



Hydrogen sulfide mediated deCoAlation of CoAlated Peroxiredoxin 6

Daniella Dörgő
Chemistry MSc student



National Institute of Oncology
Oncology Research Center
Department of Molecular Immunology and Toxicology (MITO)

Supervisor: **Prof. Péter Nagy, D.Sc.**
Scientific Director

Budapest
2024

Acknowledgements

I express my gratitude:

to my supervisor, Prof. Péter Nagy, who, as the scientific director of the National Oncology Institute and the head of the Department of Molecular Immunology and Toxicology (MITO), allowed me to join his group and supported and coordinated my work with exemplary professional humility and competence;

to my consultant, Dr. Andrea Domán, who was endlessly patient and selfless in support during the practical work and writing, and provided me with constant motivation and enthusiasm through her cheerful personality;

to Dr. Éva Dóka, who introduced me to the team and introduced me to work with her selfless and supportive help;

to Dr. Anna Lopata, who provided an incredible amount of help during the practical work, and from whom I could learn a lot professionally;

to Dr. Tamás Ditrói, who performed the LC-MS/MS measurements and there was no question he could not answer;

to the other members of MITO, Dr. Ágnes Czikora, Dr. Dorottya Garai, Dr. Katalin Erdélyi, Klaudia Galambos, Annamária Hakl, Petra Eszter Jurányi, Kornél Kerényi, Zsuzsanna Lénárt, Noémi Szántó, Dr. Laura Somodi and Réka Szatmári, who helped with all questions that arose, created a welcoming and professionally motivating atmosphere, and showed what true teamwork is like;

to Professor Dr. Gábor Magyarfalvi, who, as a teacher of the chemistry seminar at Bolyai College, connected me with the team and helped me to improve;

to Bolyai College for the professionally motivating environment;

to my Mom and Dad for supporting me from the beginning;

to my family, friends and partner, for always being able to count on them.

Table of Contents

1. INTRODUCTION	4
2. LITERATURE OVERVIEW	6
2.1. Biochemical roles of hydrogen sulfide	6
2.1.1. Biochemical properties of hydrogen sulfide	6
2.1.2. The two sides of hydrogen sulfide: toxicity and bioactivity	7
2.1.3. Hydrogen sulfide metabolism	7
2.1.3.1. Endogenous sulfide production	7
2.1.3.2. Catabolism of sulfide	8
2.1.4. Biological sulfide buffer systems	9
2.1.5. Biologically relevant reactions of H ₂ S	10
2.2. Redox homeostasis	11
2.2.1. Reactive oxygen species (ROS)	12
2.2.2. Oxidative stress	13
2.2.3. Antioxidant defense system	13
2.2.3.1. Protein persulfidation and the oxidative stress	14
2.2.3.2. Protein glutathionylation and CoAlation and the oxidative stress	16
2.3. Peroxiredoxin family	18
2.3.1. The role of peroxiredoxins	20
2.3.1.1. Possible roles of sulfide in protection against overoxidation of peroxiredoxins	21
2.3.2. Peroxiredoxin 6	23
2.3.2.1. Function and activities	23
2.3.2.2. Structure	24
3. AIM OF THE WORK	26
4. MATERIALS AND METHODS	27
4.1. Expression of recombinant wild type and C47S mutant peroxiredoxin 6	27
4.2. Purification of wild type and C47S mutant peroxiredoxin 6	28
4.3. Determination of protein concentration	29
4.4. Gel electrophoresis	29
4.4.1. Coomassie staining	30
4.4.2. Western blot	30
4.5. CoAlation of peroxiredoxin 6	31
4.6. Sulfide-mediated deCoAlation reaction and sample preparation for Western blot and bottom-up proteomic measurement	31
4.7. LC-MS/MS measurement of peptides	33
5. RESULTS	34
5.1. Characterization of the produced recombinant proteins	34
5.1.1. Purity	34
5.1.2. Determination and quantification of the expressed proteins as peroxiredoxin 6	37
5.2. CoAlation of peroxiredoxin 6	37
5.3. Sulfur species-mediated deCoAlation of Prdx6-CoA	39
5.3.1. Comparing the deCoAlation effect of inorganic sulfide and disulfide	39
5.3.2. Comparing the deCoAlation effect of sulfide for C91S and WT Prdx6-CoA	41
5.4. Identifying the products of sulfide-mediated deCoAlation of C91S and WT Prdx6-CoA by LC-MS/MS technique	42
5.4.1. DeCoAlation experiments with excess sulfide	42
5.4.2. DeCoAlation experiments with excess peroxiredoxin 6	44
6. CONCLUSIONS AND FURTHER PLANS	47
7. REFERENCES	49
SUMMARY	55

1. Introduction

Hydrogen sulfide (H_2S) is a small, gaseous molecule with a pungent smell, that has been long recognized as a toxic gas. In recent years, studying the biological role of hydrogen sulfide has become a major field within redox biology.¹ It is now clear that dedicated enzyme systems are responsible for the production and degradation of sulfide in the body. Growing evidence supports that H_2S has a regulatory function in almost all cell types and is involved in a wide variety of biochemical reactions and cellular signaling. However, its method of action on a molecular level is still a matter of intensive research. One of the possibilities is that H_2S exerts its biological effect through covalent modification of the thiol group of cysteine-containing proteins through protein-persulfidation.² Thiol proteins (Protein-SH), which contain one or more redox active cysteines in their sequence, play an important role in biological systems because the thiol group of the cysteine residue may undergo a wide variety of redox reactions and post-translational modifications (PTMs) such as sulfenylation (Protein-SOH), persulfidation (Protein-SSH), glutathionylation (Protein-SSG) etc. These PTMs may alter protein functions in response to (patho)physiological changes.³

An important layer of the biological functions of H_2S is its reported cytoprotective effect. Hydrogen sulfide is thought to counteract oxidative stress through interactions with protein cysteine residues, thus contributing to the maintenance of healthy cellular redox balance, referred to as redox homeostasis.⁴ Oxidative stress arise when oxidative processes overwhelm reducing effects inside the cell and the resulting excessive oxidation may cause serious cell damage. Oxidative stress arises when oxidative processes overwhelm reducing effects inside the cell and the resulting excessive oxidation may cause serious cell damage.⁵ Elevated hydrogen peroxide (H_2O_2) level is a possible source of oxidative stress. One of the most important groups of thiol proteins with antioxidant functions is the peroxiredoxin (Prdx) family. They are distinguished among enzymes by their extremely fast and selective reaction with hydrogen peroxide, therefore their primary role is to control physiological H_2O_2 levels.⁶

The human Prdx family covers six members (Prdx1-6), and the active center of Prdxs contains a so-called peroxidative cysteine (C_p) residue which becomes sulfenylated in its reactions with H_2O_2 during the catalytical cycle. However, the possibility of the generation of other PTMs on the peroxidative cysteine residues and their biological implications are not fully understood in every Prdx.⁷

Prdx6 is a unique member of the Prdx family because it has only one conserved cysteine in its structure, the peroxidative Cys. The rest of the group have a second Cys in their active

site, the resolving Cys (C_r), which creates intra- or intermolecular disulfide bonds with the C_p residue. Lacking the resolving cysteine, Prdx6 is usually glutathionylated intracellularly, that is, forms a mixed disulfide bond with GSH.^{8,9}

Another recently discovered post-translational protein modification similar to S-glutathionylation is CoAlation. It appears that CoAlation of thiol proteins is an oxidation induced, reversible PTM, although it is currently unclear how CoAlation and deCoAlation reactions are regulated. Reduction of Protein-S-S-CoA bonds by sulfide and/or inorganic polysulfides emerged as an interesting possibility as it could give rise to persulfidated products that may be involved in signaling processes as well as protection against oxidative stress.^{10,11}

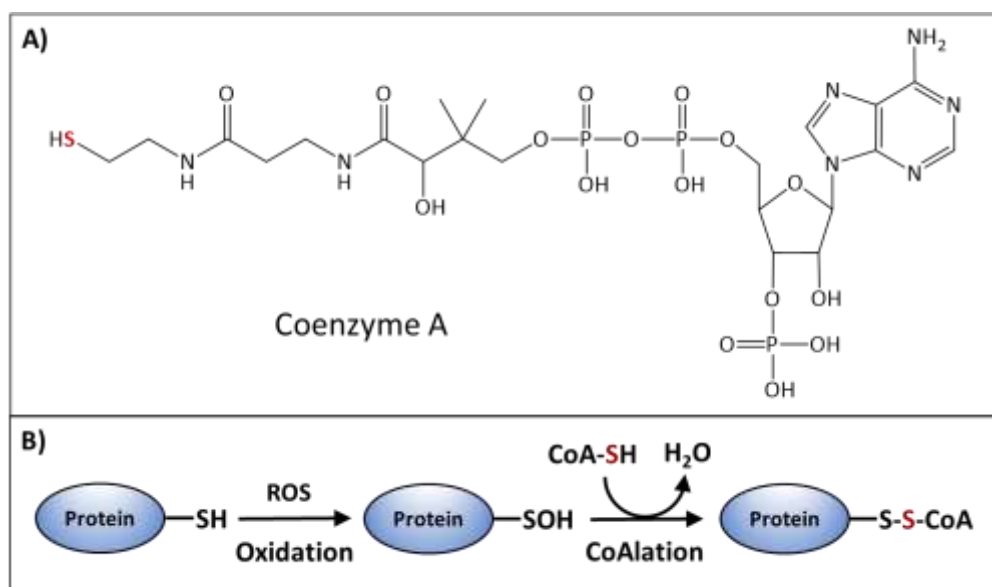


Figure 1: A) The structure of coenzyme A; B) the CoAlation reaction of cysteine-containing proteins. (Based on Baković et al., Molecular and Cellular Biochemistry, 2019)¹²

The aim of my work was to express the recombinant proteins (wild type (WT) and C47S mutant Prdx6), produce the CoAlated form of the proteins, investigate the sulfide- and disulfide-induced deCoAlation process of CoAlated Peroxiredoxin 6 (Prdx6-CoA) and determine the deCoAlation products.

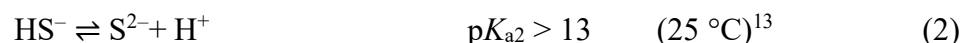
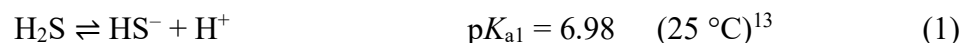
2. Literature overview

2.1. Biochemical roles of hydrogen sulfide

Nature uses an arsenal of biochemical reactions to sustain life, yet only relatively few elements are involved in these diverse reactions. Sulfur is one of these essential elements. It is ubiquitous in our environment (16th place in the Earth's crust by mass)¹³ and is also of great importance in our body (7th in humans by mass).¹³ A vital property of sulfur is that it can occur in various oxidation states (from -2 to +6), which gives it an outstanding role in redox processes. A few examples of organic sulfur derivatives are thiols (-2), disulfides (-1), sulfenic acids or sulfoxides (0), sulfinic acids (+2), and sulfonic acids (+4), where the numbers in the brackets show the respective oxidation states of the sulfur atom. Furthermore, two sulfur containing amino acids, cysteine (Cys, C) and methionine (Met, M), are found in our proteins and take part in their crucial functions, e.g. structural or functional disulfide bridges are formed through cysteine residues. These properties underline the importance of sulfur in the body. Hydrogen sulfide (H₂S) is the simplest representative of sulfur-containing biomolecules, whose biological role was obscured for a long time.¹³⁻¹⁵

2.1.1. Biochemical properties of hydrogen sulfide

Hydrogen sulfide (H₂S) is the sulfur derivative with the lowest oxidation state (-2). It is structurally similar to the water molecule, with the oxygen atom replaced by the chalcogen element right below, whose larger size results in some important physicochemical differences. Sulfur has a lower electronegativity than oxygen, resulting in a smaller dipole moment. Thus, there are no strong intermolecular forces/interactions or noncovalent interactions between H₂S molecules, and it is gaseous under standard conditions.^{13,15} It is soluble in water and may undergo two deprotonation steps:



Considering the pK_a values, hydrogen sulfide ions (HS⁻) dominate over H₂S (in an approximately 70:30 (n/n)% ratio) and sulfide ions (S²⁻) are present in a negligible amount under physiological conditions (pH = 7.4). Since the different forms (H₂S, HS⁻ and S²⁻) maintain dynamic equilibria with each other, I will use the word “sulfide” as an umbrella term for all three species in my work.¹³

2.1.2. The two sides of hydrogen sulfide: toxicity and bioactivity

The toxicity of sulfide was first described in 1713, which initiated a wave of publications on its harmful biological effects.¹⁵ Its pungent odor can be easily recognized well below the toxic limit (the LC₅₀ value being 1 μ M)¹⁶, but at higher concentrations, it causes respiratory failure due to the inhibition of cytochrome c oxidase (Complex IV) within the mitochondrial electron transport chain. H₂S poisoning can have an array of serious consequences, from irritation of conjunctive and respiratory tissue to pulmonary edema, anxiety, headache, syncope, and ultimately convulsions, paralysis of the nervous system, cardiac arrhythmia and death.^{13,15}

A study on sulfide poisoning examined the lifetime of exogenously introduced (inhaled or injected) sulfide in the brain. It was observed that the amount of sulfide in the brain increased in a dose-dependent manner. Surprisingly, sulfide was also measured in the control samples that did not receive any exogenous sulfide. This finding from 1989 was the first evidence of endogenous sulfide production.¹⁷ It took more than 250 years for us to learn about the “other side” of sulfide. This discovery led to several papers examining the biological effects of sulfide and showing that the body produces sulfide for multiple reasons.¹⁵

The dual biological aspects of sulfide can be recognized most intuitively within the electron transport chain (ETC): H₂S inhibits ETC function above the toxic threshold, however, it has a stimulatory effect on the ETC at physiological concentrations. Sulfide is also involved in neuromodulation, regulation of vascular tone, cytoprotection against oxidative stress, angiogenesis, inflammation and oxygen sensing. Sulfide exerts signaling functions in almost all cell types, therefore H₂S entered the field of redox biochemistry as the third gaseous signaling molecule after NO and CO.^{15,18–20}

2.1.3. Hydrogen sulfide metabolism

2.1.3.1. Endogenous sulfide production

In contrast to plants, bacteria and archaea, mammalian cells are unable to reduce inorganic sulfur species (such as sulfate or thiosulfate), their primary source of sulfide is the reduction of sulfur-containing amino acids (Cys and Met) via complex mechanisms. Sulfide production in the body is carried out by three enzymes: cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) with aspartate/cysteine aminotransferase (CAT/3MST) or D-amino acid oxidase (DAO/3MST) (Fig. 2).¹⁴

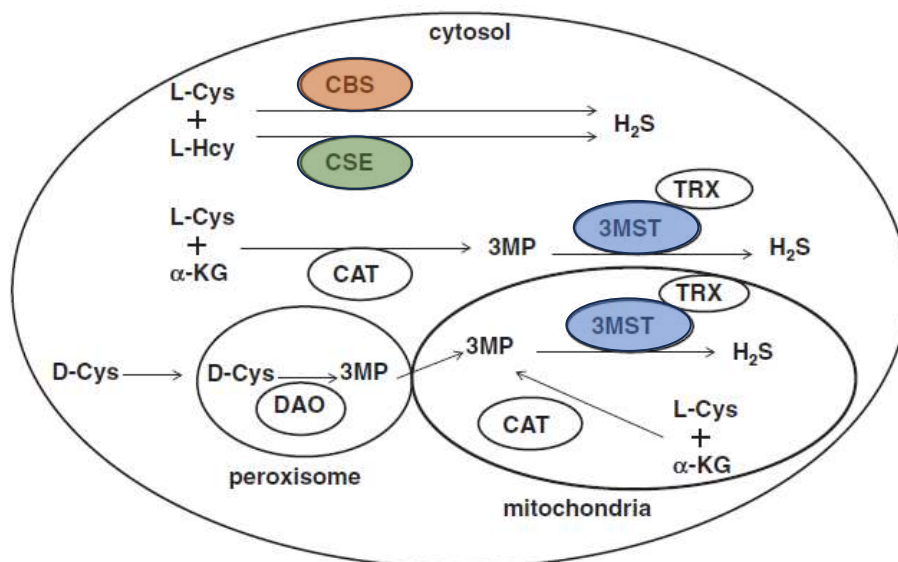


Figure 2: The main H₂S-producing enzymatic pathways in the human body: CBS (cystathionine- β -synthase), CSE (cystathionine- γ -lyase), CAT/3MST (aspartate/cysteine aminotransferase with 3-mercaptopyruvate sulfurtransferase) and DAO/3MST (D-amino acid oxidase with 3-mercaptopyruvate sulfurtransferase). The reducing agent thioredoxin (Trx). Substrates of the H₂S-producing enzymes: L-Cys (L-cysteine), L-Hcy (L-homocysteine), α -KG (alpha-ketoglutarate), D-Cys (D-cysteine) and 3MP (3-mercaptopyruvate), respectively. Reducing agent: Trx (thioredoxin) contributes to H₂S production by reducing the persulfide intermediate in the active site of 3MST. (Adapted from Kimura H.; Proc. Jpn. Acad., Ser. B; 2015)¹⁵

2.1.3.2. Catabolism of sulfide

Sulfide catabolism takes place in the so-called mitochondrial sulfide oxidation pathway (Fig. 3).¹⁴

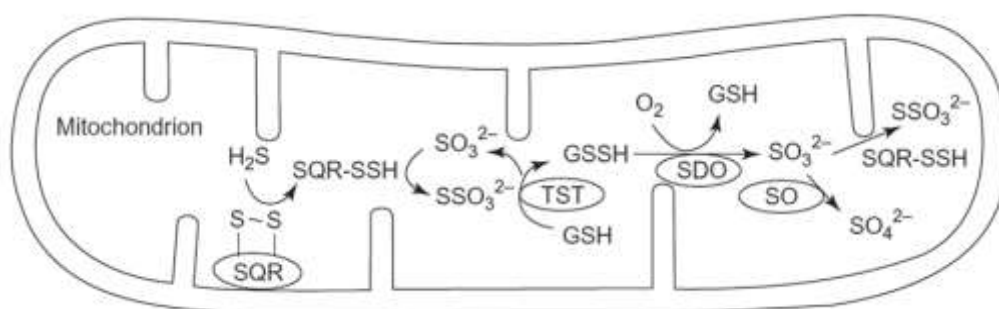


Figure 3: Mechanism of sulfide catabolism in the mitochondria. SQR: sulfide-quinone reductase, TST: thiosulfate sulfur transferase, SDO: sulfide dioxygenase, SO: sulfide oxidase, GSH: glutathione, GSSH: glutathione persulfide, SO_3^{2-} : sulfite, SO_4^{2-} : sulfate, SSO_3^{2-} : thiosulfate (Adapted from Nagy P., Methods in Enzymology, 2015)¹⁴

The initial step in this model involves the reduction of the disulfide bond in sulfide-quinone reductase (SQR) by sulfide, resulting in the formation of a persulfide derivative (SQR-SSH). This intermediate product subsequently reacts with sulfite (SO_3^{2-}) to produce thiosulfate (SSO_3^{2-}).

