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Review

# Quantification of thiols and disulfides ☆

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ABSTRACT

*Background:* Disulfide bond formation is a key posttranslational modification, with implications for structure, function and stability of numerous proteins. While disulfide bond formation is a necessary and essential process for many proteins, it is deleterious and disruptive for others. Cells go to great lengths to regulate thiol-disulfide bond homeostasis, typically with several, apparently redundant, systems working in parallel. Dissecting the extent of oxidation and reduction of disulfides is an ongoing challenge due, in part, to the facility of thiol/disulfide exchange reactions.

*Scope of review:* In the present account, we briefly survey the toolbox available to the experimentalist for the chemical determination of thiols and disulfides. We have chosen to focus on the key chemical aspects of current methodology, together with identifying potential difficulties inherent in their experimental implementation.

*Major conclusions:* While many reagents have been described for the measurement and manipulation of the redox status of thiols and disulfides, a number of these methods remain underutilized. The ability to effectively quantify changes in redox conditions in living cells presents a continuing challenge.

*General significance:* Many unresolved questions in the metabolic interconversion of thiols and disulfides remain. For example, while pool sizes of redox pairs and their intracellular distribution are being uncovered, very little is known about the flux in thiol-disulfide exchange pathways. New tools are needed to address this important aspect of cellular metabolism. This article is part of a Special Issue entitled Current methods to study reactive oxygen species — pros and cons.

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## 1. Introduction

### 1.1. Thiol-disulfide exchange

Thiol-disulfide exchange reactions play critical roles in many aspects of cellular function. In these reactions, a nucleophilic thiolate attacks one of the two sulfur atoms of the target disulfide bond (Fig. 1). Since the reactivity of the sulfhydryl group is dominated by that of its deprotonated form, we first briefly address aspects of the acidity ($pK_a$) and nucleophilicity of thiolates. The protonated forms of simple alkyl thiols are practically unreactive as nucleophiles under normal conditions; reacting some $10^{10}$-fold slower than their corresponding thiolates [1,2]. As the concentration of the thiolate is derived from the Henderson–Hasselbalch equation, the pH dependency of the reaction rate for thiol-disulfide exchanges is governed by the following equation for reaction kinetic relationship [3]:

$$k_{obs} = k/\left(1 + 10^{pKa-pH}\right).$$

Here $k_{obs}$ is the observed rate constant at a given pH, and k is the corresponding limiting rate constant for the thiolate at high pH values. Thus, $k_{obs}$ is one half of the limiting rate constant at the $pK_a$, but falls to $1/10^4$ of the maximal reactivity at 4 pH units below the $pK_a$.

Biological thiols show a very wide range of $pK_a$ values (from about 3 to 11, thus corresponding to an 8-order of magnitude shift in the deprotonation equilibrium [4]). The factors contributing to this profound modulation of thiol pKa's are under, which so profoundly modulate thiol pKa's, are under active investigation, and include solvation, electrostatic effects with neighboring charges and dipoles, as well as H-bonding interactions [5–7]. It is important to note that the $pK_a$ of thiols has two distinct effects on reactivity. Obviously, as noted above, a lower thiol $pK_a$ increases the fraction of thiol in its reactive thiolate form, however, the intrinsic reactivity of fully-formed thiolates (at the

*Abbreviations:* ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; CDAP, 1-cyano-4-dimethylamino-pyridinium; 4-DPS, 4,4′-dithiodipyridine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic) acid; EDTA, ethylenediamine tetraacetic acid; ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; HMD, heavy maleimide derivative; MBBr, Monobromobimane; ME, 2-mercato ethanol; MMTS, S-methyl methanethiosulfonate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethyleneglycol; SDS, sodium dodecylsulfate; TCEP, tris(2-carboxyethyl) phosphine; SBD-F, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate; THP, tris(2-hydroxyethyl) phosphine; TNB, 5-thio-2-nitrobenzoic acid

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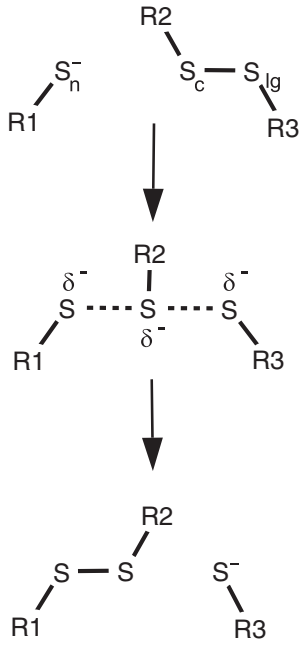


**Fig. 1.** Thiol-disulfide exchange. The attack of a thiolate (the nucleophile, S-n) on a disulfide bond takes place through a linear transition state where the central sulfur atom (Sc) will participate in a new disulfide bond and resolution of a new leaving group thiolate (S-lg). Which of the two sulfur atoms participating in the disulfide bond will eventually act as leaving group is dependent on steric, electrostatic and intrinsic acidity of the thiolate species involved.

high pH limit) typically declines with decreasing thiol $pK_a$ for a series of structurally-related thiols [8–10]. The ability of a thiol sulfur atom to retain a proton is to some extent a reflection of its intrinsic nucleophilicity, thus illustrating the correlation between nucleophilicity and $pK_a$.

