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The Role of Sulfenic Acids in Cellular Redox Signaling: Reconciling Chemical Kinetics and Molecular Detection Strategies

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Abstract

The reversible oxidation of protein cysteine residues is well recognized as an important regulatory mechanism in redox-dependent cell signaling. Cysteine oxidation is diverse in nature and involves various post-translational modifications (sulfenic acids, disulfides, etc.) and the specific functional or structural impact of these specific oxidative events is still poorly understood. The proximal product of protein cysteine oxidation by biological reactive oxygen species (ROS) is sulfenic acid (Cys-SOH), and experimental evidence is accruing for the formation of Cys-SOH as intermediate in protein cysteine oxidation in various biological settings. However, the plausibility of protein Cys-SH oxidation by ROS has often been put in question because of slow reaction kinetics compared to more favorable reactions with abundant thiol-based reductants such as peroxiredoxins (Prx) or glutathione (GSH). This commentary aims to address this controversy by highlighting the unique physical properties in cells that may restrict ROS diffusion and allow otherwise less favorable cysteine oxidation of proteins. Some limitations of analytical tools to assess Cys-SOH are also discussed. We conclude that formation of Cys-SOH in biological systems cannot always be predicted based on kinetic analyses in homogenous solution, and may be facilitated by unique structural and physical properties of Cys-containing proteins within e.g. signaling complexes.

1. Introduction

The reversible oxidation of protein cysteine residues in biological redox signaling is now widely recognized.[1–3] Cysteines are relatively underrepresented in the mammalian proteome and the sulfur atom in cysteine has wide-ranging chemical reactivity. Moreover, the apparent simultaneous evolution of increased cysteine usage in proteins in complex multicellular organisms [4] with the appearance of multiple isoforms of NADPH oxidases (NOX) and regulatory factors [5, 6] suggests the importance of reversible cysteine oxidation

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Conflicts of Interest

YJH and AVDV are coinventors on U.S. Patent No. 8,053,243 “Detections of Nitrosylated Proteins”; YJH holds U.S. Patent No. 8,877,447: “Detection of Glutathionylated Proteins”.

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as a major mechanism of redox signaling by reactive oxygen species (ROS) generated by NOX enzymes and/or by respiring mitochondria. However, experimental studies of redox-based signaling have been plagued by technical limitations of (bio)chemical approaches to selectively and quantitatively demonstrate specific redox events in biological systems in relation to functional outcomes.

The first step in the reversible oxidation of cysteine thiols (Cys-SH) by e.g. NOX-generated ROS is their initial oxidation to a sulfenic acid (Cys-SOH; Figure 1).[7, 8] This process, termed sulfenylation, is mediated by two-electron oxidation by various biological ROS, including hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), peroxynitrite (ONOO^-), and hypohalous acids (HOX), and potentially involves locally catalyzed mechanisms by metal ions.[9] The kinetics by which Cys-SH is oxidized is highly variable and is not simply dictated by the $\text{p}K_a$ of the involved Cys residue.[10–12] Second-order rate constants of H_2O_2 -dependent Cys-SH oxidation range dramatically from 10^{-3} to $10^8 \text{ M}^{-1}\text{s}^{-1}$, implying that adjacent or surrounding amino acids or other factors are capable of extensively tuning the reactivity of protein Cys-SH.[9] Similarly, the chemical properties of protein Cys-SOH species are diverse, including both nucleophilic as well as electrophilic properties, and are likely responsible for highly variable stability of this intermediate within diverse proteins.[8, 13] In general, Cys-SOH species readily react with reduced thiols to form intra- or inter-molecular disulfides that are essential for protein tertiary structure [14] as well as protein-protein interactions, respectively, or with low-molecular weight thiol compounds such as glutathione (GSH) to form mixed disulfides (*S*-glutathionylation; Cys-SSG), which may be catalyzed by glutathione *S*-transferases (GST) or other thiol transferases.[15, 16] Cellular thioredoxins (TRX) or glutaredoxins (GRX) in turn reduce these disulfide states to the initial Cys-SH, thus completing this reversible redox signaling cycle.[17, 18] In some cases, Cys-SOH can also be further oxidized to sulfinic (Cys-SO₂H) and sulfonic (Cys-SO₃H) acids, representing more irreversible modifications that may mediate redox-dependent signaling mechanisms under stress conditions (Figure 1).[19, 20]