Thiosulfate sulfur transferase (TST) facilitates the conversion of glutathione (GSH) to glutathione persulfide (GSSH) using SSO_3^{2-} through transsulfuration. Subsequently, sulfide dioxygenase (SDO or ETHE1, persulfide dioxygenase) uses GSSH to produce sulfite.²¹ SO_3^{2-} can be further oxidized to sulfate (SO_4^{2-}) by sulfide oxidase (SO) or it can be recycled in a previously mentioned catalytic reaction, where it can react with another SQR-SSH. The cycle ends when sulfur is excreted from the human body in the form of sulfate or thiosulfate. Sulfide can serve as a stimulator of the electron transport chain. This occurs through the oxidation of sulfide to thiosulfate using SQR, which consequently reduces coenzyme Q (CoQ). CoQ transfers the electron to Complex III of the electron transport chain. So, besides its canonical source, CoQ receives additional electrons through sulfide oxidation with the help of SQR, which accelerates electron transfer and energy production.^{14,22}

2.1.4. Biological sulfide buffer systems

The concentration of endogenous sulfide is a subject of stringent control, which goes beyond the laws of acid-base equilibrium. To reproduce the sulfide-induced changes in cells, a significantly higher sulfide concentration was required compared to in vitro experiments, leading to the conclusions that 1) high local sulfide concentration is required to trigger the diverse biological effects and 2) sulfide stores are present in biological systems under strict regulation to ensure selectivity and keep these effects under control.^{13,15,23} It was concluded that sulfide can be released from these buffer systems (named sulfide pools) in case of shortage. Two main types of intracellular sulfide storage systems can be distinguished: acid-labile sulfur and bound sulfane sulfur (sulfur bound to another sulfur atom, with an oxidation state of 0) (Fig. 4).^{15,24} As the term suggests, acid-labile sulfur may be released under acidic conditions, and it mainly consists of iron-sulfur clusters. Bound sulfane sulfur (Fig. 4) entails inorganic and organic per- and polysulfides, thiosulfate, polythionates, thiosulfonates, elemental sulfur and protein-associated sulfane sulfur (e.g. protein-cysteine per- and or polysulfides). The liberation of bound sulfane sulfur requires reducing agents. Sulfide pools have been a matter of intensive research and still to date raise a number of questions. They may be involved in redox signaling processes by promoting persulfidation i.e. transferring sulfane sulfur to functional cysteine residues of proteins.²⁴⁻²⁶

Sulfide is reversibly bound to the biomolecules discussed above; thus, they serve as a reserve for bioactive sulfide and have a potential regulatory capacity. The binding and release of sulfide to and from the pools are governed by Le Chatelier's principle. Any changes in the local concentrations prompt the system to restore the equilibrium. This is primordial in keeping sulfide levels below the toxic threshold.^{15,23,24}

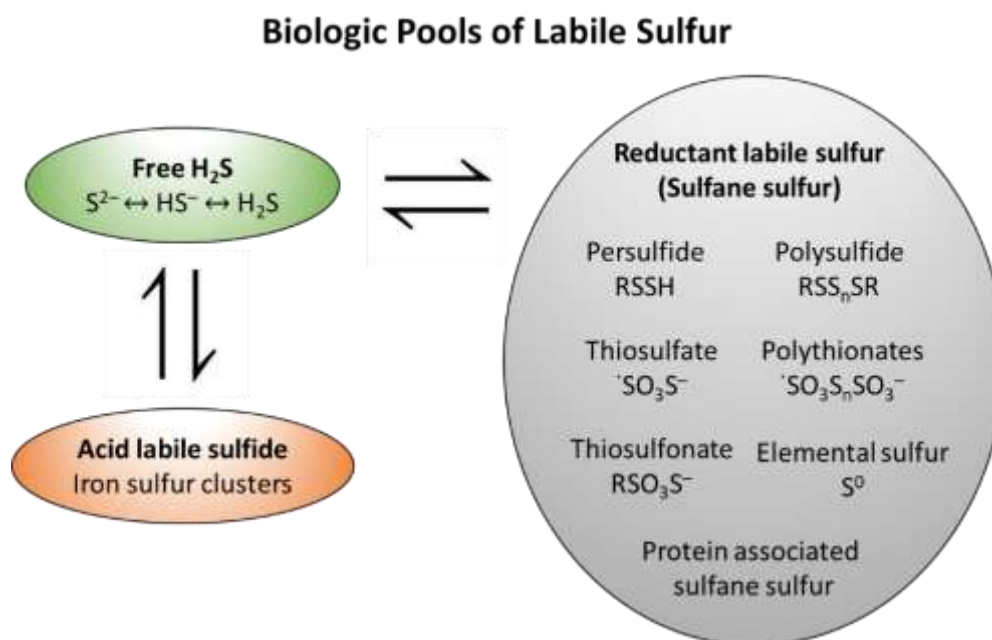


Figure 4: Sulfide buffer system: free H₂S, acid-labile sulfur and bound sulfane sulfur. (Based on Shen et al., Free Radical Biology and Medicine, 2012)²⁴

The detection of biological sulfide concentration is highly challenging. Most available methods are based on irreversible derivatization of sulfide, which removes free sulfide from the system and thus shifts the equilibrium with the pools.^{27,28} Sulfide is released from different sulfide-storing biomolecules at different rates, and the reactions are also significantly influenced by the experimental conditions (pH, incubation time, redox potential etc.). For example, acid-labile sulfur can easily be released under acidic conditions, which has no direct effect on the bound sulfide. Therefore, different methods based on sulfide labeling can be expected to give different results from the same samples.^{24,27,28} Recent studies about the steady-state concentrations of free sulfide describe values of 4-55 nM¹³ for most tissues.

In light of the dynamic regulation of free sulfide concentration, monitoring the temporal changes in sulfide concentration within living organisms emerged as a pivotal goal.

2.1.5. Biologically relevant reactions of H₂S

Within biological systems, the reactivity of H₂S can be categorized into three distinct groups of interactions (Fig. 5): (1) reacting with or scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS); (2) binding to metal centers of metalloproteins and potentially participating in subsequent redox reactions (although, the ROS scavenging function of H₂S has been disputed based on kinetic considerations)¹⁴; (3) interacting with proteins cysteine residues, leading to protein persulfidation also called as S-sulfhydration (persulfidation is the preferred term). Persulfidation is involved in signal transduction through alterations of protein functions.

This can happen via inhibition, activation, or emergence of a new function. Cys-persulfidation has grown into an accepted model of sulfide-regulated signaling processes and is the subject of intensive research in the field of redox biology.^{25,29–32}

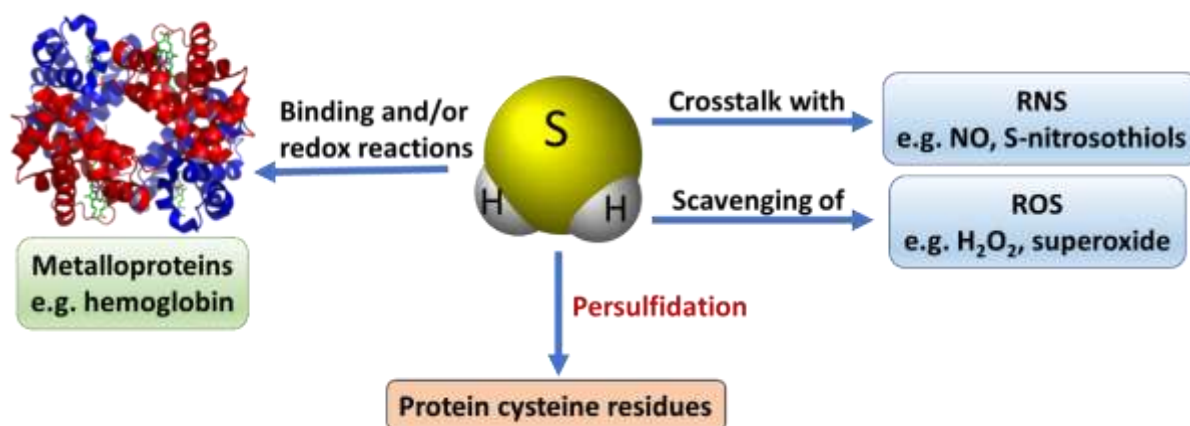


Figure 5: The main reaction types of sulfide in the body (Based on Filipovic, Handb Exp Pharmacol., 2015)²⁹

In addition to sulfide anabolism and catabolism, as well as the interactions with the described pools, these reactions also influence the physiological concentrations and signaling potential of sulfide.^{26,32,33}

2.2. Redox homeostasis

To maintain the functioning of living organisms, a large number of biochemical reactions take place every moment. Redox reactions are particularly important as oxidative and reductive reactions contribute to redox homeostasis, i.e. the balance between oxidative and reductive power within the cells, is a focal point in the field of redox biology. Redox biological research seeks answers to the main questions of how redox reactions regulate and maintain redox homeostasis, and what the consequences are if oxidative or reductive processes predominate causing oxidative or reductive stress, respectively. For this reason, the metabolism and reactions of physiologically relevant oxidizing and reducing agents are the subject of vast research.^{5,34,35}

Redox active biomolecules may be closed shell or radical type species. They may be involved in redox signaling events but their excessive amount can lead to serious biological damage. The longest-known and most studied representatives are the reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl radical ($\bullet\text{OH}$).

However, reactive nitrogen species (RNS) are also of great importance, and their research shows a rapidly growing trend. RNS include nitrogen monoxide ($\bullet\text{NO}$) and its derivatives such as nitrate (NO_3^-), nitrite (NO_2^-), and peroxynitrite (ONOO^-).

Recently, reactive sulfur species (RSS), including H_2S , have gained considerable attention in the literature. They play a major role in signal transmission and oxidative stress. ROS and RNS resemble to RSS in a sense that they are “two-sided” species because as long as normal metabolism keeps them at a low concentration, they act as signaling molecules, but if the antioxidant mechanisms fail to combat them, they can cause biological damage.^{6,26,36}

2.2.1. Reactive oxygen species (ROS)

Extensive literature is available on the formation and effects of ROS in biological systems, both in terms of the damage they cause and their involvement in cellular regulatory and signaling pathways. It is important to remember that ROS do not represent a uniform entity. While this term is suitable for describing a general class of compounds, it is counterproductive in understanding mechanisms. The term ROS generally includes the initial particle types i.e. superoxide or hydrogen peroxide which are formed by the reduction of oxygen, and their secondary reactive products such as hydroxyl radical. This complex picture and the fact that many reactions are reversible, often make it difficult to decipher which oxidant is involved in a particular process. For this reason, it is recommended to divide ROS into one-electron (radical type) and two-electron (closed shell) oxidants. Reactivity and oxidation state vary within both classes.^{26,37–39}

Superoxide ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are the main representatives of the ROS family. NADPH oxidases (NOXs) specifically produce superoxide from oxygen. Another important source of superoxide is the electron leakage of the electron transport chain, which results in superoxide generation. H_2O_2 is specifically produced within the cell by various enzymes. The major metabolic H_2O_2 sources include flavin-dependent oxidases, peroxisome acyl-coenzyme A oxidases (AOX), and superoxide dismutases (SODs). The latter group of enzymes catalyzes the disproportionation of superoxide anion radicals ($\text{O}_2^{\bullet-}$) and so NOXs indirectly participate in H_2O_2 generation. ROS can also arise from ionizing and UV radiation, as well as from the metabolism of drugs and xenobiotics. ROS are also generated in the endoplasmic reticulum during protein folding and disulfide bond formation.^{5,6}

This targeted production of H_2O_2 clearly shows that it also plays a role in important cellular functions, especially in the redox control of signaling processes. The signaling properties of H_2O_2 can be attributed to its relative stability, diffusivity, and selective reactivity.

However, whether H_2O_2 acts as a signaling molecule or as a harmful oxidant to biomolecules depends on the cellular environment, its local concentration and the kinetics of its production and elimination. For example, in the presence of free redox-active metal ions, non-toxic physiological levels of H_2O_2 may be converted into highly toxic hydroxyl radicals ($\bullet\text{OH}$) in Fenton-type reactions, which indiscriminately oxidize virtually every biomolecule they encounter.^{6,38,40}

2.2.2. Oxidative stress

Oxidative stress is a condition at the cellular level, which means a disruption in the balance of oxidizing and reducing processes in favour of oxidizing processes, e.g. as a consequence of ROS overproduction.⁴¹ This phenomenon is characteristic of aerobic organisms. Oxidative stress is a complex biochemical state, induced by the increased level of redox active and oxidizing species (and/or the lack of appropriate reductive power). It is a normal phenomenon in the body, and occurs as a result of global redox functions, but excessive or lasting imbalance can have serious consequences. The biological expense due to elevated oxidative stress is extremely diverse and includes neurological disorders, cancer, liver damage, cardiovascular and kidney diseases, inflammatory and degenerative conditions. This corroborates the importance of intense research targeting oxidative stress and defense mechanism.^{34–37,42,43}

2.2.3. Antioxidant defense system

Understanding the effects of antioxidants is crucial for elucidating the cellular effects of ROS. Antioxidants can be defined as any substance that delays, prevents or eliminates oxidative damage to the target molecule. The reaction is protective only if the product is less reactive or harmful than the parent ROS. This is particularly a problem with radicals, as the removal of one radical often creates another.^{6,42}

Four groups of antioxidants are distinguished in the literature: primary, secondary, tertiary and quaternary antioxidants. The enzymes superoxide dismutase, catalase, peroxiredoxins and glutathione peroxidase play an essential role in the cells' defense mechanism against oxidative stress and are counted as primary antioxidants. They prevent or reduce the intracellular production of oxidants. This group also includes chelator proteins which coordinate a free metal ion (e.g. Fe, Cu) and thus prevent the metal ion from catalyzing ROS production. Secondary antioxidants include ascorbic acid, vitamin E, uric acid and glutathione. The key to their mechanism of action is their reaction with ROS radicals, in which they themselves become ROS. For example, the dominant form of ascorbic acid (AcH_2) at

physiological pH is the monoanion form (ascorbate monoanion, AscH^-). AscH^- can donate an electron to the ROS radical, which becomes the ascorbate radical (semidehydroascorbate, $\text{Asc}^{\bullet-}$) during the reaction. The product is less harmful than the ROS radical and they are easily destroyed by the antioxidants of the same group. Tertiary antioxidants only come into play after ROS-induced damage took place. These are enzymes that repair the damage to biomolecules and cell membrane. They also recognize, degrade and remove oxidized or damaged proteins, DNA, and lipids to prevent their accumulation, which can be harmful to tissues. Common examples include DNA repair enzyme systems (polymerases, glycosylases, and nucleases) and proteolytic enzymes (proteases and peptidases) found in both the cytosol and mitochondria of mammalian cells. The mechanism of action of quaternary “antioxidants” is based on chemical signaling. The signal represented by the generated ROS initiates the downstream production and transport of the corresponding antioxidants.^{37,38}

With regards to sulfide, it reacts with the biologically most important ROS *in vitro*. Despite these favourable oxidation reactions, most of the antioxidant effects of sulfide observed in biological systems cannot be attributed to them. The vast majority of processes are controlled kinetically and not thermodynamically, i.e. the relative rate of the different reactions has the strongest effect on the outcome of the system. Sulfide is present in relatively low concentrations in the cellular environment, as discussed above, and ROS are consumed much more rapidly by other species, such as primary antioxidants described above (kinetic control prevails). Therefore, it is unlikely that sulfide protects against oxidative stress *in vivo* through a direct ROS scavenging capacity.^{14,44,45}

2.2.3.1. Protein persulfidation and the oxidative stress

The examination of individual reactive sulfur species presents a challenge, as they maintain dynamic equilibria with each other. Nonetheless, growing evidence suggests that compounds containing sulfane sulfur (marked in red in Fig. 6) largely contribute to the biochemical effects attributed to sulfide. Their diverse biological functions arise from their enhanced reactivity in comparison with thiols.¹³

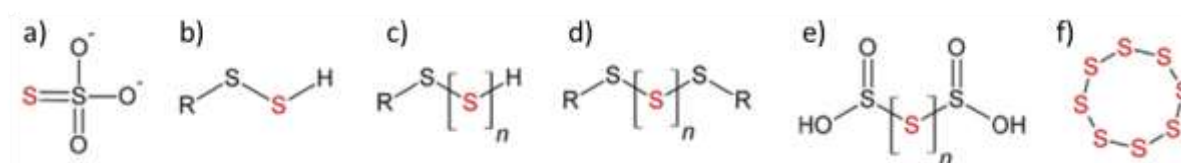


Figure 6: Shown in red font are the sulfane sulfurs in a) thiosulfate, b) persulfides, c) hydropolysulfides, d) polysulfides, e) polythionates, and f) elemental sulfur (Adapted from Alvarez et al., Arch Biochem Biophys, 2017)¹³

Persulfides are metastable compounds acting as reactive intermediates in the body and are responsible for numerous biological effects. Unlike thiols, sulfane sulfur can undergo both reduction and oxidation. As a result, persulfides may act as reducing and oxidizing agents as well. Moreover, persulfides are generally more reactive than thiols. They exhibit superior reducing capabilities and possess enhanced nucleophilicity owing to the α -effect, which indicates heightened reactivity of a nucleophile, containing a heteroatom with a non-bonding electron pair adjacent to the nucleophilic center at α -position. However, they can also act as electrophiles. An important reaction type of persulfides is transpersulfidation, during which sulfane sulfur can be transferred to another thiol. This may initiate a cascade of biochemical processes, thus contributing to signaling events.^{29–32}

Due to the diverse biological properties of persulfides, protein persulfidation (Protein-Cys-SSH) is an intriguing and highly studied oxidative post-translational modification (oxPTM). OxPTMs refer to the covalent modification of cysteine side chains by oxidation, after protein biosynthesis. Such oxPTMs include S-nitrosation (Protein-Cys-SNO), S-sulfenylation (Protein-Cys-SOH), S-acylation (Protein-Cys-Acyl) and S-glutathionylation (Protein-Cys-SSG), etc. Persulfidation may strongly affect the reactivity of several cysteine-containing enzymes; e.g. it can activate or inhibit enzyme activity (e.g. PTP1B, NFkB).^{14,29,45–47}

Recent findings revealed that persulfidation is a key player in the defense against oxidative stress (see Chapter 2.2.). During oxidative stress, the cysteines of thiol proteins (Protein-Cys-SH) can be oxidized to sulfenic acid (Protein-Cys-SOH) or overoxidized to sulfinic acid (Protein-Cys-SO₂H) and sulfonic acid (Protein-Cys-SO₃H). Overoxidation is practically irreversible because the regeneration of the protein into the active thiol form can only occur through complex enzymatic reactions, requiring a lot of time and energy. Thus, overoxidation of functional cysteine residues is biologically harmful. On the other hand, overoxidized protein persulfides i.e. perthiosulfinic acid (Protein-Cys-SSO₂H) and perthiosulfonic acid (Protein-Cys-SSO₃H) forms can be reduced at the disulfide bond, thus the regeneration of the enzyme takes place providing an opportunity to restore the enzymatic function. The overoxidized persulfide derivatives can be considered as reversible modifications and hence persulfidation may protect protein functions in case of oxidative stress.^{4,13,15}

Previously, some reports implied that protein thiols could directly react with sulfide to form persulfide. This is chemically inaccurate because both thiol and sulfide are fully reduced sulfur derivatives in the -2 oxidation state, i.e. no redox reaction can take place between them. One of the species must be in an oxidized state to allow protein persulfide formation. The most feasible protein persulfide-producing redox pathways of general Cys-containing proteins are

summarized in Figure 7. If the reduced sulfide is one reactant, the thiol must be oxidized previously. The oxidized thiol form can be sulfenic acid (Fig. 7A) or disulfide (Fig. 7B). If the protein thiol is in the reduced form, the sulfide should be oxidized previously. In this case, inorganic hydrogen disulfide could be a proper persulfidating agent (Fig. 7C). In addition to these reactions, persulfide can also be formed through radical and enzymatic mechanisms, and on the translation level as well.^{48,49}