Although it might seem unnecessary in terms of populating the thiolate, some enzymes have evolved to have $pK_a$'s far below the predominant pH of a typical cellular environment. Such low $pK_a$ values might, however, suppress oxidative side reactions that would otherwise compromise catalysis. Another reason is that marked differences in acidity allow the equilibrium constant for thiol disulfide exchange to be tuned by thiol $pK_a$ values. Thus, in the thiol-disulfide exchange reaction:

$$R1 - SH + R2 - S - S - R2 \rightleftharpoons R1 - S - S - R2 + R2 - SH$$

lowering the $pK_a$ of R2-SH with respect to R1-SH will improve the leaving-group properties of R2-SH and bias the equilibrium to the right [5,11].

Two consecutive thiol/disulfide exchange reactions accompany the overall redox reaction shown below:

$$R1 - S - S - R1 + 2\,R2 - SH \overset{K_{ox}}{\rightleftharpoons} 2\,R1 - SH + R2 - S - S - R2.$$

Knowing the stability of one disulfide, together with the magnitude of $K_{ox}$, allows the stability of the other disulfide to be directly calculated. Again the magnitude of $K_{ox}$ will be dependent on a combination of effects including steric, electrostatic and $pK_a$ values of the thiol species involved [12].

Finally, the rates of thiol-disulfide exchange reactions are influenced by the requirement for a linear arrangement of the three sulfur atoms in the transition state [13,14]. In proteins, the two sulfur atoms of the disulfide bonds often differ markedly in their accessibility to an attacking thiolate nucleophile generating a single mixed disulfide intermediate. In the event that both disulfide sulfur atoms are exposed, the outcome of disulfide exchange may largely reflect discrimination based on $pK_a$ values (see above).

### 1.2. Overall principles for thiol-disulfide detection and quantifications

Thiols are typically detected directly by virtue of their relatively high reactivity compared to most other common species in biological systems. Disulfides, on the other hand, have no strong chemical signature, and are hence most commonly detected after reduction to their corresponding thiols. Thus, the most common methodologies for thiol and disulfide quantification involve determination of free thiol concentration, followed by alkylation, reduction of disulfide bonds, and subsequent quantification of the additional exposed thiols. The processes of reduction and alkylation are thus pivotal for thiol quantification. In the determination of disulfides, the complete removal of the reducing species prior to detection is crucial so that no cross-reaction takes place between the reductant and the reagent used for thiol detection.

## 2. Quenching of thiol oxidation and exchange

### 2.1. Thiol alkylation

Alkylation of cysteine thiols with iodo, bromo or chloro substituted acetic acid or acetamide is a classic approach that has been exploited since the 1930's. The relative reaction rates between glutathione and these halogenated acetates are 100:60:1 for iodo-, bromo- and chloroacetates respectively [15]. Although iodoacetic acid or iodoacetamide are by far the most widely used haloalkanes for thiol alkylation, they can show significant reactivity towards other nucleophilic side chains. While such side-reactions may be fairly innocuous for most analytical applications, they become major problems in proteomic approaches involving the identification of reacted modified species by mass spectrometry. Thus iodoacetate treatment was shown to significantly modify lysine residues as demonstrated by mass spectroscopy [16]. The substitution of chloroacetic acid alleviated this problem but this solution cannot be adopted when thiol residues must be quenched rapidly.

Maleimides are very widely used reagents for the alkylation of thiols. The reaction represents a Michael addition of the thiolate on the electrophilic double bond of the maleimide (Fig. 2, Reaction A). The enone functionality of N-ethylmaleimide (NEM) shows an extinction coefficient of 620 $M^{-1}$ $cm^{-1}$ at 302 nm allowing reactions with nucleophiles to be conveniently followed spectrophotometrically [17]. A notable additional advantage of maleimides is that they react rapidly with thiols at neutral or slightly acidic pH values with rate constants that are some 3 to 4 orders of magnitude faster than iodoacetamide under comparable conditions [1,18,19]. Despite their utility, several reactions may complicate the use of maleimides, particularly at pH values above 7. Firstly, while maleimides are frequently characterized in the literature as irreversible thiol-modifying reagents, the adducts are subject to base-catalyzed reverse Michael reactions (Fig. 2, Reactions B and C) leading to the possible migration of the maleimide between thiol targets [20]. Further maleimide adducts, particularly those where N–R represents an aniline functionality, are prone to ring-open by hydrolysis, yielding the isomeric products shown in Fig. 2, Reaction D [21]. Such ring-opening reactions have been used to identify maleimide-labeled peptides [22,23]. In aggregate, these secondary reactions may play an important role in modulating the stability of maleimide conjugates in vivo [20,21].

Mammalian cultured cells are permeable to NEM and this has encouraged its use for quenching thiols in intact cells. However, the inclusion of a denaturant, such as SDS, may be necessary to ensure rapid labeling of all free cysteine residues because about 20% of total cellular protein thiols are not susceptible to modification by NEM under native conditions [24].

Vinyl pyridine, like NEM, reacts with thiols at the double bond and was previously widely used. Since vinyl pyridine reacts more than

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