are separated by mass and sorted along an m/z scale, and the amino acid sequence is deduced from the mass difference between the different parent ions and fragments produced. The *b* ions are designated by a subscript that reflects the number of amino acids counting from the amino-terminus, whereas *y* ions are denoted counting from the carboxyl-terminus. To calculate the mass of the *b* ion series, the mass for 1 H is added, and for the *y* series, the mass for H_3O^+ is added [122].

There are two main approaches to identifying proteins in mixtures: Either 1. By gel electrophoresis, in gel proteolysis, mass spectrometry and database searching, or 2. By proteolysis, liquid chromatography-MS/MS and database searching. In the latter approach, the capability of the tandem mass spectrometer to separate and fragment ions

individually is used to analyse the peptide mixture directly, after an initial step of liquid chromatography separation. For analysis of protein-protein interactions, non-denaturing conditions are used, in combination with, for instance, chromatography, co-immunoprecipitation, co-precipitation of affinity-tagged proteins, or protein affinity interaction-chromatography [121]. There have also been approaches developed for quantitative analysis by the mass spectrometer using isotope labelling [123, 124].

Several publicly available algorithms exist today for easy, automated identification of fragmented peptides and matching of their spectrum with a suggestion for a likely amino acid sequence [119].

THE PRESENT STUDY

1. Aims of the study

The general purpose of this study was to develop a method suitable for proteomics-based studies of proteins undergoing oxidation of cysteines by S-glutathionylation. The specific aims were to:

I.   Develop a suitable technique for rapid identification of proteins undergoing S-glutathionylation.

II.   Develop a suitable protocol for affinity purification and site-specific analysis of trypsinised, S-glutathionylated proteins.

2. Methods

2.1 Cell culture and diamide exposure

The ECV304 cell line was used in the study, and cultured in Dulbecco's Modified Eagle Medium supplemented with fetal calf serum. The cells were exposed to diamide-induced oxidative stress to produce large amounts of S-glutathionylated proteins. The free protein thiols in the stressed cells were blocked with N-ethyl-maleimide (NEM) prior to cytosolic protein preparation by ultracentrifugation. In paper 1, unexposed cells were also analysed. Furthermore, the presence of free protein thiols before and after NEM-treatment was tested by measuring reactivity of the cytosolic extract proteins with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [125].

2.2 Affinity tagging procedures

Isolated S-glutathionylated cellular proteins were de-glutathionylated using the *E. coli* mutant Grx3C14SC65Y (specific for de-glutathionylation [49] of mixed disulphide substrates [46] in the presence of NADPH and glutathione reductase. The release of glutathione during the de-glutathionylation procedure was confirmed by autoradiography.

In paper 1, the freed protein cysteines were then tagged with biotin-maleimide, and the protein mixture was applied to an affinity column. In paper 2, the freed protein cysteines were either tagged with biotin maleimide or biotin-(polyethylene oxide)-maleimide, the proteins were trypsinised overnight and the trypsinised extract was applied to the affinity column.

2.3 Affinity chromatography

Tagged proteins (paper 1) or peptides (paper 2) were isolated on a monomeric avidin affinity column, and eluted with biotin (paper 1) or acetic acid (paper 2) prior to analysis. To evaluate the efficacy of the different avidin-affinity columns used in paper 2, the column eluates were analysed by reverse-phase high-performance liquid chromatography (RP-HPLC).

## 2.4 Analysis of affinity-isolated proteins and peptides

In the case of paper 1, proteins were separated by 2-D gel electrophoresis, trypsinised, analysed by MALDI-TOF mass spectrometry, and identified by database search using the Mascot fingerprinting program. In paper 2, peptides were separated by reverse-phase nano-HPLC, analysed by ESI-quadropole-TOF mass spectrometry, and identified using the Mass Lynx 4.0 peptide sequence software and database search.

3. Results and discussion

3.1 Paper 1

A protocol was established (see Figure 15) to identify proteins undergoing oxidation of thiols by S-glutathionylation in diamide-stressed cells. Free protein thiols in intact cells were blocked with NEM (step 1) prior to preparation of cytosolic proteins (step 2). The proteins were then de-glutathionylated using Grx (the *E.coli* mutant Grx3C14SC65Y; step 3) and the freed protein thiols were tagged with biotin-maleimide (step 4). The tagged proteins were isolated by avidin-affinity chromatography (steps 6-7), and the eluted proteins were analysed by 2-D gel electrophoresis (step 9). Finally, the proteins