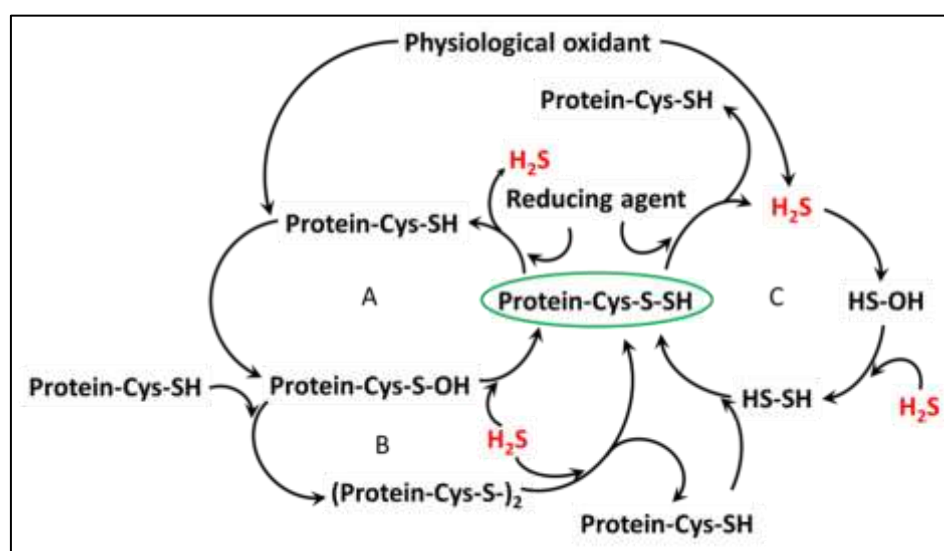


Figure 7: The main possible protein persulfide-forming redox reactions (Based on Nagy. et al, *Biochimica et Biophysica Acta*, 2014)^{28,48}

The detection of intracellular persulfide remains challenging. There are several methods for detecting sulfane sulfur species (e.g. cold cyanolysis⁵⁰ and fluorescent probes⁵¹) and/or persulfides (e.g. biotin-thiol assay⁵², qPerS-SID, dimedone-modified qPerS-SID⁵³, ProPerDP⁵⁴, tag-switch method⁵⁵, improved tag-switch method⁵⁶, dimedone-switch method⁵⁷, modified biotin-switch method² and LC-MS based proteomic methods^{58,59}) which are all under further development.¹³

2.2.3.2. Protein glutathionylation and CoAlation and the oxidative stress

A significant and widely studied PTM of cysteine-containing proteins is S-glutathionylation. Glutathione (GSH) is a tripeptide that can bind to protein cysteine residues via a mixed disulfide bond. It plays an important role in protection against oxidative stress, as the glutathionylated protein cannot be overoxidized and therefore does not permanently lose its activity and function. The disulfide bond can be broken enzymatically (Trx or GSH systems) or by small reducing molecules (H_2S , H_2S_2) in the cell, recovering the active enzyme. In the latter case (i.e. reduction by sulfide or disulfide), even protein persulfidation may occur during the reaction and protein persulfides can participate in signaling processes. Glutathione also

plays an important role in cell signaling and enzymatic production and reduction cycles in the cell. S-glutathionylation induces important biochemical processes by altering the activity of proteins.^{10,11,60,61}

In 2017, one of our collaborating groups experimentally demonstrated another post-translational protein modification similar to S-glutathionylation.¹⁰ This process is called protein CoAlation, i.e. the formation of a mixed disulfide bond between a coenzyme A molecule and a protein cysteine residue.

CoA is an essential cofactor found in all cells and synthesized from cysteine, pantothenate (vitamin B5) and ATP in a conserved pathway. It has a significant impact in organisms mainly as an acyl group carrier and a carbon activating molecule, resulting in several important thioester derivatives such as acetyl-CoA, malonyl-CoA, succinyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and a dynamic equilibrium exists between these species. The CoA pool (CoA and all its derivatives) occurs in very different amounts in diverse cell types, thus reflecting its various biochemical functions (Fig. 8).¹⁰

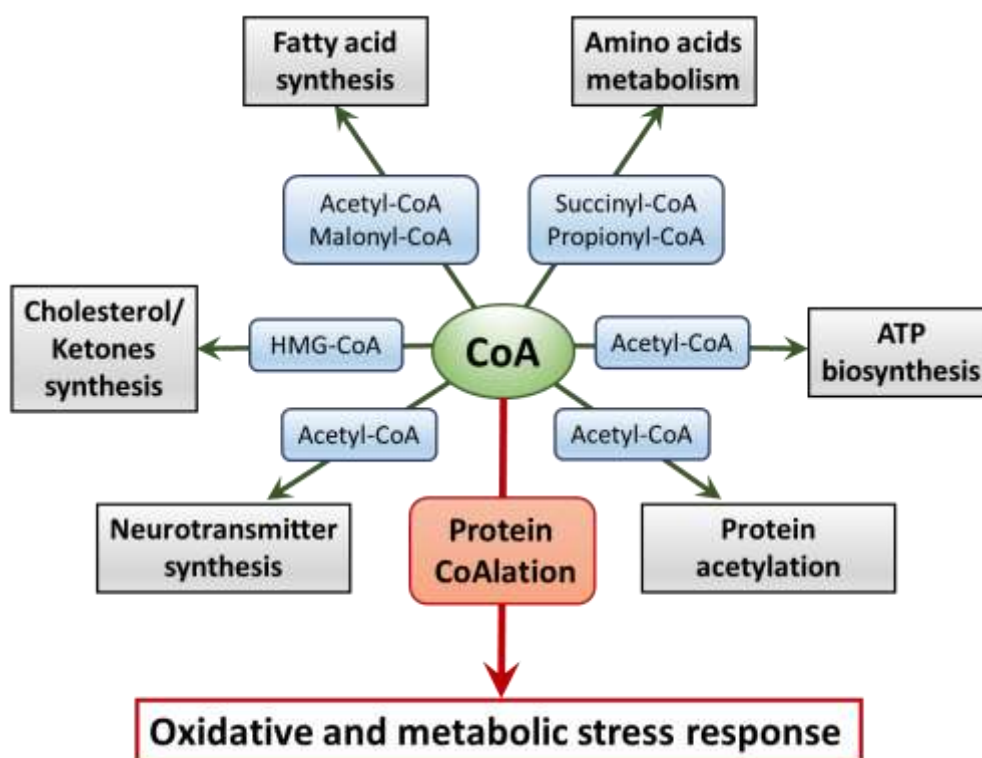


Figure 8: The main cellular functions of CoA (Based on Tsuchiya Y. et al., Biochemical Journal, 2017)¹⁰

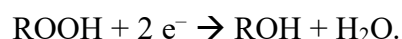
The CoA pool is indispensable in important cellular processes, such as the synthesis and oxidation of fatty acids, it is involved in Krebs cycle, ketogenesis, biosynthesis of cholesterol and acetylcholine, regulation of gene expression, cellular metabolism through protein

acetylation and more. Extracellular stimuli, stress, nutrients and intracellular metabolites play a pivotal role in regulating the CoA pool. CoA biosynthesis is reduced by insulin, glucose, pyruvate and fatty acids, while cell starvation increases CoA biosynthesis. In serious diseases such as diabetes and cancer, changes in the quantitative distribution between the different species of the CoA pool have been demonstrated, making research in this area particularly important.^{11,60,62,63}

CoAlation of cysteine-containing proteins is a reversible PTM. Our collaborators showed that in mammalian cells treated with various oxidants (H₂O₂, diamide, phenylarsine oxide (PAO), TBH, menadione), proteins were CoAlated in a dose-dependent manner. After the oxidant was removed, deCoAlation began, i.e. the disulfide bond between CoA and cysteine-containing proteins was broken. It is unclear whether this process is controlled enzymatically or by small-molecule reducing agents. Reduction by sulfide and/or disulfide may be an interesting possibility as it can lead to a persulfidated end-products that may be involved in signaling processes. In cells pre-treated with antioxidants (N-acetyl-L-cysteine (NAC), vitamin C), deCoAlation was significantly reduced. From these results, it was concluded that oxidative stress induces protein CoAlation. So similarly to GSH, CoAlation may have an important function in protection against oxidative stress. The main targets of CoAlation were proteins involved in metabolic pathways. A significant pattern difference between glutathionylated and CoAlated proteins was detected in H₂O₂-treated rat hearts, suggesting that GSH and CoA target different specific cysteines. CoAlation seems to be a reversible oxidative stress-induced process that can alter the activity and function of the target proteins and is also specific to regulatory cysteine side chains. It is currently not clear which other processes CoAlation may affect, and by which mechanisms deCoAlation occurs in the cell.^{10–12,60,62,63}

2.3. Peroxiredoxin family

Peroxiredoxins (Prdx) are cysteine-containing antioxidant proteins that are found in large quantities in almost all organisms. The main characteristic of these enzymes is their peroxidase activity, i.e. they break down organic and inorganic peroxides according to the following reaction:



The rate constant of this reaction is particularly high in case of Prdxs, which favors peroxiredoxins as H₂O₂ scavenging proteins. In human cells, there are 6 isoforms (Prdx1-Prdx6, Fig. 9), whose peroxidase activity is due to the redox active cysteine conserved in their

sequence, the so-called peroxidative cysteine (C_p). It was shown that the microenvironment and amino acids surrounding the C_p render it extremely susceptible towards H_2O_2 induced oxidation.⁶⁴ In addition to this cysteine, others may be present in the sequence, but these are not redox sensitive under physiological conditions. In addition to the peroxidative cysteine, most Prdxs have another important cysteine which participates in the catalytic cycle, the so-called resolving cysteine (C_r). Depending on the number of cysteines per monomer involved in the catalytic cycle, Prdxs can be divided into two large groups: 2-Cys Prdxs contain both peroxidative and resolving cysteines and 1-Cys Prdxs contain only peroxidative cysteine. 2-Cys-Prdxs can be further divided into typical and atypical 2-Cys Prdxs. Typical Prdxs form a homodimer in the cell, while atypical Prdxs are monomers. The only member of the 1-Cys Prdx family, Prdx6, also forms a homodimer in a cellular environment (Fig. 9). Homodimers are stabilized in cell by intermolecular forces/interactions or noncovalent interactions. Properties of human peroxiredoxins are summarized in Table 1.^{65–70}

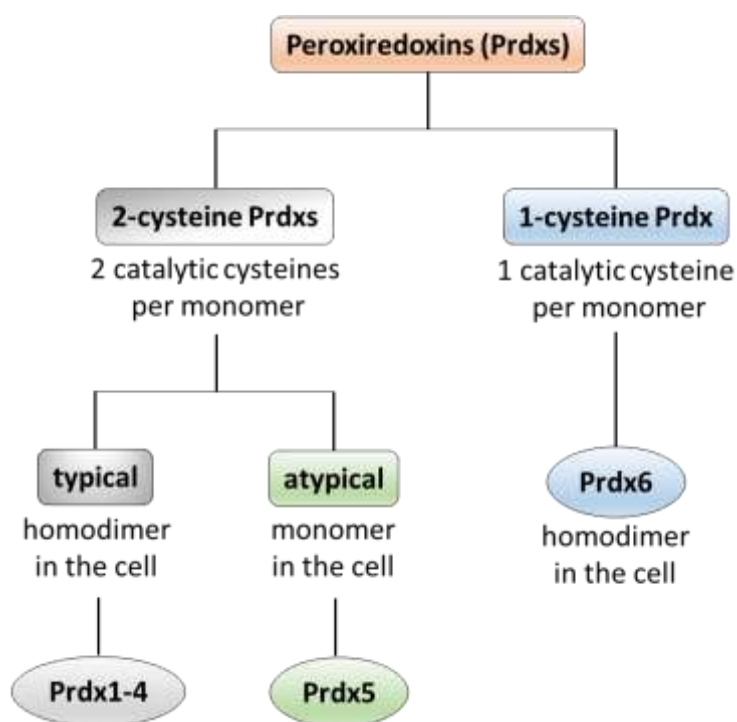


Figure 9: Classification of human peroxiredoxin isoforms (Based on Sue G. R. et al., Free Radical Biology and Medicine, 2005)⁶⁵

Peroxiredoxins also have a tendency to form higher order oligomers, in connection with their redox state. For example, typical 2-Cys Prdxs are stabilized in a reduced form as a decamer in a cellular environment. In addition to the redox state of the enzyme, oligomerization is also influenced by pH, ionic strength and Mg^{2+}/Ca^{2+} concentration.^{66,67,71,72}

Table 1: Properties of human peroxiredoxin isoforms⁶⁹

human Prdx	Prdx1	Prdx2	Prdx3	Prdx4	Prdx5	Prdx6
subtype	2-Cys	2-Cys	2-Cys	2-Cys	atypical 2-Cys	1-Cys
chain length (aa)	199	198	256	271	214	224
peroxidative Cys	Cys52	Cys51	Cys108	Cys124	Cys48	Cys47
resolving Cys	Cys173	Cys172	Cys229	Cys245	Cys152	—
noncatalytic Cys	Cys71	Cys70	Cys35, Cys45, Cys60, Cys127,	Cys52, Cys148	Cys73	Cys91
cellular location	cytosol, nucleus	cytosol, membrane	mitochondria	cytosol, Golgi	mitochondria, peroxisome, cytosol	cytosol
$k_{H_2O_2} / 10^7 M^{-1}s^{-1}$	3.8	1.3 - 10	2	2.2	0.035	3.4

* $k_{H_2O_2}$ is the second order rate constant between the given Prdx and H_2O_2 .

2.3.1. The role of peroxiredoxins

Prdxs perform multiple functions in the cell. Due to their direct reaction with ROS (reaction 1 in Fig. 10, blue box), they were previously characterized as antioxidant enzymes, but this is only one of their important properties in addition to their function as redox signaling molecules.

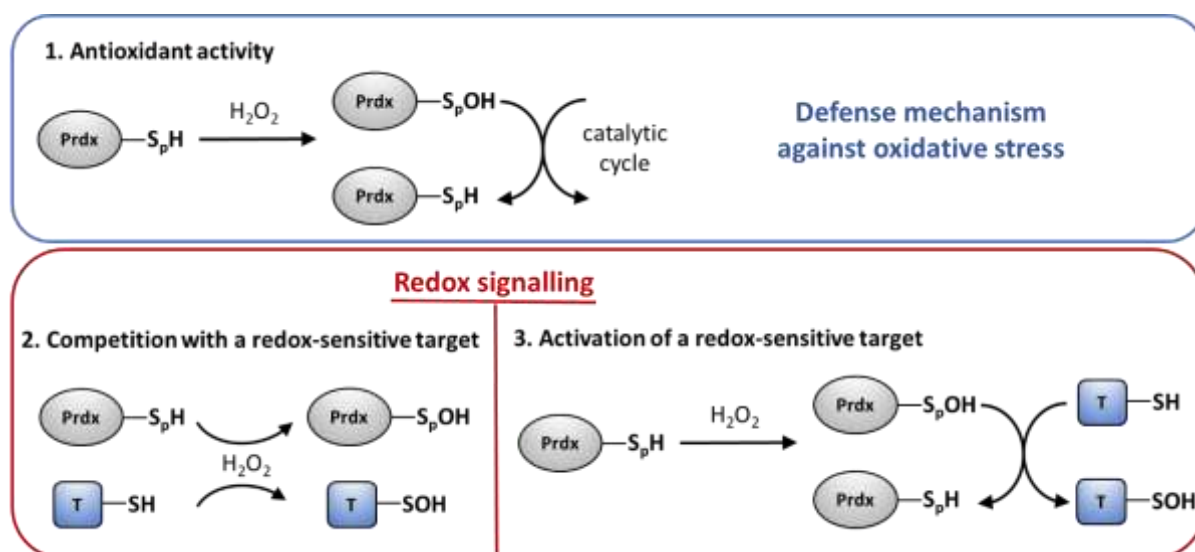


Figure 10: The roles of human peroxiredoxins Based on Winterbourn C. et al., *Molecules and Cells*, 2016)⁶⁸

Through competition, it can regulate the oxidation state of other redox-active cysteines (reaction 2 in Fig. 10, red box, left side). Reaction 3 (Fig. 10, red box, right side) is intended to demonstrate the role of the Prdx redox relay, where the oxidized Prdx is converted to a reduced form while the reduced partner is oxidized in the reaction, thereby causing redox signaling to occur. Reactants that do not react directly with peroxide can also be oxidized, but Prdx can transfer the oxidation equivalent to them.^{73,74}

2.3.1.1. Possible roles of sulfide in protection against overoxidation of peroxiredoxins

Through the catalytic cycle, Prdxs are reduced from their oxidized state back to their active thiol form. It is not entirely clear which oligomer Prdxs are stabilized under different *in vitro* conditions. Therefore, when depicting the catalytic cycles (Fig. 11), I used the monomer forms based on purely chemical considerations to illustrate the redox states. This does not influence on the chemical reactions, since in a cellular environment the reduced dimers are stabilized by a second-order interaction and in this representation, the redox state of the peroxidative cysteine is more emphasized.^{14,45,46}

The catalytic reduction of Prdxs belonging to different subtypes is different, but their responsibility is somewhat similar: to reduce the oxidized, inactive enzyme to its active form after oxidation by peroxide. Typical 2-Cys Prdxs are oxidized to sulfenic acid (Protein-Cys-SOH), which forms a homodimer with another Prdx molecule via an intramolecular disulfide bridge. It is important that in this case, the peroxidic cysteine forms a bond with the resolving cysteine. In the cell, the oxidized dimer is reduced back to its active form by the enzyme system Trx/TrxR/NADPH (thioredoxin/thioredoxin reductase/nicotinamide adenine dinucleotide phosphate) (Fig. 11A). In the case of atypical 2-Cys Prdx, the process is very similar, with the difference that Prdx does not form a homodimer with another Prdx, but is linked to Prdx5's own peroxidative and resolving cysteine via an intramolecular disulfide bridge (Fig. 11B). The Trx/TrxR/NADPH enzyme system is based on the principle that active Trx reduces the disulfide bridge of oxidized Prdx, while itself is oxidized via the formation of an intramolecular disulfide bridge within the thioredoxin domain and Prdx is reduced to the active thiol form. Since Prdx6 belongs to the 1-Cys Prdx subtype, it does not have a resolving cysteine and therefore cannot form an intra- or intermolecular disulfide bridge with a resolving cysteine after sulfenic acid formation. In the catalytic cycle of Prdx6, the formation of the disulfide bridge therefore occurs via heterodimerization with glutathione-sulfur transferase (GST π). This is followed by substitution with glutathione (GSH), and then another GSH reduces the S-S bond during the exit of a glutathione disulfide (GSSG), so that Prdx6 get its active, i.e. reduced form back (Fig. 11C).^{4,47,75}

