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Review Article

# Biochemical methods for monitoring protein thiol redox states in biological systems

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ABSTRACT

Oxidative post-translational modifications of proteins resulting from events that increase cellular oxidant levels play important roles in physiological and pathophysiological processes. Evaluation of alterations to protein redox states is increasingly common place because of methodological advances that have enabled detection, quantification and identification of such changes in cells and tissues. This mini-review provides a synopsis of biochemical methods that can be utilized to monitor the array of different oxidative and electrophilic modifications that can occur to protein thiols and can be important in the regulatory or maladaptive impact oxidants can have on biological systems. Several of the methods discussed are valuable for monitoring the redox state of established redox sensing proteins such as Keap1.



**Contents**



## 1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are catch-all terms encompassing a broad range of molecular entities that have potential to chemically oxidize biological molecules. Although in common use, we should be mindful that the individual molecular species that comprise ROS and RNS can be very different in their physicochemical properties that underlie biological responses occurring when this diverse array of species change their concentration. Thus, although we often classify this broad array of molecules together, this may not always be helpful when trying to understand the molecular events that underlie biological responses to these distinct entities. Cells

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utilize a diverse collection of oxidase enzymes to catalyze reduction–oxidation (redox) reactions in which electrons are passed from an electron donor source to molecular oxygen, so reducing it to form the ROS species superoxide, which can dismutate to form hydrogen peroxide ($H_2O_2$). Many contemporary studies focus on ROS generated from NADPH oxidase enzymes [1,2], as these enzymes have evolved to specifically generate superoxide that is functionally important. Whereas oxidant production by other oxidase enzymes involved in cell metabolism [3] can be secondary by-products which may not impact on protein function. ROS are also generated by uncoupled nitric oxide synthase (NOS) enzymes [4,5] and macrophages that utilize it in host defense [6], as well as by mitochondria when electrons become uncoupled from their electron transport chain and combine with molecular oxygen to generate superoxide [7,8].

An elevated level of oxidants within the cell (due to their increased synthesis or decreased antioxidant capacity that limits ROS scavenging) is often referred to as 'oxidative stress'. To many the term oxidative stress implicitly conveys the idea that ROS simply exert a detrimental impact on biological function. However oxidants are now known to have biological functions that are not injurious, and can be considered crucial to maintenance of homeostasis or adaptive signaling events that can limit injury. These biological responses triggered by changes in cellular oxidant concentration are commonly referred to as 'redox signaling'. In terms of oxidants causing oxidative stress, this was once considered to occur via uncontrolled oxidation of cellular biomolecules. However, another important factor in the pathogenesis of oxidant-mediated injury involves ROS dysregulating basal redox signaling pathways crucial for homeostasis, thus interfering with regulatory pathways important for the maintenance of health.

Cysteine residues are relatively uncommon in proteins compared to other amino acid, comprising only about ~2.3% of the human proteome [9]. The thiol (also known as mercaptan or sulfhydryl) –SH side chain of cysteine is susceptible to reaction with ROS or RNS species, giving rise to a range of oxidative post-translational oxidative modifications, as schematically presented in Fig. 1, that in some cases can functionally regulate the protein. At first glance this would perhaps be considered an unlikely mechanism of regulation, as an elevation in cellular ROS might be anticipated to non-selectively oxidize all manner of protein thiols, potentially triggering uncoordinated functional changes that manifest as dysfunction and development of disease. However, this concept ignores the fact that there is selectivity in the oxidative modification of protein thiols induced by ROS. This is because ROS, such as $H_2O_2$, are selective in the thiols they oxidize as a result of oxidants preferentially reacting with deprotonated ($-S^-$), nucleophilic thiolates with a low acid dissociation constant (p*K*a). Most cysteines thiols however have a p*K*a of 8–9, and so are almost fully protonated at physiological pH, making them much less reactive with oxidants and so not susceptible to oxidative modification and regulation in this way. In addition to the protein thiol p*K*a, which is lowered by proximity to proton accepting amino acids (histidine, lysine, arginine) or an increase in cytosol pH, susceptibility to oxidation may be controlled by their vicinity to oxidase enzymes.