were trypsinised and identified using MALDI-TOF mass spectrometry and database search (steps 10-12).
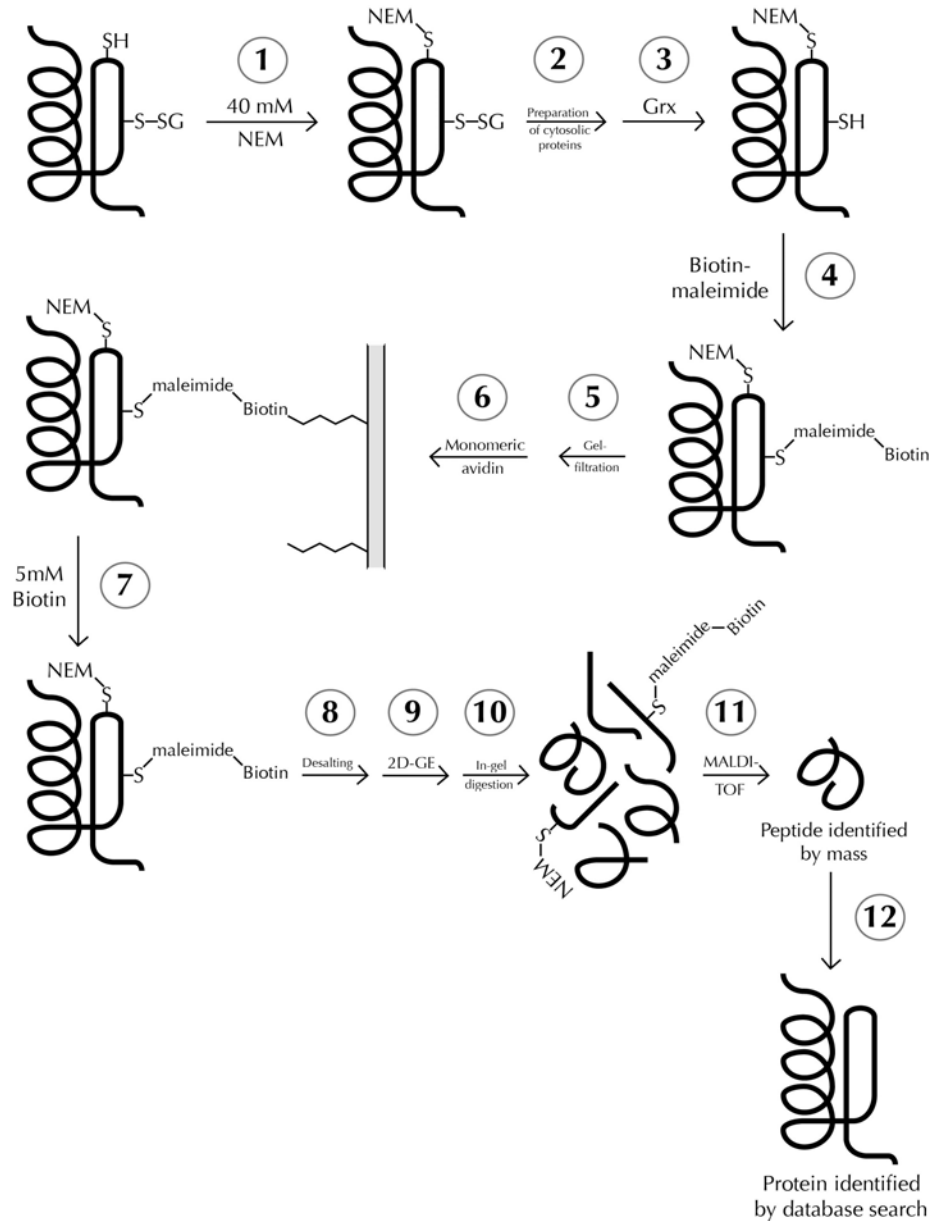
Figure 15. Scheme of protocol developed in paper 1: GSH, glutathione; NEM, N-ethyl-maleimide; Grx, glutaredoxin; 2D-GE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted time-of-flight mass spectrometry.

The different steps were validated as follows:

- Little interaction with DTNB after NEM-treatment (step 1) confirmed that the free thiols had been blocked by NEM.

- Autoradiographic analysis confirmed rapid and specific de-glutathionylation by the Grx mutant (step 3).

- The amount of cytosolic protein present in the 'GSH-stripping' incubation was proportional to the absorbance of the avidin-affinity column eluate peak (steps 6-7). Furthermore, omittance of Grx before biotin-tagging led to disappearance of this peak, confirming that the affinity capture was specific. The experimental procedure employed appeared to be well reproducible, not only in diamide-stressed cells, but also in cells undergoing constitutive metabolism.