Our understanding of protein cysteine oxidation is largely based on two types of experimental studies. First, a range of biochemical derivatization strategies or *in situ* imaging approaches have been developed with the goal to detect Cys-SOH or other intermediate oxidized forms in specific proteins, in attempts to relate these observations to altered cell biology due to ROS. These approaches typically rely on the assumption that detection probes used to visualize intermediate Cys-SH oxidations outcompete their reactions with other endogenous targets and at the same time are selective for specific intermediate oxidized forms. Given the aforementioned wide diversity in reactivity of protein cysteines and their oxidized forms, this presents a daunting challenge. As a second approach to address protein cysteine oxidation, mechanistic and kinetic studies with purified proteins have aimed at assessing the relative reactivity of various protein cysteines towards ROS as well as the structural and functional consequences of oxidative modifications. One of the major controversies in the field of redox signaling arises from the fact that experimental detection of Cys-SOH within a wide range of proteins in intact biological systems [21–23] is difficult to reconcile with the limited reactivity of most of these proteins towards e.g. H_2O_2 compared to highly reactive H_2O_2 -metabolizing proteins (Prx), or with their limited cellular abundance compared to similarly reactive abundant low-molecular

weight thiols such as GSH. Since intermediate formation of Cys-SOH is being recognized as a functionally critically intermediate in redox-dependent regulation of e.g. tyrosine kinases [24, 25], it is important to reconcile this issue. In the following paragraphs, we will briefly discuss some of the concerns regarding the selectivity of certain derivatization strategies [26, 27], and point out important considerations with respect to extrapolating experimental findings from biochemical kinetic studies with isolated proteins conducted in the test tube to intact cellular contexts.

2. Reaction of H₂O₂ with thiol proteins: reaction kinetics versus cell-based studies

Approaches with various Cys-SOH-reactive probes with different chemical reactivity and specificity have indicated widespread formation of Cys-SOH in cellular systems in response to e.g. NOX activation.[13, 28, 29] However, estimated second-order rates of H₂O₂ with most protein cysteines are slow, especially compared to much more rapid reaction rates with known H₂O₂-metabolizing enzymes, such as peroxiredoxins (Prx), glutathione peroxidases (Gpx), and catalase.[30, 31] For example, the protein tyrosine phosphatase PTP1B, a well-known redox-sensitive protein that is regulated during redox signaling [1, 32, 33], contains a catalytic Cys-SH within its active site that reacts with H₂O₂ with a second-order rate of $\sim 10 \text{ M}^{-1}\text{s}^{-1}$, many orders of magnitude slower than similar reaction of H₂O₂ with e.g. Prxs which reduce H₂O₂ with a rate of $\sim 10^7\text{--}10^8 \text{ M}^{-1}\text{s}^{-1}$. [34] Also, the thiolate form of cellular GSH (GS⁻), which reacts with H₂O₂ at rates similar to PTP1B, would likely outcompete most protein targets for direct reaction with H₂O₂ because of its estimated abundance in cells (up to 50 μM). [26, 35] Based on these considerations, it has been argued that direct reaction of H₂O₂ with most cellular protein cysteines is unlikely, and that NOX-dependent redox signaling must largely involve indirect mechanisms, such as relay mechanisms via initial direct oxidation of highly reactive targets such as Prx. [30, 36, 37] However, such considerations fail to adequately explain the increasing experimental evidence of Cys-SOH formation in many cellular proteins.

One important intrinsic flaw in efforts to extrapolate kinetic data obtained in homogenous aqueous systems in the context of cell biology is that they are typically based on estimated average concentrations of cellular reactants (e.g., H₂O₂ and its putative protein targets) and assumptions that reactions are not restricted by diffusion. [35] This is problematic as redox signaling events initiated by e.g. NOX activation typically occur within localized cell regions, as supported by experimental evidence indicating that H₂O₂ signaling is highly confined near the plasma membrane or within endosomes, depending on where NOX is by activated. [38] Indeed, cell signaling typically involves formation of scaffolded macromolecular signaling complexes in unique microenvironments, such as lipid rafts or caveolae (e.g. [39–41]), and the activation of NOX enzymes within such signaling scaffolds generates ROS within a local environment that is highly dissimilar from homogeneous aqueous solutions. [42, 43] Moreover, recruitment of redox-sensitive protein Cys-SH targets towards such NOX-containing signaling complexes during activation, as demonstrated in several experimental studies [25, 32, 44, 45], would enhance the competitiveness of such Cys-SH targets compared to bulk concentrations of e.g. GSH or other reactive cell targets for