Although protein cysteine oxidation is often mechanistically rationalized by the oxidant directly reacting with the thiol, this may not always be the case. For example, although protein-tyrosine phosphatase 1B (PTP1B) is susceptible to oxidation [10], it has been questioned how this can happen in the cellular setting [11]. This is because although the target thiol in PTP1B has a p*K*a of ~6.8 and is clearly susceptible to oxidation, it may be difficult to reconcile this happening when peroxiredoxin (Prx) proteins with very reactive low p*K*a thiols (~5.5) are present in high abundance. One possibility is that PTP1B (or other targets) only become oxidized after the Prx are oxidant-inactivated by hyper-oxidation of their peroxidatic thiols [12]. In addition, it is conceivable that a low p*K*a protein thiol such as those in Prx or thioredoxin (Trx) may become oxidized and then react with the less reactive target protein cysteine to 'pass on' the oxidation [3], as depicted in Fig. 2.

Diverse arrays of oxidative modifications are crucial to redox signaling events and are integral to all manner of cellular and

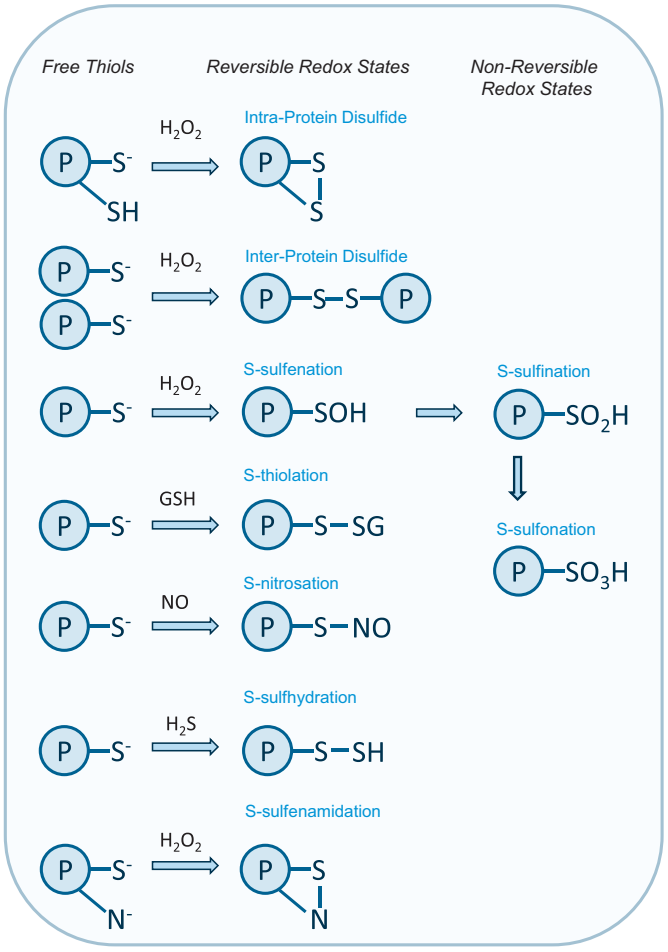


**Fig. 1.** Summary of the oxidative modifications formed in protein thiols. Protein thiols can form a variety of oxidative modifications, including reversible (intra-protein disulfides, inter-protein disulfides, S-sulfenation, S-nitrosation, S-thiolation, S-sulfhydration, S-sulfenamidation) and non-reversible hyper-oxidized (S-sulfination , S-sulfonation) redox states. Some redox states, such as S-sulfenation, S-nitrosation or S-sulfhydration, can be intermediates that transition to disulfides. Prolonged exposure to oxidants can result in irreversible modifications such as S-sulfination or S-sulfonation.

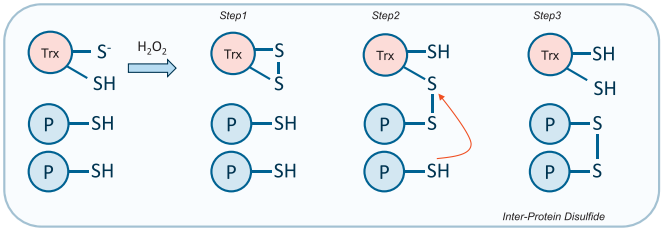


**Fig. 2.** Alternate potential mechanisms leading to disulfide formation. A protein thiol may be oxidized via another redox sensitive protein, in this example thioredoxin (Trx), first becoming oxidized. Trx has a lower p*K*a than most other proteins and so is more likely to be preferentially oxidized by $H_2O_2$ to form an intra-molecular disulfide. The Trx disulfide is then attacked by a thiol of a second protein with a higher p*K*a which is then reduced by a second thiol. Trx essentially picks-up and passes on the oxidation state to the less reactive target protein thiol.