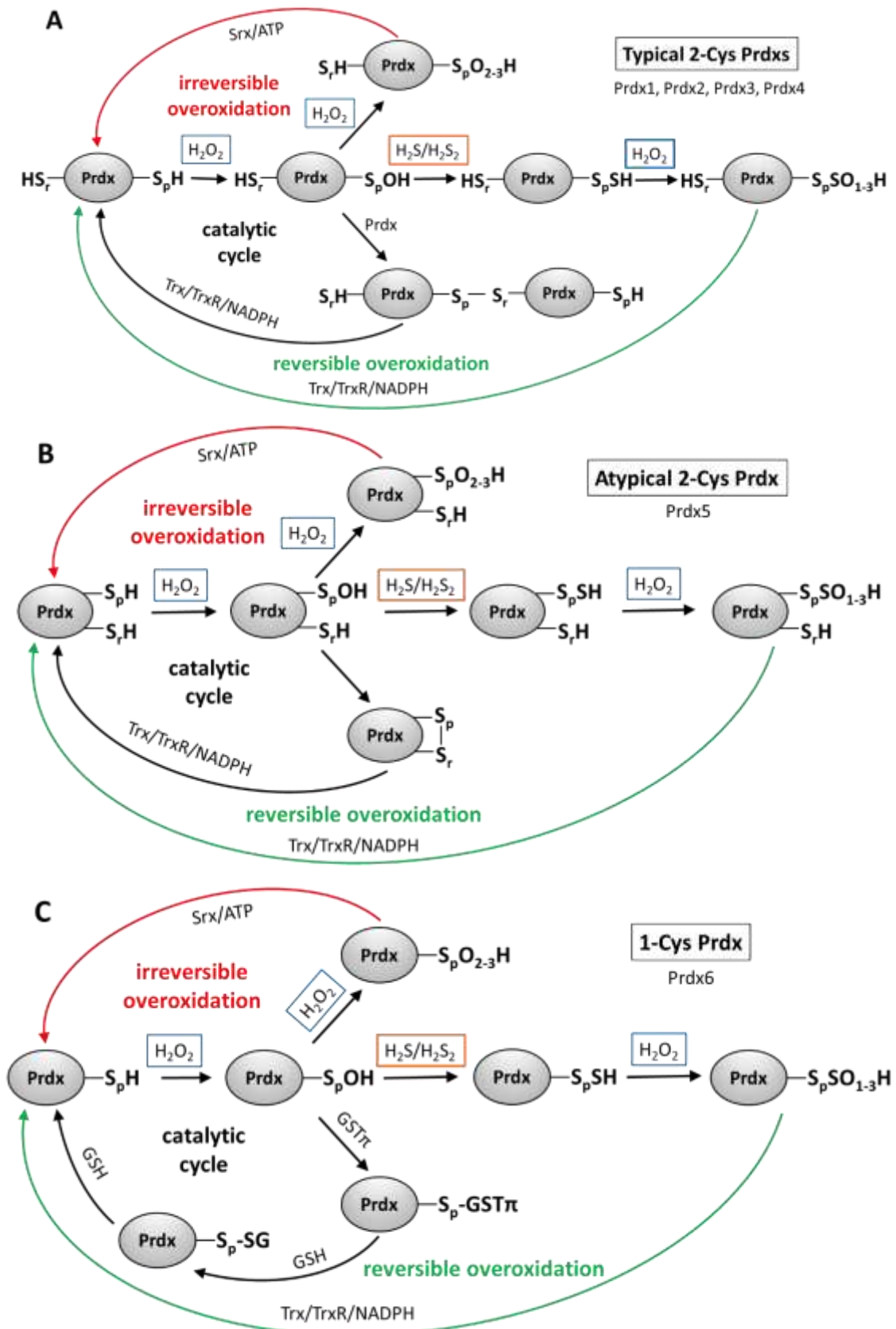


Figure 11: The catalytic cycle and the reversible and irreversible oxidation processes of the different Prdxs. A) Typical 2-Cys Prdxs (Prdx1-4). B) Atypical 2-Cys Prdx (Prdx5). C) 1-Cys Prdx (Prdx6). (Based on Nagy P., Antioxidants and Redox Signaling, 2013)⁴⁵

Figure 11 also depicts other important reactions of Prdxs, which, unlike the catalytic cycle, are the same for all isoforms: the irreversible and reversible oxidation of their peroxidative cysteine. The oxidation of peroxidative cysteine thiol (Cys-S_pH) to sulfenic acid (Cys-S_pOH) is a reversible process, the catalytic cycle of the enzyme reduces this form back to the active form. Further processes start from this sulfenic acid (Cys-S_pOH) form. If it is further oxidized to sulfinic acid (Cys-S_pO₂H) or sulfonic acid (Cys-S_pO₃H), the enzyme is temporarily or permanently stuck in these forms, because the catalytic cycle cannot reduce them back to the active form. The Cys-S_pO₂H oxidation products can be reduced by the sulfiredoxin/adenosine triphosphate (Srx/ATP) enzyme system by a slow, energy-demanding process, but the Cys-S_pO₃H form is destined to proteosomal degradation which is why the generation of sulfinic acid (Cys-S_pO₂H) and sulfonic acid (Cys-S_pO₃H) is called irreversible overoxidation. Another possible reaction starting from sulfenic acid (Cys-S_pOH) is the reaction with sulfide, during which persulfide (Cys-S_pSH) is formed. This form can be further oxidized to perthiosulfenic (Cys-S_pSOH), perthiosulfinic (Cys-S_pSO₂H) and perthiosulfonic acid (Cys-S_pSO₃H) forms. These oxidation products can be cleaved due to the presence of the disulfide bond, so the Trx/TrxR/NADPH enzyme system can reduce them back to their active form, so they are not stuck in an overoxidized state. Thus overoxidation becomes reversible due to persulfidation, i.e. the Prdxs can be easily reduced to their active form again, which is why they can react with new peroxide molecules. In the case of Prdxs, persulfidation may play a protective role against oxidative stress along this process. Preliminary data support the relevance of this mechanism for Prdx2.⁴

2.3.2. Peroxiredoxin 6

2.3.2.1. Function and activities

Prdx6 is a unique member of the Prdx family because it has only 1 conserved cysteine in its structure. It has multiple enzymatic activities: peroxidase activity like other Prdxs, phospholipid hydroperoxide GSH peroxidase (PHGPx), phospholipase A₂ (PLA₂) and lysophosphatidylcholine acyl transferase (LPCAT) activities. Prdx6 can reduce phospholipid hydroperoxides (PLOOH) to alcohols (PLOH) (PHGPx activity), hydrolyze glycerophospholipids at the sn2 position (PLA₂ activity), and lysophosphatidylcholine with a free fatty acid (CoA derivative) can be acylated by Prdx6 (LPCAT activity) (Table 2).⁷⁶

The combination of these enzymatic activities provides a complete cell membrane repair function to Prdx6. In the direct reaction with peroxide (peroxidase activity), Prdx6 competes with GPx enzymes, and other Prdxs and catalases. Furthermore, by transferring the oxidative equivalent, Prdxs may be able to oxidize proteins that other oxidants cannot.

The combination of these effects is still far from being fully mapped, and a deeper understanding of the involvement of Prdx6 in redox homeostasis and cell signaling is the subject of intensive research. Prdx6 stands out among the Prdxs due to its complex cell membrane repair property, which represents an another level of protection against oxidative stress (see tertiary antioxidants).^{76,77}

Table 2: Enzymatic activities of Prdx6⁷⁶

Activity	Abbreviation	Catalyzed reaction
peroxidase		ROOH → ROH
phospholipid hydroperoxidase (similar to glutathione peroxidase 4)	PHGPx	reduce phospholipid hydroperoxides (PLOOH) to alcohols (PLOH)
phospholipase A ₂	PLA ₂	hydrolyze glycerophospholipids at the sn2 position
lysophosphatidylcholine acyl transferase	LPCAT	acylate lysophosphatidylcholine with a free fatty acid (CoA derivative)

2.3.2.2. Structure

Human Prdx6 is a 25 kDa protein containing 224 amino acid residues. As discussed in chapter 2.3.1.1., its catalytic cycle does not involve the interaction of two monomers, despite this, the native (reduced) protein has a dimeric crystal structure. The monomers in the dimer are in an antiparallel position and are stabilized by hydrogen bonds and contain the so-called "thioredoxin fold" similarly to the other Prdxs. While the reduced form is mostly dimer in solution, the oxidized form is more likely to be stabilized in a monomeric state, but it also crystallizes as a dimer. However, this can be modified by changing the crystallization conditions. The formation of higher oligomers (e.g. decamers) has been investigated, but there is not enough experimental data to prove these structures and to confirm their physiological significance.^{76–78}

However, the peroxidase activity is of great physiological importance and is studied well on the Prdx family. Since Prdx6 is the last discovered human Prdx, many preliminary results could be obtained from research on 2-Cys Prdxs. In the case of 2-Cys Prdxs, in addition to the peroxidative cysteine, an amino acid triad consisting of proline, threonine and arginine (Pro, Thr, Arg) strongly supports the peroxidase activity. These were also detected in the structure of Prdx6 around the peroxidative cysteine (Cys47). Pro is thought to protect the thiolate from

undesired reactions and to position Thr and Arg for hydrogen bonding. Arg is said to position and activate the thiolate. Thr and Arg presumably jointly activate the oxygen atom of the peroxide substrate (the electrophilic center). The combination of these amino acids forms an exceptionally favourable active site for the breakdown reaction of peroxide. The structure of the human Prdx6 dimer is shown in Figure 12.⁷⁶

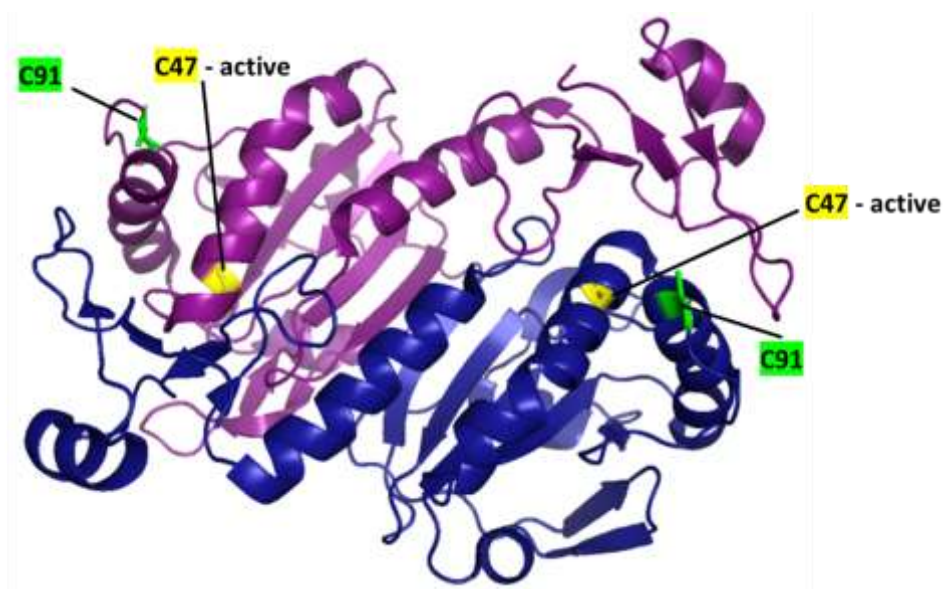


Figure 12: Homodimer formed by two antiparallel chains is shown, with the peroxidative cysteine 47 (Cys47) on each chain labelled in yellow and the non-active cysteine 91 (Cys91) labelled in green. (Prdx6 structure (5b6n) was downloaded from UniProt. For the visualization of the structural features of proteins, the PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC) was used.

3. Aim of the work

The goal of this project was to investigate the molecular mechanism and biochemical significance of the CoAlation of peroxiredoxin 6 (Prdx6) and to explore the potential role of hydrogen sulfide in the reverse process, namely the deCoAlation of Prdx6-CoA. The study was conducted in a collaborative manner, in close cooperation with the research group of Prof. Ivan Gout (University College London, Structural & Molecular Biology). Our laboratory contributed to the project by addressing the possibility of H₂S-mediated deCoAlation. There is an article in progress based on this project which will contain cellular and animal results as well, and to the best of our knowledge, it will be the first scientific article about the CoAlataion of Prdx6.

My personal tasks related to this work included the expression and purification of the wild type (WT) and C47S mutant human Prdx6 and the investigation of sulfide and disulfide induced deCoAlation process. We also had access to the C91S mutant Prdx6, which allowed us to investigate the CoAlation of individual cysteines of the protein. Our primary motivation in this study was to find out whether the deCoAlation step occurs through persulfidation of Prdx6.

4. Materials and methods

4.1. Expression of recombinant wild type and C47S mutant peroxiredoxin 6

The expression of the recombinant human WT and C47S Prdx6 proteins was carried out in BL21(DE3)pLysS *Escherichia coli* bacterial strain. The wild type (WT) form corresponds to the normal, natural structure of the protein, while the Cys47 residue is mutated to serine in the C47S mutant. For this purpose, appropriate plasmids were created that can be taken up by the bacterial cell in such a way that it incorporates into the bacterial genome. The WT plasmid was created by molecular cloning and it was subsequently used to create the C47S mutant by site-directed mutagenesis. The plasmids were provided by Prof. Gout in UCL. These DNA constructs consist of two parts: the DNA section coding the protein that we want to express (insert) and the part responsible for amplification (vector). An important part of the vector is the promoter region, which is responsible for regulating protein expression. Because the selective marker gene of the plasmid encodes the information on antibiotic resistance, an antibiotic is needed to remove cells that do not contain the DNA construct (in this case, ampicillin).

The production of Prdx6 follows the basic steps of traditional recombinant protein expression and purification. The main steps are transforming the corresponding plasmid, plating the bacteria on an antibiotic-containing plate, inoculating the solid colonies into a liquid culture, inducing protein expression and finally purification. The competent bacterial cells used for the expression (Thermo EC0114), normally deep frozen at -80°C were partially thawed on ice. 1 μL (50-100 ng) of the corresponding plasmids (WT PRDX6-pET21b(+)) and C47S PRDX6-pET21b(+) for WT and C47S Prdx6, respectively, was added to 50 μL of bacterial culture, then incubated on ice for 25 minutes. The appropriate plasmids were provided as a kind gift from Prof. Ivan Gout, University College London). This was followed by a heat shock at 42°C for 45 seconds and then an ice shock for 1 minute. The transformed cells were incubated in 200 μL of rich medium (Super Optimal Broth with Catabolite repression, SOC, Thermo Fisher) at 37°C for 1 hour with shaking at 250 rpm. The cells were centrifuged at 700 g for 5 minutes and the supernatant was discarded. After centrifugation, the culture was plated on agar plates (LB broth with agar (Miller)) containing 0.1 mg/ml ampicillin. The agar plates containing ampicillin were incubated overnight at 37°C , afterwards they can be stored at 4°C until further use. Then Terrific Broth (TB broth) was prepared out of 144 g of Terrific Broth, 2 mL of antifoam and 20 g of glycerin in 3600 mL of MilliQ or ultrapure water (ultrapure water that is ultra-low in organics,

hereinafter referred to as MQ water. The solution was sterilized in an autoclave and 400 ml 10x TB saline and 0.1 mg/ml ampicillin were added under a biosafety cabinet. A selected single bacterial colony was inoculated into 100 mL of the TB medium and incubated overnight at 37 °C with shaking at 220 rpm. Then 35 mL of the culture is transferred to 2000 mL of new TB broth medium without ampicillin and further shaking at 37 °C at 220 rpm.

After 3 hours, we checked the optical density at 600 nm (OD₆₀₀) using a TECAN Spark® Multimode Microplate Reader, which provided information about the amount of bacteria based on the turbidity of the culture. The expression of the target protein only begins when the promoter region is induced. Production should be initiated at an optimal bacterial population, which corresponds to OD₆₀₀ between 0.6 and 1.0. The bacterial culture was then induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma).

4.2. Purification of wild type and C47S mutant peroxiredoxin 6

The extraction of the produced WT and C47S Prdx6 from the bacteria started by centrifugation at 5500 g and 4 °C for 30 min . The pellets could be stored at –80°C until use. The bacterial pellets (separately for WT and C47S Prdx6) were lysed in 30 mL of lysis buffer (20 mM tris(hydroxymethyl)aminomethane (Tris, SIGMA), pH 7.50, 150 mM NaCl (AnalAR NORMAPUR), 1 mM DTT (dithiothreitol, reducing agent), 10 mM imidazole (for column equilibration), 1 mM PMSF (phenylmethylsulfonyl fluoride, a protease inhibitor, dissolved in ethanol), 1 mM benzamidine, 1 mM phenanthroline (dissolved in ethanol) and 1x PIC (protease inhibitor cocktail for preventing the digestion of the protein by the internal proteases of the bacteria). The lysates were then rotated at 4 °C for homogenization (approx. 1-2 hours). This can be facilitated with an additional step, using the Potter S Homogenizer vessel and tight-fitting plungers. Finally, the lysates were sonicated in a Fisherbrand™ Sound Enclosure for Models 505/705 Sonic Dismembrators device/machine with the following settings: amplitude: 40; 2 sec ON – 2 sec OFF cycles for 3x2 min for the WT protein and 4 min + 2 min for the C47S mutant. Then the cell remnants were separated from the lysate by centrifugation at 4 °C, 20 000 rpm, 40 min using fortified vials.

The produced Prdx proteins were separated from the bacterial lysate using the IMAC (Immobilized Metal Affinity Chromatography) method. A His-Tag, which consists of six histidines, is attached to the recombinant protein and it binds strongly to transition metal ions (usually Ni²⁺), attached to a solid phase (resin). In our case, the purification of the WT and C47S Prdx6 was carried out using the fast protein liquid chromatography method (FPLC, ÄKTA Pure-25 M instrument) on a 20 mL HisPrep FF 16/10 column. The protein flow was

monitored with an UV detector at 280 nm (A_{280}), making use of the intrinsic absorption of aromatic residues (Tyr and Trp). Prewashing (cleaning and conditioning of the column) was carried out with 2 column volumes of MQ water at a rate of 5 mL/min and then with 2 column volumes of binding buffer (50 mM Tris, 250 mM NaCl, 25 mM imidazole pH 7.50) at a rate of 5 mL/min. Then the lysate was applied followed by washing with binding buffer at a rate of 5 mL/min until the A_{280} signal became stable. Elution was performed with the same buffer, containing 400 mM imidazole, at a rate of 3 mL/min. Fractions were taken in 3 mL increments. The imidazole binds more strongly to the nickel on the column than the His-tag, displacing and thus eluting Prdx6. Purity of the eluted fractions was examined on a Coomassie-stained gel (see details below) and the fractions with sufficient purity were pooled together.

The imidazole remaining in the combined fractions was removed by dialysis. For this purpose, the pooled fractions were filled into a dialysis membrane sealed with dialysis tweezers and placed in 1 L of dialysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.50) at 4 °C, with a magnetic stirrer. The WT Prdx6 fractions were kept under these conditions for 2 hours, the C47S Prdx6 fractions for 3 hours, then the dialysis buffer was changed with the omission of DTT and the samples were dialyzed overnight at 4 °C. The next day the buffer was replaced with fresh dialysis buffer without DTT and the samples were aliquoted after 3 hours. Aliquots were snap-frozen in liquid nitrogen and stored at -80°C until use.

4.3. Determination of protein concentration

I measured the protein concentration using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer at 280 nm. The extinction coefficient was calculated by the ProtParam webserver and was $22,460 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ in every case.

4.4. Gel electrophoresis

The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel) method was used to separate proteins by molecular weight in a Bio-Rad MINI-PROTEAN Tetra Cell instrument. 15-well and 10-well 12% pre-cast gels (Bio-Rad) were used. An adequate amount of protein was loaded into each well with 1x sample buffer (diluted from 4x Laemmli Sample Buffer, (BioRad) in MQ water) and separated in 1X SDS-PAGE SDS Running Buffer buffer (25 mM Tris, 192 mM glycine and 0.1% (w/V) SDS, pH 8.3) at 100-140 V. All samples were subjected to heat denaturation at 95 °C for 5 min prior to the electrophoresis. Proteins can be separated under reducing or non-reducing conditions, which means the presence or absence of 5% (V/V) mercaptoethanol (Sigma) or DTT in the loaded samples. These reducing agents cleave disulfide

bridges thus modifying the electrophoretic mobility of the proteins. The choice of reducing or non-reducing gels depends on the nature of the experiment and the information we search for, and it is indicated on the figures showing gels.

The purpose of the gel electrophoresis was to check the purity of the produced proteins with subsequent Coomassie staining or identification of Prdx6 with the Western blot technique (see sections below).

