**Final report for project KH_126766 entitled 'Cooperative actions of Trx/GSH systems and the CARS2 system in mediating protein polysulfidation'.**

References to publications that emerged from this project are highlighted in red.

## Introduction

This project was proposed and funded based on the excessive interest raised by our paper titled *'A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems*', published in Science Advances, in 2016. This study reserved its position as a landmark in the field, it has received close to 100 independent citations since the publication date. It is worth to be mentioned that Science Advances is currently ranked 3rd in the SciMago science journal ranking in the natural sciences category, after Nature and Science.

With the aid of this grant 15 research articles were published since the starting date, of which 9 has primary authorship from our group: 11 original research contributions (cited along the text), 1 review article (Nagy *et al.*, 2020), 2 book chapters (Doka *et al.*, 2019, Garai *et al.*, 2019) and an editorial of a special issue (Nagy *et al.*, 2019). The accumulated impact factor of these papers is 118.549.

## Major findings

What we consider the major outcome of this project, is that we found experimental evidence for the protective function of protein persulfidation against oxidative stress. It has been long hypothesized that protein persulfide groups may exhibit antioxidant functions due to their elevated nucleophilicity (Ono *et al.*, 2014) and thus contribute to the observed cytoprotective effects of hydrogen sulfide ((Nagy, 2015) and references therein). However, no systematic assessment of this idea has been carried out before. We published a detailed study titled *'Control of protein function through oxidation and reduction of persulfidated states*' in Science Advances in 2020 (Doka *et al.*, 2020), which already drew the attention of the experts, shown by a number of citations promptly after publication. We perceive this paper as a direct continuation of our earlier article which created the base of our KH grant. The highlights of this study are the following results: we conducted quantitative, LC-MS based analysis of the low- and high molecular weight persulfide species in mouse liver tissue samples conditionally lacking key enzymes from the glutathione and thioredoxin systems. The data confirmed the regulatory role of the Trx/GSH systems in persulfide levels as well as other oxidized sulfur species. They also implicated that the so-called thioredoxin like protein of 14 kDa (TRP14) has specific persulfide reductase roles in distinct signaling pathways.

The prevalence of differently oxidized persulfide species (-SSOH, $-SSO_2H$ and $-SSO_3H$) was assessed on multiple systems from isolated proteins to tissue homogenates. Reversible persulfide oxidation was found on the peroxidative cysteine of recombinant peroxiredoxin 2 (Prx2) and cell culture experiments revealed the role of thioredoxin reductase 1 (TrxR1) in the modulation of this modification. This could

be a fundamental observation in redox biology, taking into account that Prx2 is actively involved in peroxide scavenging due to its exceptional reactivity with $H_2O_2$ (Nagy *et al.*, 2011) as well as transduction of peroxide mediated redox signals via protein-protein interactions (Stöcker *et al.*, 2018). We obtained a postdoctoral excellence fellowship from NKIFH to follow up on the implication of active site persulfidation of Prx2 in its antioxidant and redox relay functions.

Furthermore, reversible persulfide oxidation was found to be essential in the regulation of protein phosphatase 1B (PTP1B) activity with related consequences in epidermal growth factor receptor (EGFR) induced tyrosine phosphorylation signaling pathways. In vitro enzymatic assays regarding PTP1B phosphatase activity revealed the active role of the members thioredoxin system in the reactivation of the enzyme after subsequent polysulfide and peroxide treatments. Similarly to Prx2, persulfide and oxidized persulfide species were detected on the active site cysteine. Cell culture studies were conducted in A431 squamous carcinoma cell line with high EGFR expression, showing that polysulfide treatment promoted ligand binding induced tyrosine phosphorylation signal as well as the phosphorylation of PTP1B target site on EGFR. It was concluded that phosphatase capacity is more affected by persulfidation than kinase activity. Similar tendencies were found in TRP14 knockdown HEK293 cells and upon selenium supplementation, suggesting that the Trx system, perhaps mainly TRP14, regulates tyrosine signaling in a dynamic manner.

We reckon that persulfidation acts as a preemptive mechanism of cytoprotection, namely that a certain part of the thiol proteome reside in the persulfidated states under normal conditions which are reductively activated by the thioredoxin system in case of an excessive oxidation event.

The exact nature of the persulfide targets by the different thioredoxin components are yet to be elucidated. In fact, we requested an extension of the funding period with the intention to carry out functional proteomic studies on the persulfidome of WT compared to full body knockout TRP14-null deep frozen mouse liver samples (the latter animal model being described first in (Doka *et al.*, 2020)). Important developments have been made during this time frame to perform these experiments. However, due to experimental limitations we found during our systematic method development (discussed below), we are still optimizing our sulfur proteomics/metabolomics methods. The suggested improvements of the ProPerDP method (Doka *et al.*, 2016) are under development: we are aiming to perform tryptic digest preceding the biotin-streptavidin pull-down step, in order to minimize artifactual hits and increase the selectivity of the method. In the meantime we published a book chapter with detailed protocols regarding the application of the ProPerDP method on various biological systems (Doka *et al.*, 2019).