locally generated H_2O_2 , even if it needs to first pass a membrane through an aquaporin channel.[46, 47] Such localized reactions are further facilitated by restricting passive diffusion within such signaling complexes (see below). In fact, the “concentrations” of H_2O_2 or a given protein target within a signaling complex is impossible to define without knowledge of the “volume” in which H_2O_2 is allowed to diffuse, but are likely much greater than estimated cellular concentrations of specific proteins, or even the highest estimates of steady-state cellular H_2O_2 concentrations (e.g. [24]), and will enable even kinetically less favorable reactions.

Although structural data defining specific cellular signaling complexes are not known, it can be hypothesized from the considerations above that selective Cys-SH oxidation in a signaling complex is a controlled kinetic process, and the factors that control the kinetics of H_2O_2 -dependent oxidation of a Cys-SH residue are highlighted in the following general bimolecular reaction rate expression (Equation 1) [48]:

$$-\partial[H_2O_2]/\partial t = 4\pi r^2 \Delta r \nu \rho [H_2O_2] [Cys-SH] \exp\left(\frac{-E_a}{RT}\right) \quad (\text{Equation 1})$$

where E_a reflects the activation energy, $4\pi r^2$ denotes a spherical volume with radius r , ν is the frequency of collision, ρ takes into account steric effects, R is the gas constant, T is temperature, and $[H_2O_2]$ and $[Cys-SH]$ denote the concentrations of H_2O_2 and protein target Cys-SH, respectively. To best understand the differences between cell-based and homogeneous solution kinetics, is it important to consider how these various parameters may vary between homogenous aqueous solutions and intracellular environments (Table I). For example, reaction of H_2O_2 with a protein Cys-SH in a homogeneous solution will depend predominantly on relative concentrations of $[H_2O_2]$ and $[Cys-SH]$ with relatively little impact from solvent-dependent collisions or diffusion. As mentioned above, localized production of H_2O_2 in a signaling complex would result in higher local “concentrations” (or molar equivalents) of H_2O_2 near the target protein Cys-SH, which would result in enhanced reaction rates compared to expected rates based on global cell concentrations of these reactants. As is also evident from Equation 1, localized production of H_2O_2 near a target Cys-SH within a signaling complex can be expected to enhance collision frequency (ν), and also alter steric effects (ρ), although this is largely speculative in the absence of structural data. It is also possible that protein-protein interactions in a signaling complex, or other post-translational modifications occurring in such a complex, induce structural alterations that affect the reactivity of Cys-SH (by lowering pK_a or transition state energies) towards H_2O_2 . Nevertheless, based on these considerations, H_2O_2 -mediated protein Cys-SH oxidation within controlled environments, such as a signaling complex in which NOX is activated, may be much faster than is estimated from measurements in homogeneous solutions in which reactivity is determined by reactant concentrations and molecular diffusion.[48, 49] Indeed, estimates based on second-order kinetics from homogenous systems are too simplistic for understanding (bio)chemical events within such highly localized cell regions, and cannot necessarily predict localized redox reactions within specialized cell regions in which H_2O_2 is generated.

Another aspect that is frequently overlooked in biochemical studies is that the biophysical properties of intracellular regions differ widely from aqueous solutions, even though they consist primarily of water (60% by volume, but > 90% based on molecular number). In fact, cells possess gel-like properties in which water molecules are more structurally ordered due to extensive interactions with lipid membranes and cell proteins,[50] which has profound implications for diffusion of cellular constituents which is highly different from that in aqueous solution. This applies primarily to proteins and ions but also to neutral small solutes such as H₂O₂. Indeed, experimental studies with polyvinyl alcohol hydrogels have shown that diffusivity of H₂O₂ within such hydrogels is about 4–5 times lower compared to aqueous solution.[51] Based on this, the gel-like properties within cells would be expected to limit diffusion of H₂O₂ thereby allowing for enhanced local concentrations near its production site. Analogous to the structural organization of water molecules due to interactions with e.g. protein surfaces [50], the similar physical properties of H₂O₂ would suggest that its intracellular transport may be governed largely by dynamic interactions with proteins rather than by passive diffusion. Similarly, transmembrane transport of H₂O₂ is likely not mediated passive diffusion but rather by interactions with aquaporins that mediate H₂O₂ transmembrane passage.[52, 53]