4.4.1. Coomassie staining

Coomassie staining is non-selective gel staining method, all proteins are visible on the gel after staining, because of non-covalent binding of the dye to the proteins (mainly the aromatic residues). Therefore in our experiments, it was suitable for quality control of the purified protein fractions.

After running the gel, the fixation step took place in a fixing solution consisting of 40% (V/V) ethanol, 10% (V/V) glacial acetic acid and 50% (V/V) MQ water for 15 minutes. I then dyed the gel with Coomassie Brilliant Blue R 250 dye (Bio-Rad) for 30 minutes and then washed the background away with MQ water.

4.4.2. Western blot

WB is a highly specific protein analysis technique, which is based on immobilization of the protein on a polymer membrane and identification using selective antibodies. For immunoblotting purpose, the proteins were transferred from the gel to nitrocellulose membrane using the “2 Mini-Gels” or “1 Midi-Gel” program of the Bio-Rad Trans-Blot Turbo Transfer System semi-dry blotting system for 7 minutes. The membranes were blocked for 1 h at room temperature in TTBS buffer (TBS: 20 mM Tris, 150 mM NaCl + 0.1% (V/V) Tween20) containing 5% (w/V) nonfat dry milk powder and 0.5% (w/V) bovine serum albumin (BSA), to avoid non-specific binding of the antibodies. The membranes were then incubated with the primary antibody diluted with TTBS overnight at 4 °C. Finally, I washed with TTBS for 3x10 minutes, then incubated with the secondary antibody diluted in TTBS for 1 hour at room temperature and then washed for 3x10 minutes. For protein visualization, I used Clarity Max ECL Western Blot substrate (Bio-Rad) with an instrument called Syngene G:BOX. I used the antibodies in the following dilutions: anti-Prdx6 1:2000 (Antibodies, A85194), anti-CoA 1:6000 (provided by our collaborator, Prof. Ivan Gout), anti-goat HRP 1:2000 (Dako, P0449) and anti-mouse HRP 1:5000 (Dako, P0447). Antibody labeling is selective; only target proteins that are specifically labeled with the antibody become visible. I evaluated the membranes densitometrically using the GelAnalyzer software, which quantified the ratio of black and white pixels and assigned the signals an intensity value in an arbitrary unit.

4.5. CoAlation of peroxiredoxin 6

For the experiments, I used the following human Prdx6 proteins: WT, C47S mutant (expressed in our laboratory as described above) and C91S mutant (expressed in Prof. Gout's laboratory at UCL). For the 1X PBS buffer required for the experiment, I dissolved 1 PBS tablet (phosphate buffered saline tablet, Sigma) in 200 mL of MilliQ water and adjusted the pH to 7.40. I bubbled the buffer with N₂ for 10 minutes to remove the dissolved oxygen. Then I pre-reduced the Prdx6 proteins WT, C47S and C91S at the desired concentration (approximately ~150 μ M) to reach the active enzyme form prior to the reactions. The pre-reduction was carried out with a final concentration of 10 mM dithiothreitol (DTT, Sigma) on ice for 30 minutes. Excess DTT was removed by 7K MWCO Zeba Spin Desalting Columns (Thermo Scientific) by centrifugation at 1500 g for 2 minutes. I preconditioned the spin columns with 3x300 μ L of MQ water and then with 300 μ L of N₂-treated 1X PBS buffer at 1500 g for 1 minute. I diluted the required amount of CoA (Coenzyme A, from Prof. Gout) in 1X PBS. Nitrogen was bubbled through this solution. Prdx6 was incubated with CoA for 5 min at 25°C in a final concentration ratio of 1:3 in the case of C91S Prdx6 (C91S Prdx6:CoA) and 1:6 in the case of WT Prdx6 (WT Prdx6:CoA). I then added 10 equivalents of peroxide based on the concentration of Prdx6 to the reaction mixture and incubated the mixture for another 30 minutes at 25 °C. After the reaction, the excess CoA was removed using a spin column according to the above-described method. I measured the concentration of the product using NanoDrop and then stored it at -80°C until use.

4.6. Sulfide-mediated deCoAlation reaction and sample preparation for Western blot and bottom-up proteomic measurement

CoAlated Prdx6 (Prdx6-CoA) was diluted to a concentration of 1.55 mg/mL in 100 mM Na-phosphate buffer (80 mM Na₂HPO₄·12 H₂O, 20 mM NaH₂PO₄·2 H₂O, 100 μ M DTPA dissolved in MQ water, pH = 7.40). Then I prepared the sulfide reagent as follows: I prepared a 500 mM stock solution of sodium sulfide (Na₂S·9 H₂O, Sigma) dissolved in freshly degassed MQ water. H₂S concentration was checked first at 230 nm, then DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Sigma) was added to it and the absorbance was measured at 412 nm to verify the concentration.⁷⁹ Stock solution was and stored on ice and diluted in 100 mM Na-phosphate buffer to the desired concentration immediately before the reaction. When sodium disulfide (Na₂S₂, Dojindo) was used instead of sulfide, it was dissolved in solid form in freshly degassed MQ water, stored on ice, and then diluted in 100 mM Na-phosphate buffer to the desired concentration right before use.

To ensure that this amount of sulfide does not affect the physiological pH and thereby the kinetics, the pH of the buffer was measured before and after adding the sulfide reagent in the appropriate concentration (Table 3).

Table 3: The pH of the buffer before and after adding the sulfide reagent

condition	w/o sulfide	200 μ M sulfide
pH	7.42	7.43

The pH is not affected by sulfide under these conditions, so the reaction is investigated under physiological pH. For the reaction, I mixed the sulfide reagent with Prdx6-CoA at a volume ratio of 1:1 under the following reaction conditions: continuous mixing at 250 rpm, 25.0 °C.

For Western blot measurements, I took samples at each time point, alkylated them with 100 mM NEM (N-ethyl-maleimide, Sigma), and incubated them at 95°C for 5 minutes. The loaded protein amount was 0.7-1.4 μ g/well.

For bottom-up proteomic measurement, at each time point, I took 15 μ L samples from the reaction mixture and stored them on ice in the mixture of 75 μ L 5 mM HPE-IAM (β (4-hydroxyphenyl)ethyliodoacetamide, Merck) solution⁸⁰ and 10 μ L 100 mM Na-phosphate buffer. HPE-IAM solution: HPE-IAM dissolved in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1.0% (V/V) NP-40, 0.5% (w/V) sodium deoxycholate, 1.0 mM EDTA, 0.1% (w/V) SDS and 0.01% (w/V) sodium azide at pH 7.4). The 100 mM HPE-IAM stock solution was freshly prepared in DMSO (dimethyl sulfoxide, Sigma) and diluted 20x in RIPA buffer. I stored the samples on ice until the final sample was collected and after that I incubated all samples at 37 °C for 20 minutes. After that, I prepared the sample following the steps of the EasyPep Mini MS Sample Prep Kit (Thermo) skipping the reduction and alkylation steps. Using the enzyme reconstitution solution in the kit, I prepared a solution with a concentration of 0.2 mg/mL Trypsin/LysC (trypsin/LysC protease mixture), then added 10 μ g of protease mixture (50 μ L) to each sample and then incubated at 37 °C for 1.5 hours (digestion step). Digestion was quenched by adding 50 μ L of the digestion stop solution provided in the kit to each sample and then mixing thoroughly with a vortex mixer. Then I applied all the digests separately to the Peptide Clean-up columns conditioned according to the instructions of the kit, centrifuged at 1500 g for 2 min, and then washed the bound peptides with 300 μ L Wash Solution A and 2x300 μ L Wash Solution B. I performed the elution with 300 μ L Elution Solution at 1500 g for 2 min. The solvent was removed using a SpeedVac Vacuum Concentrator (Thermo) with the following parameters: heat: 40 °C, heat time: 30 min, run time: 1 h. The pellet was either redissolved and measured immediately or stored at -80 °C until measurement. Before the LC-MS/MS

measurement, I dissolved each pellet in 40 μL sample solvent (0.1% (*V/V*) formic acid and 5% (*V/V*) acetonitrile in MQ water) and placed them in an ultrasonic bath for 2 minutes. After complete dissolution, the samples were transferred into appropriate HPLC autosampler vials.

4.7. LC-MS/MS measurement of peptides

The measurement and data evaluation was done by Dr. Tamás Ditrói, a senior member of the research group. Peptides were separated on an Ultimate 3000 RSLCnano (Thermo Scientific) system with a Thermo EASY-Spray column (75 μm x 50cm, 2 μm) using an injection volume of 2 μL and a binary gradient of 0.1% (*V/V*)FA/H₂O (A) and 0.1% (*V/V*) FA/ACN (B). The 120 min long gradient started from 3% B and was increased linearly to 40% with a flow rate of 250 nL/min. Ions were introduced into a Thermo Exploris mass spectrometer (Thermo Scientific) that recorded the full MS spectrum between and the MS/MS spectrum using 35% HCD energy for 4 preset precursor masses. The target masses were 758.3611 (peptide+HPE), 774.3472 (peptide-persulfide+HPE), 543.3038 (control peptide 1) and 504.2821 (control peptide 2), all calculated with a charge of +2. Peak were identified and confirmed by assessing their MS/MS fragments, quantitative data was generated from extracted-ion chromatograms (XIC) with a 10 ppm *m/z* window set for the precursors.

5. Results

5.1. Characterization of the produced recombinant proteins

5.1.1. Purity

During the purification of WT and C47S Prdx6 using the FPLC method, the proteins were monitored using UV detection at 280 nm (absorption maximum of aromatic residues), which is a common characteristic of proteins. The chromatograms are shown in Figure 13.

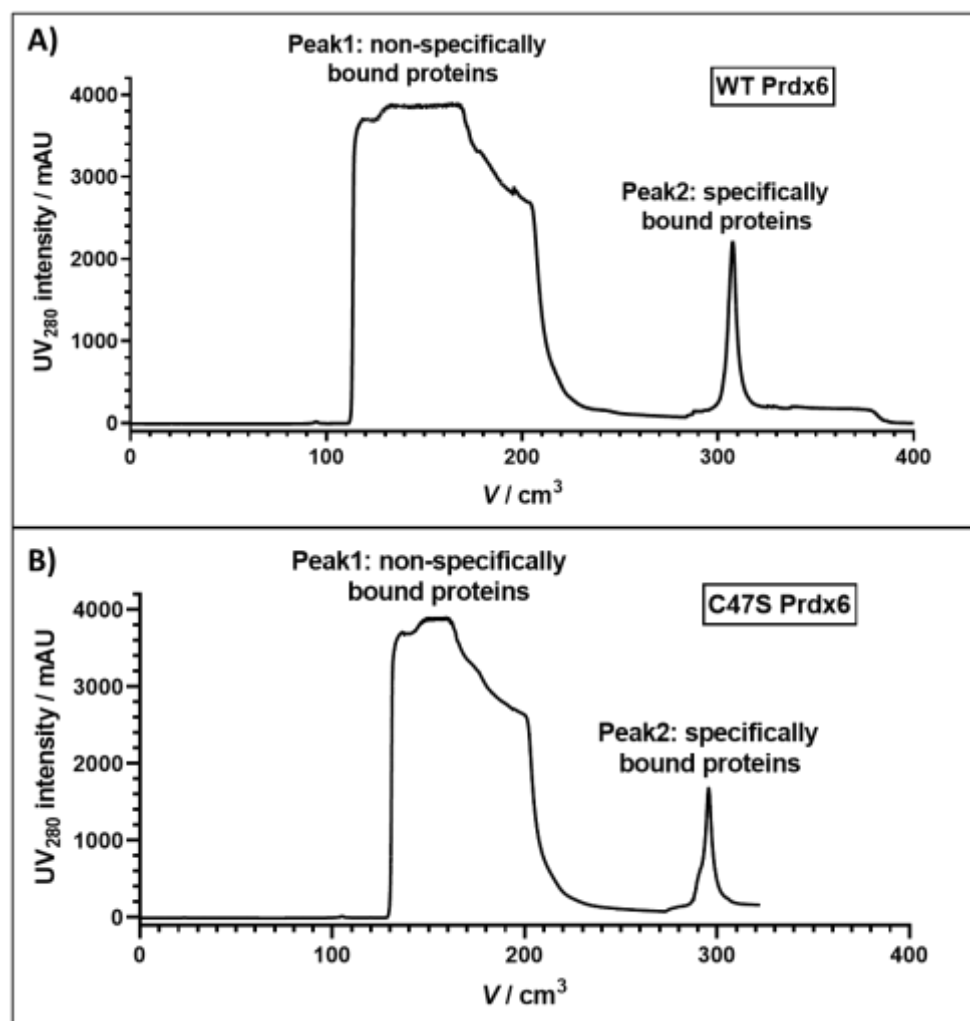


Figure 13: The chromatograms recorded during the purification of WT Prdx6 and C47S Prdx6, respectively. Purification of A) wild type (WT) B) C47S mutant human peroxiredoxin 6 with Fast Protein Liquid Chromatography (FPLC). Peak1: flow-through, e.g. non-bound and non-specifically bound proteins. Peak2: specifically bound proteins. WT and C47S Prdx6 are expected in the fractions corresponding to Peak2.

Peak1 in Fig. 13 denoted all proteins in the lysates that are non-bound or non-specifically bound to the nickel containing column. The His-tag on the recombinant WT and C47S proteins provides strong, specific binding, so our expected products leave the column at

higher elution volume, presumably in Peak2. This fraction might contain some other contaminating proteins which are bound to the His-tag (or the products) by intermolecular forces/interactions or noncovalent interactions.

The SDS-PAGE technique was used to monitor the protein purification, to identify the target proteins and to characterize their purity. Figure 14 shows the Coomassie-stained gels of the samples taken in the main steps of Prdx6 purification (as described in the Materials and Methods section (4.2.)).

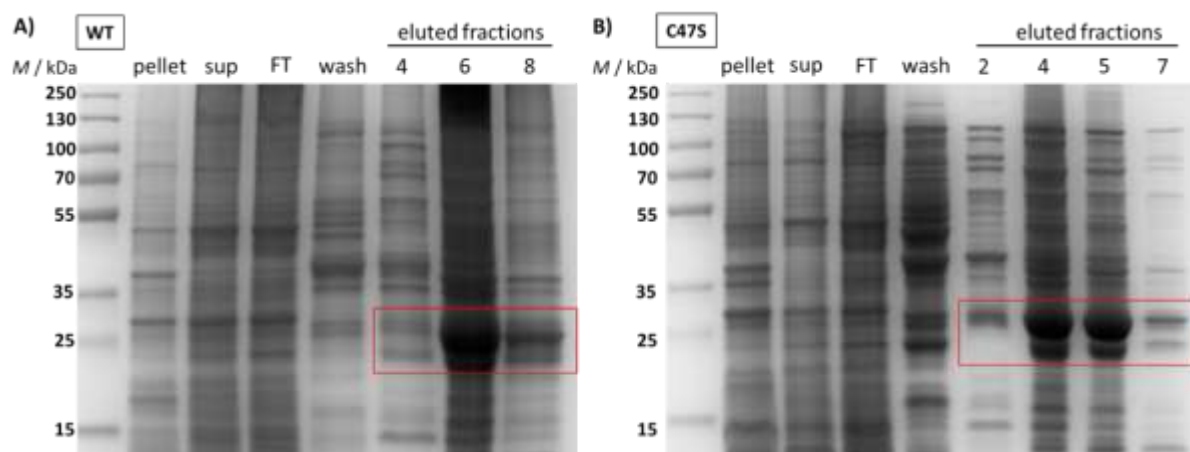


Figure 14: Monitoring of the purification steps of A) WT and B) C47S-Prdx6. The headers of each lanes (pellet, sup, FT, wash and the numbers) are described in the text. The red frames denote the expected Prdx6-containing bands at 25 kDa. Sample load: 12 μ l/well. *Pellet*, *sup* and *FT* were diluted 5-fold prior to loading, the *wash* and *eluted fractions* were loaded undiluted. Samples were run under non-reducing conditions.

After the lysis is completed, the remaining cell debris is separated from the bacterial culture by centrifugation. The *pellet* lane corresponds to the protein content of the debris and *sup* is the supernatant, i.e. all the soluble proteins produced by the bacteria. The cleared lysates are then loaded to the FPLC column, and a series of washing and elution steps are performed (as depicted in Fig. 13). *FT* is the flow-through, i.e. the portion of the lysate that was not bound to the column (corresponding to Peak1); *wash* involves non-specifically bound proteins that come down by washing (related to fractions between Peak1 and Peak2); and the *eluted fractions* are expected to contain the WT or C47S Prdx6 (Peak 2). Peak2 was sampled into 3 ml fractions, numbered from 1 to 10. An aliquot of the fractions with the indicated numbers were loaded on the gels. A protein ladder or marker with proteins of known molecular weight (*M*; leftmost lane) helps us to assign the mass to the observed bands. Vertical columns on the gels are traditionally called lanes or wells, while horizontal spots are called bands. Finally, the intensity of the bands is proportional to the amount of contained protein, which allows to qualitative comparison and semi-quantitative determination of protein content.

Prdx6 has a molecular weight of 25 kDa, so we expect a band with that size on the gel in the case of both the WT and the mutant isoform. The *pellet*, *FT* and *wash* samples do not show a strong band at 25 kDa for neither the WT, nor the C47S protein (Fig. 14), indicating that Prdx6 was not lost during these steps. These samples contain many other proteins arising from the lysate. The band of *sup* at 25 kDa is not particularly strong, which is probably the result of weak induction, so the expression of Prdx6 was low. The *eluted fractions* are expected to contain mainly the WT (Fig. 14A) or C47S (Fig. 14B) Prdx6 protein. The bands at 25 kDa are strong in the region of interest in both cases (denoted with the red frame), suggesting the presence of the target proteins as main component. In the periferal eluted fractions no. 4 and 8 (WT) and 2;7 (C47S) the band at 25 kDa is weaker, corresponding to the bell-shaped elution curve (Peak 2).

I pooled the elution fractions (WT: 4-8 and C47S: 2-7) and checked the purity of the products on a Coomassie-stained gel, under reducing and non-reducing conditions as well (Fig. 15).

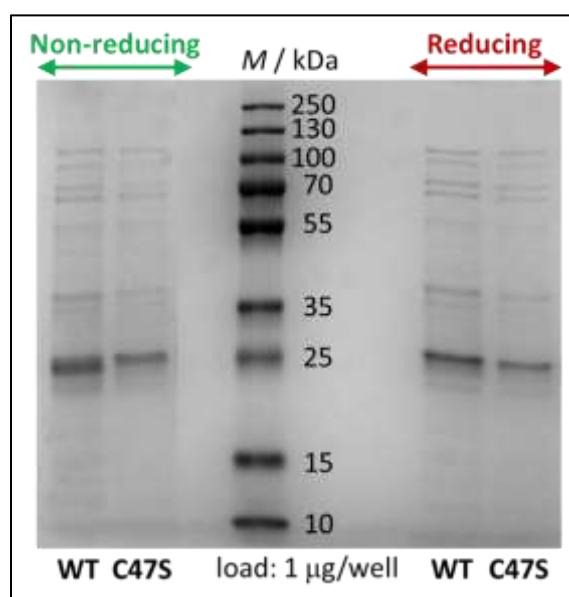


Figure 15: Purity of WT and C47S Prdx6 proteins. Reducing agent: 1.4 mM 2-mercaptoethanol.

The main component of the purified products is a protein with a mass of 25 kDa, presumably the target proteins: WT and C47S Prdx6. Reduction with 2-mercaptoethanol does not cause any changes in the migration pattern, i.e. suggesting that the proteins do not contain any disulfide bonds.