The primary prerequisite of the advancement of the persulfide field is the selective and sensitive detection of reactive sulfur species. During this project we made important contributions to this endeavor, further developed detection methods to promote the understanding of the related biological

events. Due to the enhanced reactivity of Cys per/polysulfide modifications they are fairly unstable in biological systems and their analysis requires electrophile derivatization for downstream measurements. Therefore, practically all available detection techniques (Doka *et al.*, 2016, Krishnan *et al.*, 2011, Mustafa *et al.*, 2009, Zhang *et al.*, 2014) rely on an initial alkylation step, where persulfides are converted into more stable mixed disulfide forms (reaction 1).

$$RSS_nH + Alk \rightarrow R\text{-}SS_n\text{-}Alk \quad (1)$$

The exploitation of this type of labeling is far from straightforward, because of the manifestation of the 'observer effect', i.e., the fact of the observation itself causes a disturbance of the system. Indeed, the introduction of the alkylating agents (iodoacetamides, maleimides, sulfonates etc) alters the distribution of the per/polysulfide species they are meant to stabilize by multiple means. First of all, harsh electrophiles such as maleimides (NEM) or monobromobimane (MBB) have a direct decomposing effect of longer polysulfur chains (Akaike *et al.*, 2017). We conducted detailed LC-MS based analyses and discussed the potential mechanisms of this alkylation induced destruction (Bogdándi *et al.*, 2018). According to the latter study, another effect of alkylation on persulfide detection is the perturbation of the dynamic equilibria between low- and high molecular weight thiol (including $H_2S$) and persulfide species (reaction 2),

$$P\text{-}SS_nH + R\text{-}SH \rightleftharpoons P\text{-}SS_xH + R\text{-}SS_{(n-x)}H \quad (2)$$

and their simultaneous alkylation is governed by the Curtin-Hammett principle (Bogdándi *et al.*, 2018). Meaning that all of the species present are targetable by the applied alkylating agent and the final product distribution will reflect the relative reactivities of each molecule rather than the relevant speciation originally present in the system. Simply speaking, a highly reactive intermediate with low occurrence may falsely suggest itself as the primary species present, completely misleading the observer. Such a situation easily occurs in a complex biological system, calling for cautious interpretation of experimental readouts. We published a review article to draw attention to such pitfalls of alkylation based investigations of redox active sulfur species, with special emphasis on studies reflecting intracellular redox homeostasis (Nagy *et al.*, 2020). The redox state of peroxiredoxins are often used to mirror the redox conditions of the cells (i.e. oxidative stress) and we point out that preferential alkylation of Prx cysteines or the coupled reducing machinery might lead to misinterpretation of the results. Another approach uses dimedone based labeling techniques which were long claimed to be selective to -SOH (sulfenic acid) species, the immediate oxidation product of thiol proteins. We present arguments that not only dimedone is reactive towards -SSOH forms as well, its reactivity is suitable to shift hydrolysis equilibria of persulfide species, thus hindering the distinction between -SSH and -SOH groups.

In a number of the presented studies (Doka *et al.*, 2020, Bogdándi *et al.*, 2018, Bianco *et al.*, 2019, Lin *et al.*, 2019, Mellis *et al.*, 2021) we applied an aromatic derivative of iodoacetamide, β-(4-

Hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) (Akaike *et al.*, 2017), because recent findings revealed that tyrosine and other phenolic moieties stabilize persulfide groups via inhibition of their hydrolysis and electrophilic degradation (Hamid *et al.*, 2019). With the application of HPE-IAM based LC-MS analysis of low molecular weight (CysSSH, GSSH, etc) and protein bound per/polysulfidation we gathered further information about the intra/extracellular persulfide traffic, speciation and biological implications (Bianco *et al.*, 2019, Lin *et al.*, 2019).