The method unequivocally identified 43 mostly novel cellular protein substrates for S-glutathionylation. These included protein chaperones, cytoskeletal proteins, cell cycle regulators, and enzymes of intermediate metabolism. Uniquely, the method could be used to identify proteins undergoing S-glutathionylation both during constitutive metabolism and during diamide-induced stress. Comparisons of patterns of S-glutathionylated proteins extracted from cells undergoing diamide-induced oxidative stress and during constitutive metabolism revealed that diamide predictably increased overall cellular S-glutathionylation, but that although some substrates were common for both treatments, there were also some protein substrates that failed to undergo enhanced S-glutathionylation during oxidative stress. Several of the proteins discovered to be S-glutathionylated have also been found in other, radiolabelling-based, proteomics studies of stressed cells [69, 71].

The selectivity of the analytical approach is based on the ability of Grx to identify GSH-protein mixed disulphides and reduce them. However, as native Grx can recognise constitutive protein disulphides and reduce these also [41], this would produce artefacts in the NEM-biotinylation procedure. The Grx mutant used (*E.coli* Grx3C14SC65Y) has a mutated active site residue that dictates reduction of GSH-protein mixed disulphides [46] and is, furthermore, unable to undergo auto-glutathionylation at the 65 position, which

otherwise would steer the enzyme towards S-glutathionylation [38]. The use of the Grx mutant therefore ensures high selectivity and high-throughput of the established protocol.

Although redox modification of proteins is supposed to play a central role in regulating cellular function, suitable techniques for the analysis of the redox status of protein in complex mixtures are lacking in the literature. The method developed in this study is high-throughput, and more importantly, can specifically identify S-glutathionylated proteins from cells with minimal disruption of cellular function. Therefore, the method can successively be used to study post-translational redox modification of cellular proteins – a possibly important biochemical control mechanism in coordinating cellular function.
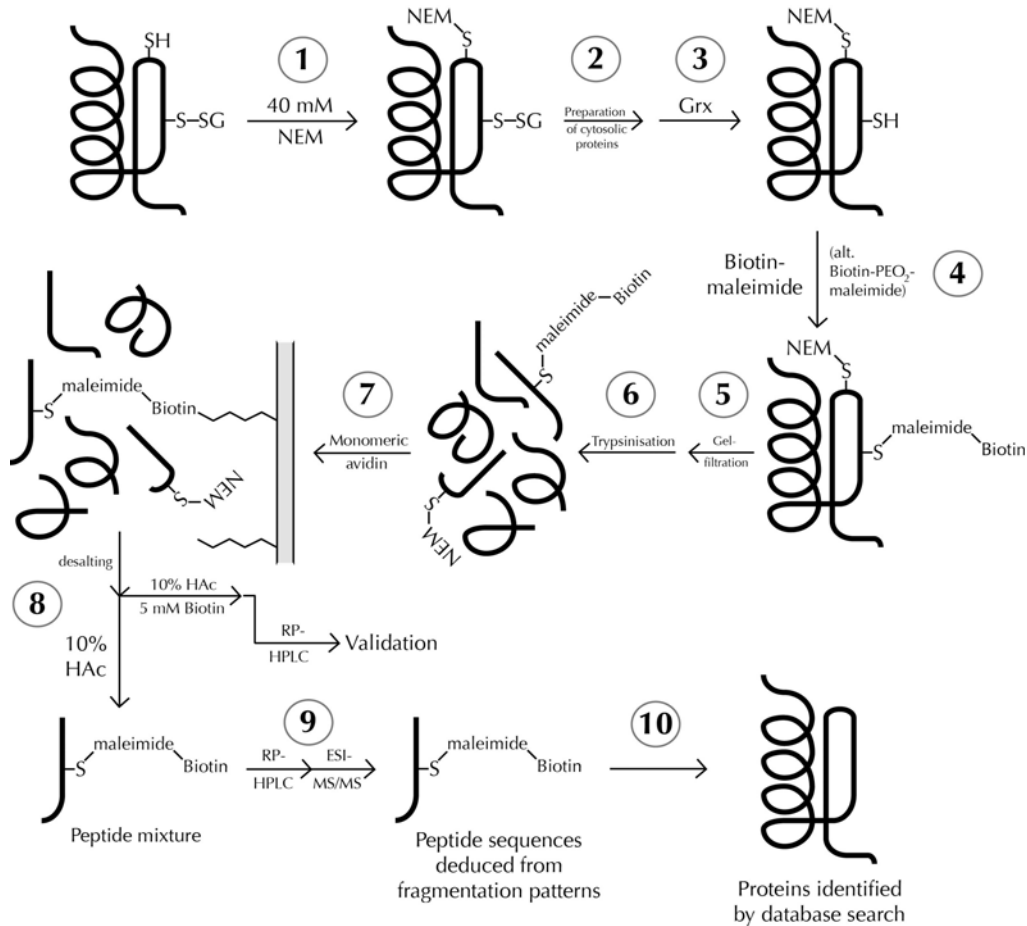
3.2 Paper 2

The protocol of paper 1 was further developed (see Figure 16) to allow for affinity purification and analysis of trypsinised S-glutathionylated proteins under non-reducing conditions, in an attempt to avoid the cumbersome 2-D gel electrophoresis step and at the same time identify the sites of S-glutathionylation by tandem mass spectrometry.