5.1.2. Determination and quantification of the expressed proteins as peroxiredoxin 6

In order to be certain about the nature of our target proteins, the WT and C47S Prdx6 proteins were identified by Western blot (WB) technique. In the immunoblotting experiment, I used an antibody that selectively binds to human Prdx6, so the signals visible on the membrane can only belong to Prdx6 (Fig. 16). Both WT and C47S protein expression were proved successful.

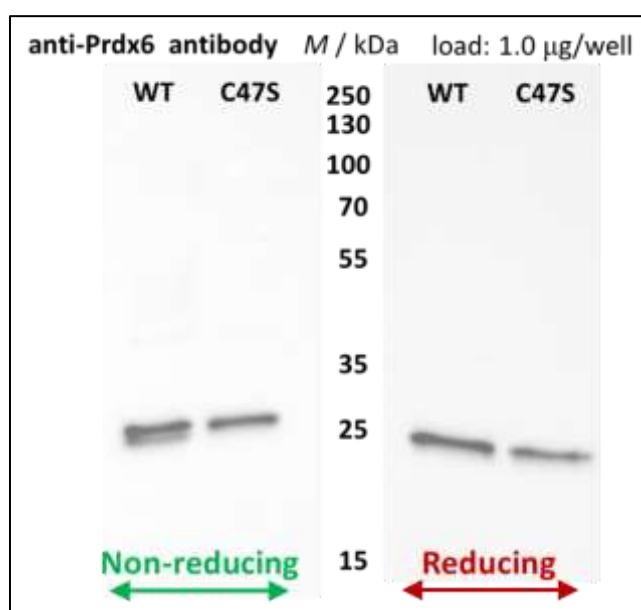


Figure 16: Identification of WT and C47S Prdx6 proteins by WB technique. Reducing agent: 1.4 mM 2-mercaptoethanol.

In the case of WT Prdx6, I obtained 15 mL of protein solution with a concentration of 5.35 mg/mL (205 µM), based on NanoDrop measurement, while in the case of C47S Prdx6, the final solution had a volume of 20 mL with a concentration of 3.44 mg/mL (132 µM).

5.2. CoAlation of peroxiredoxin 6

Human Prdx6 contains 2 cysteines: Cys47 (peroxidative, physiologically active) and Cys91 (physiologically inactive). Both cysteines are present in WT Prdx6, but in the C47S mutant Cys47 is mutated to serine, while in the C91S mutant Cys91 is mutated to serine, creating single-Cys variants, which allow individual investigations for each cysteine. For the CoAlation of the WT Prdx6 and the C91S and C47S Prdx6 mutants, the proteins were first reduced with DTT. Interestingly, two bands appeared on the gel of the C91S Prdx6 mutant before reduction (Fig. 17A, non-reducing). The signal visible at 55 kDa is probably the result of dimerization, which disappears after reduction, indicating a reversible modification (Fig. 17B, reducing). It suggests that the apparent dimer is connected by a disulfide bond.

The reducing gel shows that this dimerization is reversible and the subsequent CoAlation experiments were carried out on pre-reduced proteins. Therefore, this phenomenon presumably doesn't affect our results.

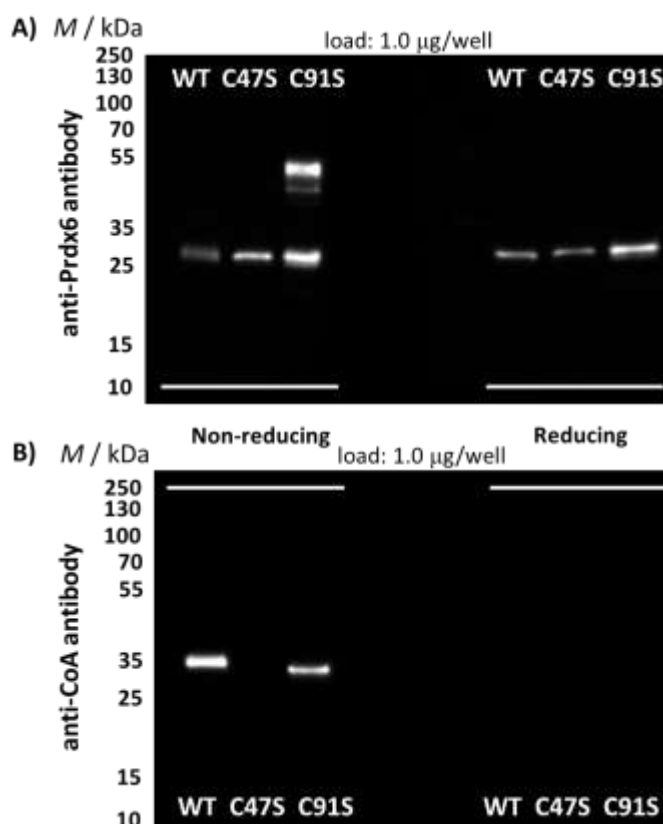


Figure 17: The CoAlation of Prdx6. A) WT, C47S and C91S Prdx6 in the presence (reducing) or absence (non-reducing) of a reducing agent. B) similar samples as in A) after the CoAlation reaction took place. Prdx6 was treated with 3 molar equivalents of CoA and 10 molar equivalents of H₂O₂ for 30 min at 25 °C. Reducing agent: 1.4 mM 2-mercaptoethanol.

In the CoAlation reaction reduced proteins were incubated with CoA and H₂O₂ was subsequently added to the mixture, to induce protein CoAlation. Protein CoAlation was confirmed with WB, using a CoA selective antibody (Fig. 17B). Non-reducing gels show that WT and C91S Prdx6 are CoAlated, whereas C47S Prdx6 is not. CoAlation was observed only when the peroxidative cysteine of Prdx6 was present (WT and C91S). CoA signals disappear on a reducing gel, which indicates the reversibility of CoAlation. These results suggest that CoA interacts with the active cysteine (Cys47) with the formation of a mixed disulfide bond. Based on these findings, we further investigated the deCoAlation of WT and C91S Prdx6.

5.3. Sulfur species-mediated deCoAlation of Prdx6-CoA

According to our idea, small reducing sulfur species may promote the decoalation of CoAlated Prdx6. To confirm this, we monitored the decrease in CoAlated C91S mutant and WT Prdx6 using various qualities and quantities of sulfide reagents.

5.3.1. Comparing the deCoAlation effect of inorganic sulfide and disulfide

First, we aimed to study if hydrogen sulfide or hydrogen disulfide may induce the deCoAlation of Prdx6-CoA species. Thus, we monitored the reaction of the WT and C91S proteins with sulfide and inorganic disulfide using WB. Taking into account that Cys47 is the peroxidative cysteine of Prdx6 and Fig. 17 suggested that only Cys47 is affected by CoAlation, we primarily focused on the reactivity of the C91S mutant, because it only contains Cys47 and allows its independent investigation. It is subsequently compared to the reactivity of the WT protein, to account for the potential contribution of Cys91. Using the anti-CoA antibody, I followed the decomposition of C91S Prdx6-CoA over time (Fig. 18).

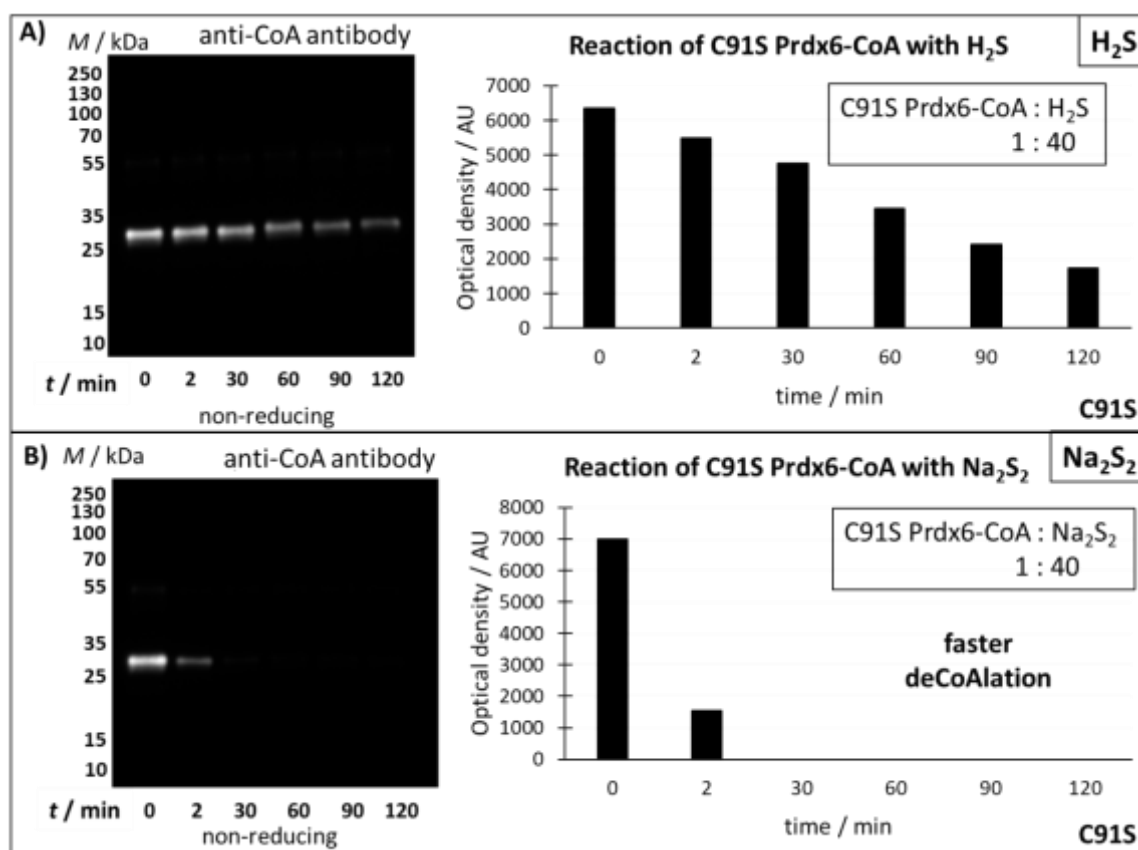


Figure 18: Investigation of the deCoAlation of CoAlated C91S Prdx6 (C91S Prdx6-CoA) over time upon the addition of A) sulfide or B) inorganic disulfide. Densitometric evaluation of the corresponding membrane are shown beside the blot. The concentration of sulfur species is 200 μ M. Representative of 3 independent repeats.

The experiment proved that H_2S and Na_2S_2 can induce the deCoAlation reaction of C91S Prdx6-CoA, because the CoA signal intensity decreases over time. The reaction with Na_2S_2 was found to be faster compared to that with H_2S . In the first case, no detectable amount of C91S Prdx6-CoA remains after 30 minutes (Fig. 18B), while upon the addition of H_2S , C91S Prdx6-CoA is detectable even after 120 minutes (Fig. 18A). Hence, Na_2S_2 was found to be a more efficient reducing agent in the *in vitro* deCoAlation reaction of C91S Prdx6-CoA.

Figure 19 shows the results of the analogous experiment conducted with WT Prdx6-CoA. Similarly, the signal of WT Prdx6-CoA decreases in time in the presence of H_2S and Na_2S_2 , and the reducing effect of Na_2S_2 (Fig. 19B) is significantly faster than that of H_2S (Fig. 19A).

The two experiments suggested that both sulfide and disulfide can mediate the deCoAlation reaction of CoAlated WT and C91S Prdx6 and disulfide was found to be more effective. To represent physiologically more relevant conditions, the effect of sulfide was investigated in the following experiments at a lower concentration.

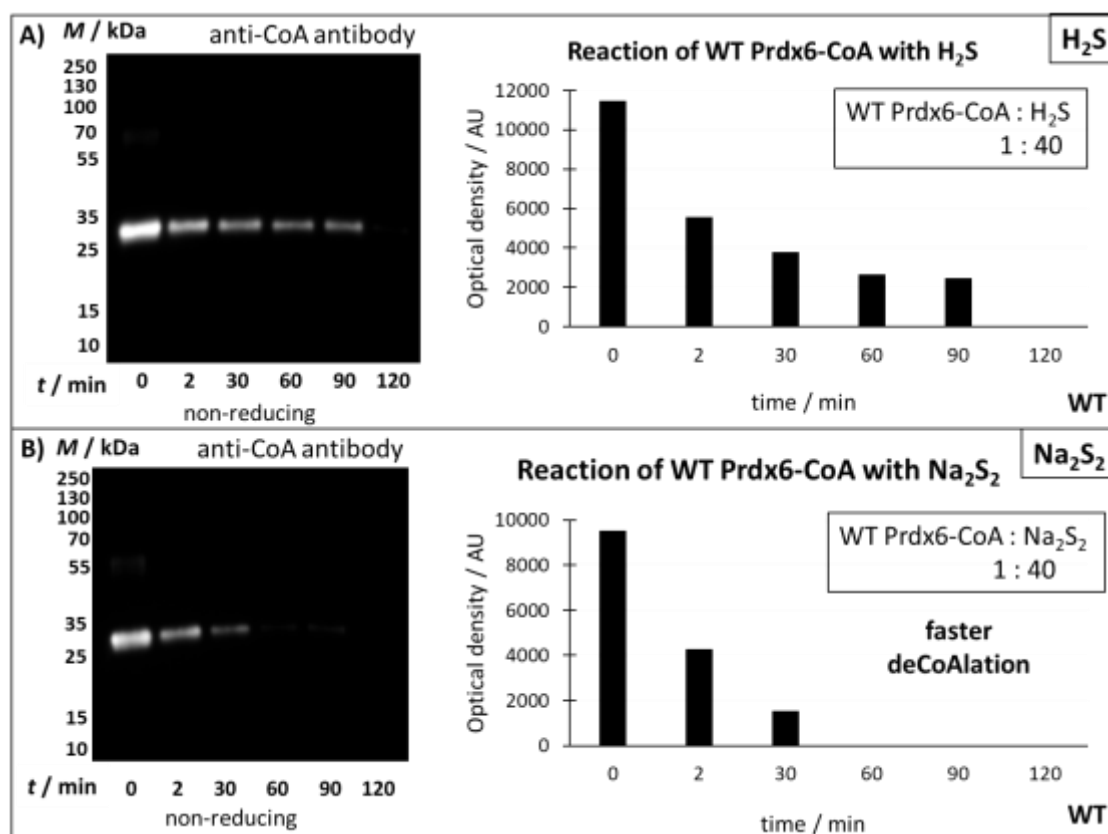


Figure 19: Investigation of the deCoAlation of CoAlated WT Prdx6 (WT Prdx6-CoA) over time, in the presence of A) sulfide and B) disulfide. Densitometric evaluation of the corresponding gels are shown beside the gels. The concentrations of the sulfur species were 200 μM each. Representative of 3 independent repeats.

5.3.2. Comparing the deCoAlation effect of sulfide for C91S and WT Prdx6-CoA

Similar to the previous experiments (5.3.1. Comparing the deCoAlation effect of sulfide and disulfide), we monitored the decreasing amount of C91S and WT Prdx6-CoA during the sulfide-mediated deCoAlation reaction. However, in this case, the sulfide concentration is lower to simulate a physiologically more relevant condition. Using the anti-CoA antibody, I followed the decomposition of C91S and WT Prdx6CoA over time (Fig. 20).

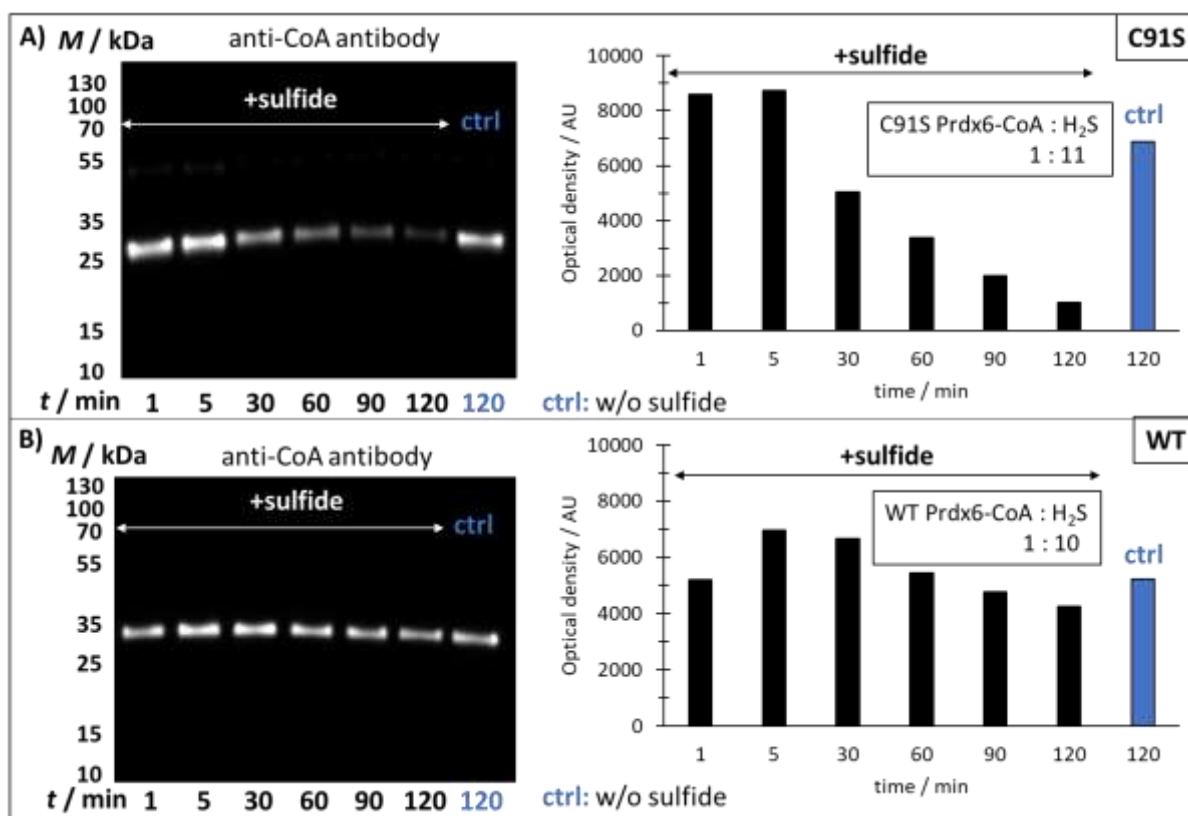


Figure 20: Investigation of the deCoAlation of CoAlated A) C91S mutant and B) WT Prdx6 (C91S and WT Prdx6-CoA) over time, in the presence of sulfide. Densitometric evaluation of the corresponding gels are shown beside the gels. The concentration of sulfur species is 200 μ M. Representative of 3 independent repeats.

Some decomposition of C91S and WT Prdx6-CoA was indeed observed even at close to physiological sulfide concentrations (Fig. 20). The control samples demonstrate that deCoAlation is sulfide-mediated because, without sulfide, the CoA signals at the endpoint of the reaction are stronger compared to when sulfide is present. The fact that the reaction did not go to completion may not be a problem under *in vivo* conditions, because the dynamic endogenous reproduction of sulfide, which is consumed in the reaction, can keep the catalytic cycle of Prx6-CoA deCoAlation running. DeCoAlation is more pronounced in the case of the C91S protein (Fig. 20A) because it contains only Cys47, whereas in the WT protein (Fig. 20B),

reactivity may also be influenced by Cys91. To get deeper insight into the deCoAlation reactions, the products were further analyzed with LC-MS/MS.