The key interface of the interaction between persulfide synthase and reductase capacities of the cells, which was the underlying motivation of our project, is hydrogen sulfide ($H_2S$) in biology. Currently a major question of the field aims to find out whether $H_2S$ is a by-product of CARS mediated translational polysulfide generation (Akaike *et al.*, 2017) with subsequent reduction by the thioredoxin system or else, protein per/polysulfides are the downstream effector molecules in sulfide mediated signaling (Paul *et al.*, 2012). In order to clarify these issues, we need to gain further information about the relevant concentrations and functional modalities of hydrogen sulfide. The state-of-the art detection method for $H_2S$ is the monobromobimane (MBB) (Wintner *et al.*, 2010), yet discrepancies were to be found in the literature in both the performance and results of the techniques. To this end, we carried out a systematic study to assess the effect of the experimental parameters on the final results and suggested an optimized version of the MBB technique (Ditroi *et al.*, 2019), readily usable by the field. Since then, this improved protocol was used in multiple systems with direct biological relevance, disease models and drug development (Kozich *et al.*, 2019, Wallace *et al.*, 2020). Most recently, we contributed to an international collaboration led by Prof. Guenter Schwarz, who is a world-renowned expert of molybden cofactor (Moco) and sulfite oxidase (SUOX) deficiencies, both conditions affecting mitochondrial sulfide catabolism (Kohl *et al.*, 2019). Our recent shared study attempted to understand sulfite production mechanisms in the cell, given the toxicity of sulfite accumulation (Mellis *et al.*, 2021). It was found that glutamate oxaloacetate transaminases (GOTs), are major contributors to sulfite production from cysteine sulfinic acid and cytosolic GOT1 is the primary enzyme responsible for the process. We participated in the project by determination of hydrogen sulfide, cysteine thiol and persulfide and glutathione thiol and persulfide levels from SUOX, GOT1/SUOX and GOT2/SUOX knockout HEK293T cell lines in order to elucidate how these proteins contribute to $H_2S$/persulfide homeostasis. A major finding of this study was that although GOT1&2 are important elements of oxidative Cys catabolic pathways, their sulfide producing Cys catabolic functions (in combination with 3MST) is important in cellular protection against sulfite accumulation in SO deficiency.

Our group conducts research regarding various mechanism of interaction between hydrogen sulfide and biomolecules. Apart from its widespread cross-talk with cysteine thiol and persulfide intermediates, $H_2S$ is known to affect metalloproteins by coordinating to their active site metal centers and also via the formation of a plethora of redox active molecules along NO mediated signaling

pathways (Nagy, 2015). A unique sulfur-nitrogen containing molecule was recently identified as biologically active sulfane sulfur carrier, named S-nitrosopersulfide ($SSNO^-$) (Bogdandi *et al.*, 2020, Cortese-Krott *et al.*, 2015). Its biochemical characterization is currently being undertaken. $SSNO^-$ can be generated in vitro by the reaction of $H_2S$ with an NO donor such as S-nitroso-N-acetylpenicillamine (SNAP). The molecule has intriguing chemical properties, so far it is the only sulfane sulfur containing (persulfide-like) species that eludes reduction by the thioredoxin and glutathione systems (Bogdandi *et al.*, 2020). Functional aspects incorporate S-nitrosylation and S-persulfidation of thiol proteins, by the continuous release of inorganic polysulfides, NO and other byproducts. Interestingly, it seems like rather than readily persulfidating protein targets, $SSNO^-$ exhibits delayed persulfidating effect following the time scale of its spontaneous decomposition. Finally $SSNO^-$ was found to induce sustained activation of the transient receptor potential ankyrin 1 (TRPA1) channel, responsible for the sensation of various external irritations.

Metalloproteins were also investigated with regards to the biochemical actions of sulfide and persulfide, a book chapter was published by our group presenting newly developped experimental techniques for the monitoring of sulfide induced inhibition of myeloperoxidase (MPO) (Garai *et al.*, 2019). We collaborated in a project showing that persulfide species efficiently reduce oxygenated myoglobin to ferric (Fe(III)) form which is rapidly reduced further to the ferrous (Fe(II)) isoform promoting oxygen binding (Álvarez *et al.*, 2020). These data corroborate the enhanced nucleophilicity of persulfides compared to thiols and concomitantly show that the interaction with metal centers is an important, yet undescribed, novel avenue in the biological meaning on protein persulfidation.