As the avidin-column previously used (Paper 1) is no longer produced, the two commercial columns whose biotinylated protein binding capacity was deemed the highest were tested on a trypsinised mixture of biotinylated S-glutathionylated proteins (steps 7-8). One of these columns bound several peptides reversibly, and the peptides could be eluted with acetic acid, which made the eluate suitable for mass spectrometry analysis. The absorbance of the elution pool was proportional to the amount of digested protein applied to the avidin column.

Mass spectrometry analysis of the eluate (steps 9-10) revealed several peptides, two identifiable which contained a cysteine residue and a particular fragmentation pattern that could be interpreted as N-ethylmaleimide-biotin binding. This pattern could be used as a selection tool for the mass spectrometer to identify tagged proteins in mixed samples, which lessens the demand for sample purification. The peptides were identified as elongation factor 1-α-1 and heat shock protein 60, and their S-glutathionylation sites were identified as Cys^{411} and Cys^{447}, respectively. However, most peptides with an amino

acid sequence of at least 7-8 found by the mass spectrometer lacked this N-ethylmaleimide-biotin binding fragmentation pattern.

Figure 16. Scheme of the modified protocol utilised in paper 2: GSH, glutathione; NEM, N-ethyl-maleimide; Grx, glutaredoxin; PEO$_2$, polyethylene oxide; HAc, acetic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; ESI-MS/MS, electrospray ionisation tandem mass spectrometry.

The protocol was modified to reduce unspecific binding of peptides. NaCl washing of the avidin column prior to acetic acid elution (step 8) removed a large number of peptides, and comparisons of the elution profiles by RP-HPLC showed seemingly no difference between biotin-eluted peptides and acetic acid eluted peptides. In order to improve the binding characteristics to the avidin column by the biotin-tagged peptides, a water-soluble and larger variant of the biotin-maleimide tag (biotin-(polyethylene oxide)-maleimide) was used to biotinylate the proteins prior to trypsinisation and avidin column

application (step 4). Mass spectrometry analysis of the affinity-isolated eluate led to the identification of a third peptide, derived from gamma-actin, and its S-glutathionylation site was Cys[217]. However, the tag did not improve the affinity for the avidin column.

The main problem with this protocol appears to be the affinity capture procedure, and other studies utilising similar strategies for peptide isolation confirm that the use of commercially available avidin columns for peptide-capture leads to significant quantitative losses and unspecific and/or irreversible binding of the biotinylated peptides [123, 124, 126, 127]. Further developments within the field of peptide affinity capture are needed before the full potential of this protocol can be realised.

4. Conclusions and future perspectives

S-glutathionylation is an expanding field within cell signalling research. Oxidative stress has been shown to be involved in several human diseases, such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, hypersensitivity, autoimmunity and acute respiratory distress syndromes [3], and glutathione has been shown to be centrally involved in the disease progression of such severe diseases as Parkinson's disease [128], cystic fibrosis [129], and AIDS [130]. Despite this fact, suitable methods for thorough investigation of the possible cell signalling processes that involve glutathione modification of proteins do not exist.

In this study, for the first time, a method has been developed for high-throughput identification of S-glutathionylated proteins without interference with cellular function. This protocol has also been modified to include identification of the S-glutathionylation sites of the proteins involved, although difficulties in peptide affinity capture interfere with specificity and high-throughput analysis.

Recent developments within mass spectrometry [123, 124] allow for quantification of protein expression using radiolabelling in tandem mass spectrometry analysis. The use of this technique in combination with site-specific analysis of protein modification would be a powerful tool in proteomics research, and ideal for the investigation of cellular S-glutathionylation processes. As the interest for biotin-affinity capture of peptides prior to MS-analysis appears to be increasing, hopefully the demand

for better specificity will lead to the creation of efficient procedures for biotinylated peptide capture. Perhaps then, the role of S-glutathionylation in cell signalling can be properly investigated, revealing the full extent of the complexity of redox regulation of cellular functions.

ACKNOWLEDGEMENTS

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