5.4. Identifying the products of sulfide-mediated deCoAlation of C91S and WT Prdx6-CoA by LC-MS/MS technique

We wanted to examine the decomposition products of the deCoAlation process of Prdx6-CoA. For the LC-MS/MS measurement, WT or C91S Prdx6-CoA was treated with H₂S as described above (similar to the data shown in Fig. 20) and samples were taken from the reaction mixture at various time points. Samples were alkylated with HPE-IAM to quench the reaction and digested for bottom-up proteomics measurement.⁵⁸ This LC-MS/MS technique is suitable for tracking the peroxidative cysteine-containing tryptic peptide in its thiol and persulfidated form too. I performed the measurements first on C91S Prdx6-CoA, a mutant that only contains a single cysteine (Cys47). After this, I repeated these experiments with WT Prdx6-CoA and detected the same cysteine-containing tryptic peptide (DFTPV⁴⁷CTTELGR) consistent with the WB results (Fig. 17), which demonstrated this is the CoAlated site. The results were normalized to a stable control peptide in the sample to account for potential differences in digestion efficiency. Thereafter, the results were normalized to the initial sample directly prior to the addition of sulfide ($t = 0$), so the relative increases in thiol (Cys-SH) and persulfide (Cys-SSH) species are comparable.

5.4.1. DeCoAlation experiments with excess sulfide

At first, I followed the two possible cysteine-containing products over time in the deCoAlation reaction of WT and C91S Prdx6-CoA in the presence of excess sulfide, as in the preliminary WB experiments. Figure 21 shows the results in the case of C91S Prdx6-CoA.

The thiol form of the tryptic peptide slightly increases during the reaction (Fig. 21A). In contrast, the persulfide form of the tryptic peptide (Fig. 21B) increases sharply, indicating C91S Prdx6-SSH formation. The observed slight increase of the thiol form can arise from two reasons: (1) during the deCoAlation reaction CoA also could be persulfidated and so Prdx6 is converted to the thiol form or (2) degradation of C91S Prdx6-SSH to C91S Prdx6-SH may lead to the observed increase of the thiol form. Considering the sharp increase in the persulfidated peptide form and the labile nature of persulfide species it is more likely that the Prdx6 deCoAlation reaction preferably produces C91S Prdx6-SSH as opposed to CoA-persulfide.

To ensure that the deCoAlation reaction is driven by sulfide, I performed control experiments similar to the previous ones in the absence of sulfide (Fig. 21A and 21B, the column diagrams on the right). The relative increases clearly show that in the case of thiol

(Cys-SH) there is no or negligible increase (average 2-fold) between the control samples and the reaction samples (Fig. 21A). In the case of persulfide (Cys-SSH), the relative peak ratios are on average 23-fold compared to the ctrl samples (Fig. 21B). This means that without sulfide no deCoAlation occurs and the main reaction yields Prdx6 persulfide.

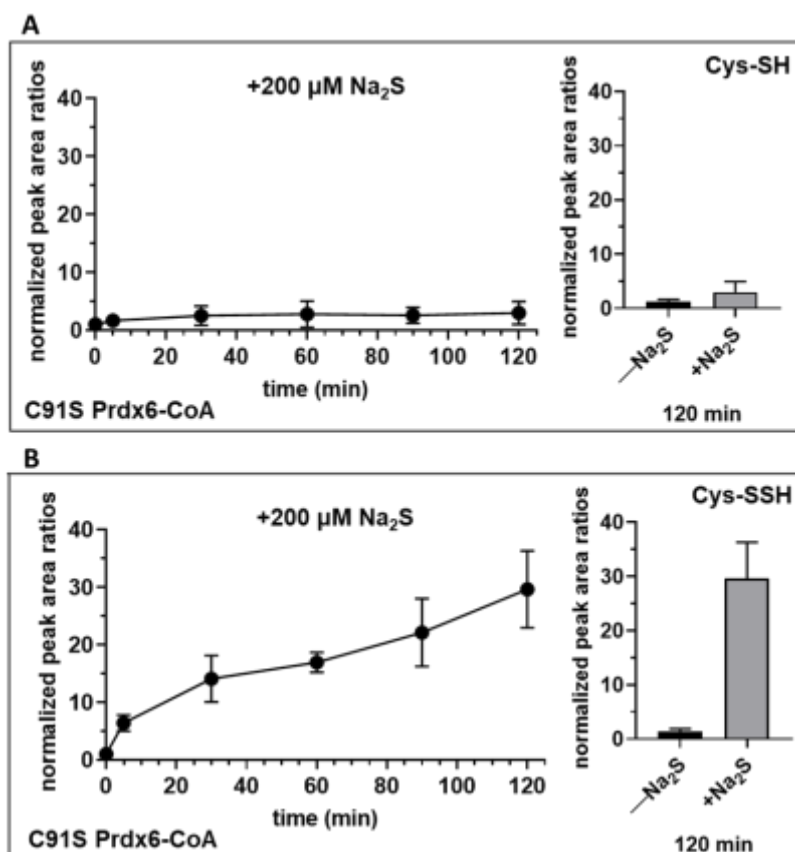


Figure 21: Following the sulfide-driven deCoAlation products of CoAlated C91S mutant Prdx6 (C91S Prdx6-CoA) with LC-MS/MS technique. Na_2S was used in 9-fold excess over C91S Prdx6-CoA. A) Thiol-containing tryptic peptide (Cys-SH) and B) Persulfide-containing tryptic peptide (Cys-SSH). The sequence of the tryptic peptide: DFTPV⁴⁷CTTELGR. The column diagrams on the right represent the products after 120 minutes of reaction, with (+) or without (—) Na_2S . The samples were alkylated with HPE-IAM and digested with Try/Lys-C. The results were normalized to the initial sample without Na_2S to represent the relative changes. The experiment was repeated in triplicates ($n = 3$), error bars represent standard deviation (SD).

I have repeated the previous experiment in the case of WT Prdx6-CoA (Fig. 22). Experiments with WT Prdx6-CoA under the same conditions demonstrate that the persulfidation reaction of WT Prdx6 is dominant. The amount of thiol (Cys-SH) can be considered constant during the reaction (Fig. 22A), while the amount of persulfide (Cys-SSH) increases in the presence of H_2S . The amount of peptides without sulfide did not increase in the case of thiol (Fig. 22A) or in the case of persulfide (Fig. 22B), i.e. WT Prdx6-CoA deCoAlation also occurs by sulfide.

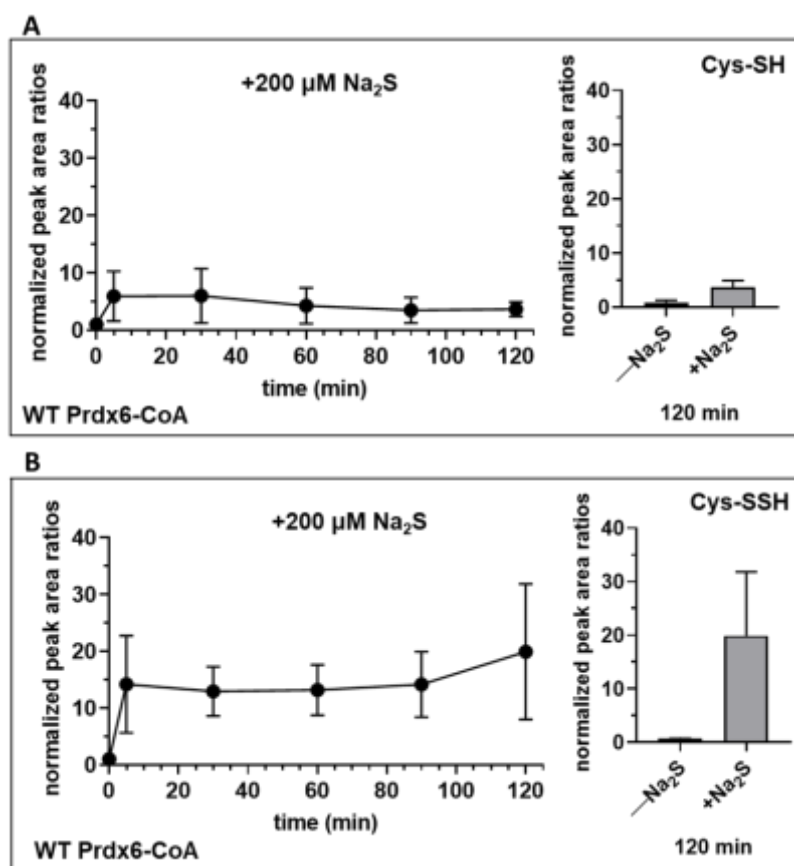


Figure 22: Following the sulfide-driven deCoAlation products of CoAlated WT Prdx6 (WT Prdx6-CoA) with LC-MS/MS technique. Na₂S was used in 8-fold excess over C91S Prdx6-CoA. A) Thiol-containing tryptic peptide (Cys-SH) and B) Persulfide-containing tryptic peptide (Cys-SSH). The sequence of the tryptic peptide: DFTPV⁴⁷CTTELGR. The column diagrams on the right represent the products after 120 minutes of reaction, with (+) or without (—) Na₂S. The samples were alkylated with HPE-IAM and digested with Try/Lys-C. The results were normalized to the initial sample without Na₂S to represent the relative changes. The experiment was repeated in triplicates ($n = 3$), error bars represent standard deviation (SD).

The deCoAlation effect is more prominent in the case of C91S Prdx6-CoA. This agrees with the previous WB results (Fig. 20).

5.4.2. DeCoAlation experiments with excess peroxiredoxin 6

The 200 μ M H₂S used in the previous experiment (see Chapter 5.4.1.) is multiple orders of magnitude higher than normal physiological conditions, so I repeated the experiment with 1 μ M H₂S, which is closer to physiological sulfide levels. It resulted in an excess of C91S Prdx6-CoA (31.5 equivalents) with regards to H₂S, (Fig. 23). Due to the decrease in sulfide concentration, I followed the reaction for a longer time (210 min) assuming a lower reaction rate. Figure 23 contains the results of an experiment where control samples (containing no H₂S) are marked with light blue, while the reaction samples (containing 1 μ M H₂S) are marked with grey.

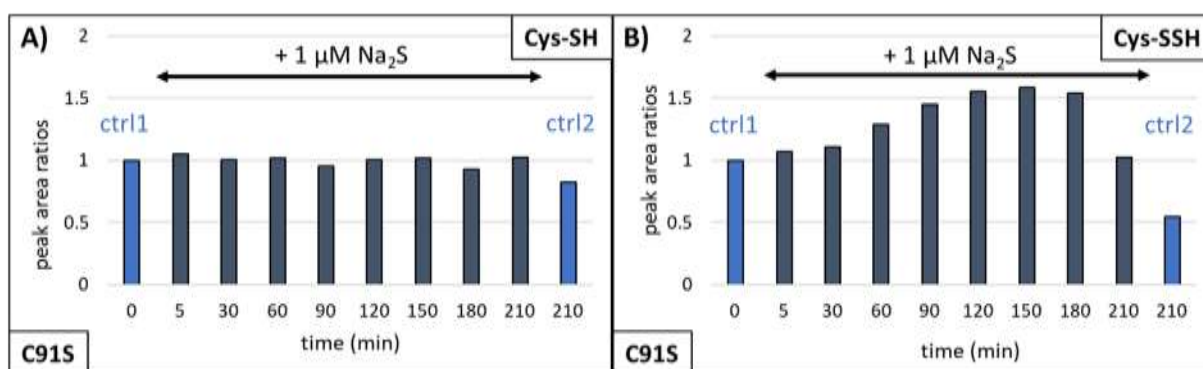


Figure 23: H₂S-driven deCoAlation products of CoAlated C91S mutant Prdx6 (C91S Prdx6-CoA) with LC-MS/MS technique. A) Thiol-containing tryptic peptide (Cys-SH). B) Persulfide-containing tryptic peptide (Cys-SSH). The concentration ratio of C91S Prdx6-CoA and Na₂S is 31.5:1. Control samples containing no Na₂S are marked in light blue. The sequence of the tryptic peptide: DFTPV⁴⁷CTTELGR. I normalized the results to the initial sample directly prior to the addition of sulfide ($t = 0$ min). The experiment was repeated in triplicates ($n = 3$), error bars represent standard deviation (SD).

The thiol form of the tryptic peptide (Fig. 23A) shows a constant level, i.e. their amount is almost constant during the reaction. The persulfide form of the tryptic peptide (Fig. 23B) shows an increase in the first 180 minutes and then begins to drop. This decrease is probably caused by the decomposition of persulfide, since it is not a stable compound. But at the same time, the thiol form (the expected product) does not show similar increase. The experiment proved that closer to physiological conditions H₂S-driven deCoAlation also occurs, resulting in C91S Prdx6 persulfidation. The control samples confirm that deCoAlation does not occur without the presence of H₂S, as we cannot detect an increase in the amounts of the degradation products.

I repeated the experiment with WT-Prdx6-CoA (Fig. 24). The results clearly show that the reaction associated with persulfidation of WT Prdx6 is dominant, as the relative increase of persulfide (Fig. 24B) is greater compared to that of thiol (Fig. 24A). Based on the ctrl samples, it can also be said here that WT Prdx6 CoAlation does not occur without the presence of sulfide. The deCoAlation reaction is more prominent in the case of the C91S mutant than in the WT protein, as revealed by previous experiments (Fig. 20, Fig. 21, and Fig. 22).

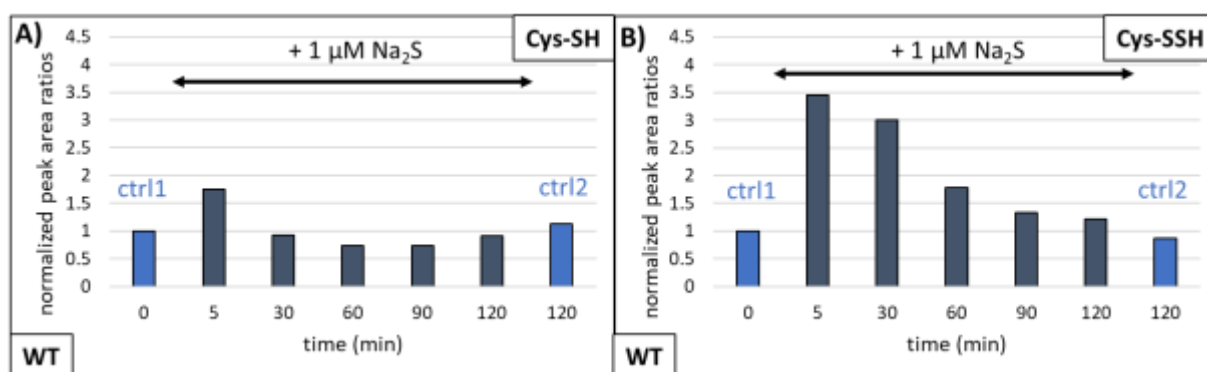


Figure 24: H_2S -driven deCoAlation products of CoAlated WT mutant Prdx6 (WT Prdx6-CoA) with LC-MS/MS technique. A) Thiol-containing tryptic peptide (Cys-SH) and B) Persulfide-containing tryptic peptide (Cys-SSH). The concentration ratio of WT Prdx6-CoA and Na_2S is 26.8:1. Control samples containing no Na_2S are marked in light blue. The sequence of the tryptic peptide: DFTPV⁴⁷CTTELGR. I normalized the results to the initial sample directly prior to the addition of sulfide ($t = 0$ min). The experiment was repeated in triplicates ($n = 3$), error bars represent standard deviation (SD).

The experiments prove that the sulfide-driven CoAlation of Prdx6-CoA takes place even at concentrations similar to physiological conditions with the main product being Prdx6 persulfide. Interestingly, here we observed a peak in the formation of the persulfidated product at the first 5 min timepoint with a decline over time. Because no significant concomittant increase in the thiol form was detected further product analyses are needed to investigate the fate of the persulfidated Prdx6 over time in this reaction system.

6. Conclusions and further plans

During my research, I successfully expressed two different forms of the human Prdx6 protein: WT and C47S mutant. I identified the products as Prdx6 using the WB technique and checked their purity with SDS-PAGE and Coomassie-staining. I subjected the WT, C47S and C91S Prdx6 proteins to the CoAlation reaction and then used the WB technique to show that only the WT and C91S mutant have CoAlated forms, and the C47S mutant does not. From this I could conclude that only the redox active peroxidative cysteine (Cys47) of Prdx6 can be CoAlated.

I studied the H₂S- and Na₂S₂-driven deCoAlation reaction of the C91S and WT Prdx6-CoA using the WB technique. I found that in the case of C91S and WT Prdx6-CoA, the deCoAlation reaction occurs faster in the presence of Na₂S₂, but it occurs in the presence of both sulfur species. To investigate whether Prdx6 deCoAlation involves persulfidation of Prdx6, I subjected the C91S and WT Prdx6-CoA H₂S-driven deCoAlation reactions to LC-MS/MS based product analyses. The LC-MS/MS measurements and the analysis of the data were carried out by an analytical chemist in the research group. From these data, I concluded that deCoAlation does not occur without the presence of H₂S and that deCoAlation leads to the formation of C91S and WT Prdx6 persulfide. I repeated the experiment under more physiologically relevant conditions where C91S and WT Prdx6-CoA were in excess compared to H₂S. These results proved that H₂S has a deCoAlation effect even under these conditions, no decomposition occurs without H₂S, and Prdx6 is persulfidated during the deCoAlation process. The effect was more significant in the case of C91S mutant than in the WT protein. However, the extent and physiological relevance of these reactions should be confirmed in cellular systems. Figure 25. summarizes the CoAlation and H₂S-driven deCoAlation process. Based on the results we suggest that the main product in the deCoAlation reaction of Prdx6-CoA is Prdx6-SSH, however the other possibility, i.e. Prdx6-CoA decomposition to CoA-persulfide (CoA-SSH) and the thiol form of Prdx6 (Prdx6-SH) cannot be fully ruled out. This reaction may also be biologically relevant, as it may be the source of persulfidated Prdx6 (Prdx6-SSH), which posttranslational modification may provide protection against oxidative stress and affect the enzymatic functions of Prdx6. In addition, CoAlation and persulfidation may provide an alternative Prdx6 recovery pathway in the cell. Persulfidated proteins can be easily reduced to the active thiol form in the cellular environment, for example, persulfidated Prdx6 may be reduced by the Trx/TrxR/NADPH enzyme system. However, this suggested alternative Prdx6 recovery pathway can only be supported by further *in vivo* and *in vitro* experiments.

In the future, I would like to carry out a more precise analysis of the H₂S-driven deCoAlation reaction for WT Prdx6-CoA to follow the other cysteine-containing peptide as well to demonstrate that Cys47, i.e. the peroxidative cysteine, is involved in the redox reactions. I would also like to perform all previous LC-MS/MS experiments with Na₂S₂ to see what effect the more reducing sulfide reagent has on the persulfidation of C91S and WT Prdx6.

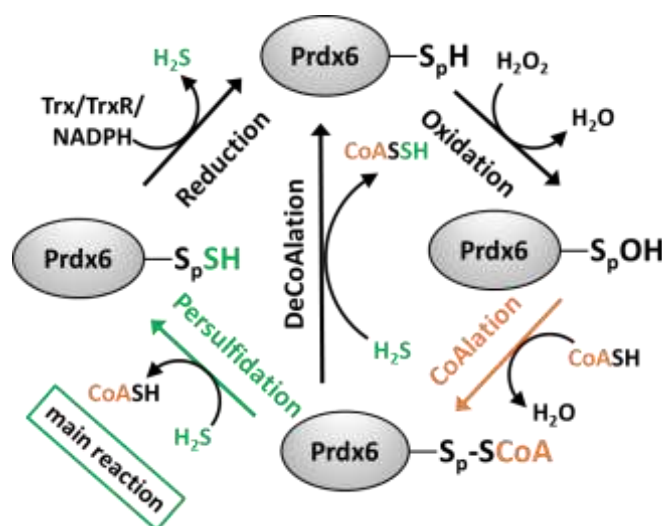


Figure 25: The suggested deCoAlation reaction of the human Prdx6. In the future, I would like to continue the investigation of the H₂S-driven deCoAlation reaction of WT Prdx6-CoA to follow the other cysteine-containing peptide and strengthen that only Cys47, i.e. the peroxidative cysteine, is involved in these redox reactions.