## References


1. P. Nagy, E. Doka, T. Ida, T. Akaike, Measuring Reactive Sulfur Species and Thiol Oxidation States: Challenges and Cautions in Relation to Alkylation-Based Protocols. *Antioxid. Redox Signal.* **33**, 1174-1189 (2020).
2. E. Doka, E. S. J. Arner, E. E. Schmidt, P. Nagy, "ProPerDP, a Protein Persulfide Detection Protocol" in Vascular Effects of Hydrogen Sulfide. Methods in Molecular Biology, J. Beltowski, Ed. (Humana Press, New York, NY, 2019), vol. 2007, pp. 51-77.
3. D. Garai, Z. Palinkas, J. Balla, A. J. Kettle, P. Nagy, "Measurements for Sulfide-Mediated Inhibition of Myeloperoxidase Activity" in Vascular Effects of Hydrogen Sulfide. Methods in Molecular Biology, J. Beltowski, Ed. (Humana Press, New York, NY, 2019), vol. 2007, pp. 179-203.
4. P. Nagy, G. Schwarz, S. Kopriva, Highlighted mechanistic aspects in the chemical biology of reactive sulfur species. *Br. J. Pharmacol.* **176**, 511-513 (2019).
5. K. Ono *et al.*, Redox chemistry and chemical biology of $H_2S$, hydropersulfides, and derived species: Implications of their possible biological activity and utility. *Free Radic. Biol. Med.* **77**, 82-94 (2014).
6. P. Nagy, Mechanistic chemical perspective of hydrogen sulfide signaling. *Methods Enzymol.* **554**, 3-29 (2015).
7. R. Greiner *et al.*, Polysulfides Link $H_2S$ to Protein Thiol Oxidation. *Antioxid. Redox Signal.* **19**, 1749-1765 (2013).
8. E. Doka *et al.*, Control of protein function through oxidation and reduction of persulfidated states. *Sci. Adv.* **6**, eaax8358 (2020).
9. E. Doka *et al.*, A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. *Sci. Adv.* **2**, e1500968 (2016).
10. P. Nagy *et al.*, Model for the exceptional reactivity of peroxiredoxins 2 and 3 with hydrogen peroxide: a kinetic and computational study. *J. Biol. Chem.* **286**, 18048-18055 (2011).
11. S. Stöcker, M. Maurer, T. Ruppert, T. P. Dick, A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nat. Chem. Biol.* **14**, 148-155 (2018).
12. N. Krishnan, C. Fu, D. J. Pappin, N. K. Tonks, $H_2S$-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci. Signal.* **4**, ra86 (2011).
13. A. K. Mustafa *et al.*, $H_2S$ Signals Through Protein S-Sulfhydration. *Sci. Signal.* **2** (2009).
14. D. H. Zhang *et al.*, Detection of Protein S-Sulfhydration by a Tag-Switch Technique. *Angew. Chem. Int. Edit.* **53**, 575-581 (2014).
15. T. Akaike *et al.*, Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. *Nat. Commun.* **8**, 1177 (2017).
16. V. Bogdándi *et al.*, Speciation of reactive sulfur species and their reactions with alkylating agents: do we have any clue about what is present inside the cell? *Br. J. Pharmacol.* **176**, 646-670 (2018).
17. C. L. Bianco *et al.*, The reaction of hydrogen sulfide with disulfides: formation of a stable trisulfide and implications for biological systems. *Br. J. Pharmacol.* **176**, 671-683 (2019).
18. J. Lin *et al.*, The Uptake and Release of Polysulfur Cysteine Species by Cells: Physiological and Toxicological Implications. *Chem. Res. Toxicol.* **32**, 447-455 (2019).
19. A.-T. Mellis *et al.*, The role of glutamate oxaloacetate transaminases in sulfite biosynthesis and H2S metabolism. *Redox Biol.* **38**, 101800 (2021).
20. H. A. Hamid *et al.*, Polysulfide stabilization by tyrosine and hydroxyphenyl-containing derivatives that is important for a reactive sulfur metabolomics analysis. *Redox Biol.* **21**, 101096 (2019).
21. B. D. Paul, S. H. Snyder, $H_2S$ signalling through protein sulfhydration and beyond. *Nat. Rev. Mol. Cell Biol.* **13**, 499-507 (2012).

22. E. A. Wintner *et al.*, A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. *Br. J. Pharmacol.* **160**, 941-957 (2010).
23. T. Ditroi *et al.*, Comprehensive analysis of how experimental parameters affect H2S measurements by the monobromobimane method. *Free Radic. Biol. Med.* **136**, 146-158 (2019).
24. V. Kozich *et al.*, Metabolism of sulfur compounds in homocystinurias. *Br. J. Pharmacol.* **176**, 594-606 (2019).
25. J. L. Wallace *et al.*, A proof-of-concept, Phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug. *Br. J. Pharmacol.* **177**, 769-777 (2020).
26. J. B. Kohl, A. T. Mellis, G. Schwarz, Homeostatic impact of sulfite and hydrogen sulfide on cysteine catabolism. *Br. J. Pharmacol.* **176**, 554-570 (2019).
27. V. Bogdandi *et al.*, Nitrosopersulfide (SSNO(-)) Is a Unique Cysteine Polysulfidating Agent with Reduction-Resistant Bioactivity. *Antioxid. Redox Signal.* 10.1089/ars.2020.8049 (2020).
28. M. M. Cortese-Krott *et al.*, The key bioactive reaction products of the NO/H2S interaction are S/N hybrid species, polysulfides, and nitroxyl. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E4651-4660 (2015).
29. L. Álvarez *et al.*, The reactions of hydropersulfides (RSSH) with myoglobin. *Arch. Biochem. Biophys.* **687**, 108391 (2020).