7. References

1. Cirino, G., Szabo, C. & Papapetropoulos, A. Physiological roles of hydrogen sulfide in mammalian cells, tissues, and organs. *Physiological Reviews* **103**, 31–276 (2023).
2. Mustafa, A. K. *et al.* H₂S Signals Through Protein S-Sulfhydration. *Science Signaling* **2**, (2009).
3. Romero-Aristizabal, C., Marks, D. S., Fontana, W. & Apfeld, J. Regulated spatial organization and sensitivity of cytosolic protein oxidation in *Caenorhabditis elegans*. *Nature Communications* **5**, 5020 (2014).
4. Dóka, É. *et al.* Control of protein function through oxidation and reduction of persulfidated states Irreversible oxidation of Cys residues to sulfinic/sulfonic forms typically impairs protein function. We found that persulfidation (CysSSH) protects Cys from irreversible oxidative loss of function by the formation of CysSSO 1-3. *Science advances*, **6**, eaax8358. (2020).
5. Sies, H. *et al.* Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. *Nature Reviews Molecular Cell Biology* **23**, 499–515 (2022).
6. Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nature Chemical Biology* **4**, 278–286 (2008).
7. Hall, A., Nelson, K., Poole, L. B. & Karplus, P. A. Structure-based Insights into the Catalytic Power and Conformational Dexterity of Peroxiredoxins. *Antioxidants & Redox Signaling* **15**, 795–815 (2011).
8. Arevalo, J. A. & Vázquez-Medina, J. P. The role of peroxiredoxin 6 in cell signaling. *Antioxidants* **7**, 172 (2018).
9. Fisher, A. B. Antioxidants special issue: Peroxiredoxin 6 as a unique member of the peroxiredoxin family. *Antioxidants* **8**, 107 (2019).
10. Tsuchiya, Y. *et al.* Protein CoAlation: A redox-regulated protein modification by coenzyme A in mammalian cells. *Biochemical Journal* **474**, 2489–2508 (2017).
11. Gout, I. Coenzyme A, protein CoAlation and redox regulation in mammalian cells. *Biochemical Society Transactions* **46**, 721-728 (2018).
12. Baković, J. *et al.* A key metabolic integrator, coenzyme A, modulates the activity of peroxiredoxin 5 via covalent modification. *Molecular and Cellular Biochemistry* **461**, 91–102 (2019).
13. Cuevasanta, E., Möller, M. N. & Alvarez, B. Biological chemistry of hydrogen sulfide and persulfides. *Archives of Biochemistry and Biophysics* **617**, 9–25 (2017).
14. Nagy, P. Mechanistic chemical perspective of hydrogen sulfide signaling. *Methods in Enzymology* **554**, 3–29 (2015).
15. KIMURA, H. Hydrogen sulfide and polysulfides as signaling molecules. *Proceedings of the Japan Academy, Series B* **91**, 131–159 (2015).

16. Nicholls P, K. JK. Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system. *Canadian Journal of Biochemistry* **60**, 613–623 (1982).
17. Warenycia, M. W. *et al.* Acute hydrogen sulfide poisoning. *Biochemical Pharmacology* **38**, 973–981 (1989).
18. Wallace, J. L. & Wang, R. Hydrogen sulfide-based therapeutics: Exploiting a unique but ubiquitous gasotransmitter. *Nature Reviews Drug Discovery* **14**, 329–345 (2015).
19. Módis, K., Panopoulos, P., Coletta, C., Papapetropoulos, A. & Szabo, C. Hydrogen sulfide-mediated stimulation of mitochondrial electron transport involves inhibition of the mitochondrial phosphodiesterase 2A, elevation of cAMP and activation of protein kinase A. *Biochemical Pharmacology* **86**, 1311–1319 (2013).
20. Aroca, A., Gotor, C., Bassham, D. C. & Romero, L. C. Hydrogen Sulfide: From a Toxic Molecule to a Key Molecule of Cell Life. *Antioxidants* **9**, 621 (2020).
21. Kabil, O. & Banerjee, R. Characterization of Patient Mutations in Human Persulfide Dioxygenase (ETHE1) Involved in H₂S Catabolism. *Journal of Biological Chemistry* **287**, 44561–44567 (2012).
22. Paul, B. D., Snyder, S. H. & Kashfi, K. Effects of hydrogen sulfide on mitochondrial function and cellular bioenergetics. *Redox Biology* **38**, 101772 (2021).
23. Nagy, P., Dick, T., Greiner, R., Pálinkás, Z. & Nagy, A. P36 Redox-, coordination- and solution-chemistry of sulfide in relation to some of its biological actions. *Nitric Oxide* **31**, S51 (2013).
24. Shen, X., Peter, E. A., Bir, S., Wang, R. & Kevil, C. G. Analytical measurement of discrete hydrogen sulfide pools in biological specimens. *Free Radical Biology and Medicine* **52**, 2276–2283 (2012).
25. Paul, B. D. & Snyder, S. H. H₂S signalling through protein sulfhydration and beyond. *Nature Reviews Molecular Cell Biology* **13**, 499–507 (2012).
26. Ida, T. *et al.* Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 7606–7611 (2014).
27. Ditrói, T. *et al.* Comprehensive analysis of how experimental parameters affect H₂S measurements by the monobromobimane method. *Free Radical Biology and Medicine* **136**, 146–158 (2019).
28. Nagy, P. *et al.* Chemical aspects of hydrogen sulfide measurements in physiological samples. *Biochimica et Biophysica Acta - General Subjects* **1840**, 876–891 (2014).
29. Filipovic M. R. Persulfidation (S-sulfhydration) and H₂S. *Handbook of experimental pharmacology* **230**, 29–59 (2015).
30. Vignane, T. & Filipovic, M. R. Emerging Chemical Biology of Protein Persulfidation. *Antioxidants & Redox Signaling* **39**, 19–39 (2023).
31. Filipovic, M. R., Zivanovic, J., Alvarez, B. & Banerjee, R. Chemical Biology of H₂S Signaling through Persulfidation. *Chemical Reviews* **118**, 1253–1337 (2018).

32. Yadav, P. K. *et al.* Biosynthesis and Reactivity of Cysteine Persulfides in Signaling. *Journal of the American Chemical Society* **138**, 289–99 (2016).
33. Kasamatsu, S. *et al.* Redox signaling regulated by cysteine persulfide and protein polysulfidation. *Molecules* **21**, 1721 (2016).
34. Ursini, F., Maiorino, M. & Forman, H. J. Redox homeostasis: The Golden Mean of healthy living. *Redox Biology* **8**, 205–215 (2016).
35. Tretter, V., Hochreiter, B., Zach, M. L., Krenn, K. & Klein, K. U. Understanding Cellular Redox Homeostasis: A Challenge for Precision Medicine. *International Journal of Molecular Sciences* **23**, 106 (2021).
36. Rahal, A. *et al.* Oxidative stress, prooxidants, and antioxidants: The interplay. *BioMed Research International* **2014**, 761264 (2014).
37. Ighodaro, O. M. & Akinloye, O. A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine* **54**, 287–293 (2018).
38. Lismont, C., Revenco, I. & Fransen, M. Peroxisomal hydrogen peroxide metabolism and signaling in health and disease. *International Journal of Molecular Sciences* **20**, 3673 (2019).
39. Murphy, M. P. *et al.* Unraveling the Biological Roles of Reactive Oxygen Species. *Cell Metabolism* **13**, 361–366 (2011).
40. Willem H Koppenol. Comparative Analysis of Hydrogen Peroxide and Peroxynitrite Reactivity with Thiols. *Hydrogen Peroxide Metabolism in Health and Disease* (eds. Vissers, M. C. M., Hampton, M. B. & Kettle, A. J.) 49–79 (2017).
41. Sies, H., Berndt, C. & Jones, D. P. Oxidative Stress. *Annual Review of Biochemistry* **86**, 715–748 (2017).
42. Schmidt, H. H. H. W. *et al.* Antioxidants in Translational Medicine. *Antioxidants & Redox Signaling* **23**, 1130–1143 (2015).
43. Kurutas, E. B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutrition Journal* **15**, 71 (2016).
44. Munteanu, C., Turnea, M. A. & Rotariu, M. Hydrogen Sulfide: An Emerging Regulator of Oxidative Stress and Cellular Homeostasis—A Comprehensive One-Year Review. *Antioxidants* **12**, 1737 (2023).
45. Nagy, P. Kinetics and mechanisms of thiol-disulfide exchange covering direct substitution and thiol oxidation-mediated pathways. *Antioxidants and Redox Signaling* **18**, 1623–1641 (2013).
46. Greiner, R. *et al.* Polysulfides link H₂S to protein thiol oxidation. *Antioxidants and Redox Signaling* **19**, 1749–1765 (2013).
47. Fukuto, J. M. *et al.* Biological hydropersulfides and related polysulfides – a new concept and perspective in redox biology. *FEBS Letters* **592**, 2140–2152 (2018).

48. Nagy, P. *OXIDATÍV STRESSZ és REDOXIJELÁTVITEL Betekintés a redoxibiológia molekuláris világába.*
http://opac.mtak.hu/F?func=direct&local_base=MTA01&doc_number=001045517
(2015).
49. Akaïke, T. *et al.* Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. *Nature Communications* **8**, 1177 (2017).
50. Wood J. L. Sulfane sulfur. *Methods in enzymology* **143**, 25–29 (1987).
51. Shieh, M. *et al.* Shining a light on SSP4: A comprehensive analysis and biological applications for the detection of sulfane sulfurs. *Redox Biology* **56**, 102433 (2022).
52. Gao, X.-H. *et al.* Quantitative H₂S-mediated protein sulfhydration reveals metabolic reprogramming during the integrated stress response. *Elife* **4**, (2015).
53. Bibli, S.-I. *et al.* Mapping the Endothelial Cell S-Sulfhydrome Highlights the Crucial Role of Integrin Sulfhydration in Vascular Function. *Circulation* **143**, 935–948 (2021).
54. Dóka, É. *et al.* A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. *Science Advances* **2**, (2016).
55. Zhang, D. *et al.* Detection of Protein S-Sulfhydration by a Tag-Switch Technique. *Angewandte Chemie International Edition* **53**, 575–581 (2014).
56. Wedmann, R. *et al.* Improved tag-switch method reveals that thioredoxin acts as depersulfidase and controls the intracellular levels of protein persulfidation. *Chemical Science* **7**, 3414–3426 (2016).
57. Zivanovic, J. *et al.* Selective Persulfide Detection Reveals Evolutionarily Conserved Antiaging Effects of S-Sulfhydration. *Cell Metabolism* **30**, 1152-1170.e13 (2019).
58. Kasamatsu, S. *et al.* High-Precision Sulfur Metabolomics Innovated by a New Specific Probe for Trapping Reactive Sulfur Species. *Antioxidants & Redox Signaling* **34**, 1407–1419 (2021).
59. Takata, T. *et al.* Methods in sulfide and persulfide research. *Nitric Oxide* **116**, 47–64 (2021).
60. Tossounian, M. A. *et al.* Profiling the Site of Protein CoAlation and Coenzyme A Stabilization Interactions. *Antioxidants* **11**, 1362 (2022).
61. Ghezzi, P. Regulation of protein function by glutathionylation. *Free Radical Research* **39**, 573–580 (2005).
62. Filonenko, V. & Gout, I. Discovery and functional characterisation of protein CoAlation and the antioxidant function of coenzyme A. *BBA Advances* **3**, (2023).
63. Petrone, O. *et al.* Changes of the Protein CoAlation Pattern in Response to Oxidative Stress and Capacitation in Human Spermatozoa. *International Journal of Molecular Sciences* **24**, (2023).

64. Peskin, A. V. *et al.* Modifying the resolving cysteine affects the structure and hydrogen peroxide reactivity of peroxiredoxin 2. *Journal of Biological Chemistry* **296**, 100494 (2021).
65. Sue, G. R., Ho, Z. C. & Kim, K. Peroxiredoxins: A historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radical Biology and Medicine* **38**, 1543–1552 (2005).
66. Wood, Z. A., Schröder, E., Robin Harris, J. & Poole, L. B. Structure, mechanism and regulation of peroxiredoxins. *Trends in Biochemical Sciences* **28**, 32–40 (2003).
67. Cox, A. G., Winterbourn, C. C., & Hampton, M. B. Measuring the redox state of cellular peroxiredoxins by immunoblotting. *Methods in enzymology* **474**, 51–66 (2010).
68. Winterbourn, C. C. & Peskin, A. V. Kinetic approaches to measuring peroxiredoxin reactivity. *Molecules and Cells* **39**, 26–30 (2016).
69. Portillo-Ledesma, S. *et al.* Differential kinetics of two-cysteine peroxiredoxin disulfide formation reveal a novel model for peroxide sensing. *Biochemistry* **57**, 3416–3424 (2018).
70. Peskin, A. V. *et al.* The high reactivity of peroxiredoxin 2 with H₂O₂ is not reflected in its reaction with other oxidants and thiol reagents. *Journal of Biological Chemistry* **282**, 11885–11892 (2007).
71. Elko, E. A. *et al.* Oxidation of peroxiredoxin-4 induces oligomerization and promotes interaction with proteins governing protein folding and endoplasmic reticulum stress. *Journal of Biological Chemistry* **296**, 100665 (2021).
72. Barranco-Medina, S., Lázaro, J. J. & Dietz, K. J. The oligomeric conformation of peroxiredoxins links redox state to function. *FEBS Letters* **583**, 1809–1816 (2009).
73. Stöcker, S., Maurer, M., Ruppert, T. & Dick, T. P. A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nature Chemical Biology* **14**, 148–155 (2018).
74. Delaunay, A., Pflieger, D., Barrault, M.-B., Vinh, J. & Toledano, M. B. A Thiol Peroxidase Is an H₂O₂ Receptor and Redox-Transducer in Gene Activation. *Cell* **111**, 471–481 (2002).
75. Nagy, P. *et al.* Model for the exceptional reactivity of peroxiredoxins 2 and 3 with hydrogen peroxide: A kinetic and computational study. *Journal of Biological Chemistry* **286**, 18048–18055 (2011).
76. Fisher, A. B. Peroxiredoxin 6 in the repair of peroxidized cell membranes and cell signaling. *Archives of Biochemistry and Biophysics* **617**, 68–83 (2017).
77. Fisher, A. B. *et al.* A novel lysophosphatidylcholine acyl transferase activity is expressed by peroxiredoxin 6. *Journal of Lipid Research* **57**, 587–596 (2016).
78. Kim, K. H., Lee, W. & Kim, E. E. K. Crystal structures of human peroxiredoxin 6 in different oxidation states. *Biochemical and Biophysical Research Communications* **477**, 717–722 (2016).

79. Vasas, A., Dóka, É., Fábián, I. & Nagy, P. Kinetic and thermodynamic studies on the disulfide-bond reducing potential of hydrogen sulfide. *Nitric Oxide* **46**, 93–101 (2015).
80. Hamid, H. A. *et al.* Polysulfide stabilization by tyrosine and hydroxyphenyl-containing derivatives that is important for a reactive sulfur metabolomics analysis. *Redox Biology* **21**, (2019).

Summary

Hydrogen sulfide mediated deCoAlation of CoAlated Peroxiredoxin 6

Daniella Dörgő, MSc student in Chemistry

Department of Molecular Immunology and Toxicology (MITO), National Institute of Oncology (NIO), Budapest

Supervisor: **Prof. Péter Nagy, D.Sc.**, Scientific Director (NIO)

In recent years, studying the biological role of hydrogen sulfide (H_2S) has become a major field within redox biology. H_2S may exert its biological effect through covalent modification of the thiol group of cysteine-containing proteins (Protein-SH) one of which is the most important the protein-persulfidation (Protein-SSH). The oxidative post-translational modifications (oxPTMs) of thiol proteins, such as protein-persulfidation, may alter their reactivity and/or functionality, thus they are potentially involved in signaling and regulatory processes within cells.[1] Another important oxPTM is protein CoAlation, which means a disulfide bond formation between a coenzyme A (CoA) molecule and the thiol group of a cysteine-containing protein (Protein-S-S-CoA). It seems to be a reversible, regulatory cysteine side chain-specific and oxidative stress-induced process.[2] Among the cysteine-containing proteins, there is an important enzyme family, namely peroxiredoxins (Prdxs) which are often called the redox sentinels of the cell. They rapidly react with hydrogen peroxide (H_2O_2) thus preventing the accumulation of oxidative compounds. Prdxs together with reducing agents are key defense factors against oxidative stress and central hubs in redox signaling processes. Prdx-persulfidation may provide protection against oxidative-stress-induced irreversible Prdx overoxidation and inhibition.[3]

The goal of our collaborative project is to investigate the molecular mechanism and biochemical significance of the CoAlation of peroxiredoxin 6 (Prdx6-SH), a member of the Prdx enzyme family and to explore the potential role of hydrogen sulfide in the reverse process, namely the deCoAlation of Prdx6-S-S-CoA. Within the proposed model, after H_2O_2 -induced Prdx6 oxidation, Prdx6-SOH reacts with a CoA molecule, forming a disulfide-bound (Prdx6-S-S-CoA). Subsequent reducing reactions with H_2S (deCoAlation) may yield both the thiol and the persulfide forms of Prdx6 (Prdx6-SH or Prdx6-SSH, respectively). My research focuses on the potential Prdx6 persulfidation reaction in the redox cycle of Prdx6 through the deCoAlation of Prdx6-S-S-CoA. My thesis successfully established that upon the reactions of Prdx6-S-S-CoA with H_2S , the primary deCoAlation product is Prdx6-SSH, which may be involved in cellular signaling processes. In my work, I used the following chemical and biological methods: recombinant protein production, fast protein liquid chromatography (FPLC), purity analysis and identification of protein modifications by gel electrophoresis (Coomassie staining and Western blotting), quantitative measure of proteins (UV-VIS spectroscopy) and liquid chromatography tandem mass spectrometry (LC-MS/MS). During my research, I successfully expressed two different forms of the human Prdx6 protein: WT and C47S mutant. I subjected the WT, C47S and C91S Prdx6 proteins to the CoAlation reaction and from this I concluded that only the redox active peroxidative cysteine (Cys47) of Prdx6 can be CoAlated. I studied the H_2S - and Na_2S_2 -driven deCoAlation reaction of the C91S and WT Prdx6-CoA using the WB technique, and product analysis was performed in the case of the H_2S -driven deCoAlation reaction with LC/MS-MS technique. From these data, I concluded that deCoAlation does not occur without the presence of H_2S and that deCoAlation leads to the formation of C91S and WT Prdx6 persulfide in the case of sulfide- and protein excess as well.

[1] Paul, B., Snyder, S. *Nature Reviews Molecular Cell Biology*, 13, 499–507 (2012)

[2] Gout, I., *Biochemical Society Transactions*, 46(3), 721-728 (2018)

[3] Dóka, É., Ida, T., Dagnell, M., Abiko, Y., Luong, N. C., Balog, N., Takata, T., Espinosa, B., Nishimura, A., Cheng, Q., Funato, Y., Miki, H., Fukuto, J. M., Prigge, J. R., Schmidt, E. E., Arnér, E. S. J., Kumagai, Y., Akaike, T., Nagy, P., *Science advances*, 6(1), eaax8358 (2020)