Modeling studies of cellular distribution of H₂O₂ or its reactions with target proteins (e.g [54, 55]) suggest that these are confined primarily by the presence of abundant antioxidant systems (based on reaction kinetics), but the aforementioned considerations indicate that cellular distribution of H₂O₂ may also be controlled by dynamic protein interactions that govern transmembrane passage and restrict intracellular diffusion, which is typically not considered. Based on these considerations, we propose that mechanisms of cellular redox signaling cannot be fully explained based on aqueous diffusivity and reaction kinetics based on measurements in homogeneous aqueous solutions. Instead, we propose an alternative model of NOX-dependent redox signaling, in which proteins are oxidized not merely based on their overall abundance or relative reactivity, but rather in a more sequential manner based largely on H₂O₂ production rate and recruitment of protein targets to a signaling complex (Figure 2). For example, considering a situation in which NOX is activated within a signaling complex in response to e.g. growth factor signaling, a first molecule (equivalent) of H₂O₂ will react with its most proximal and reactive target (e.g. Prx), after which a second (equivalent) H₂O₂ molecule is capable of oxidizing the next available target and so on. This mechanism ensures confined protein Cys-SH oxidation and avoids random and unwanted oxidative events at more remote regions, and also suggests that the extent of oxidative modification of different protein cysteines and sphere of protein oxidation can be controlled by regulating the rate and/or duration of NOX-dependent H₂O₂ formation, as well as by dynamics of protein target recruitment and activation of cellular reducing systems. Notably, even though Prx may form a major initial target because of their highly favorable kinetics and cellular abundance, their initial oxidation may not only control redox signaling by potential relay mechanisms [56, 57], but their transient inactivation may also allow for additional localized oxidative events depending on the strength and duration of the oxidant signal, and the presence of reducing systems that regenerate Prx.[58, 59] Hyperoxidation of Prx by a “second hit” of H₂O₂ would extend Prx inactivation, thus allowing for additional oxidative modifications of other nearby proteins. In addition, local depletion of Prx reducing

systems within such a signaling complex may also control the sphere of H₂O₂-mediated oxidative events. Conversely, this model also predicts that cellular oxidative events by *unregulated* H₂O₂ generation (e.g. from exogenous sources or due to redox cycling of toxic chemicals) are highly different, as such randomly generated H₂O₂ will primarily react with the most reactive and abundant antioxidant targets (e.g. Prx, Gpx, catalase) based on their highly favorable reaction kinetics, to avoid oxidative modifications of other critical proteins. In fact, the relatively limited reactivity of cysteine-containing protein targets involved in cell signaling may have evolved purposely to avoid their undesirable oxidation by such unregulated H₂O₂ production.

3. Chemical detection of sulfenic acid: is it in fact sulfenyl amide?

The previous section argues that H₂O₂-mediated oxidation of protein substrates may be much more feasible in specific cases than would be predicted based on second order reaction kinetics. A full understanding of thiol-based redox signaling requires the precise detection of intermediates generated during thiol-based redox signaling (Figure 1) and development of adequate molecular tools to probe their abundance in biological systems.[2, 60] A variety of diverse molecular probes have been generated to detect reduced Cys-SH,[61, 62] and various oxidized forms including Cys-SOH,[21, 28], Cys-SO₂H,[63, 64] as well as Cys-SSG,[65] which were in some cases also validated using alternative approaches (e.g. mass spectrometry, antibody-based approaches). However, because of the chemical diversity of protein Cys-SOH,[9] selective and quantitative detection of Cys-SOH intermediates is highly complicated. Currently, many investigators employ chemical probes containing a 1,3-diketone scaffold (e.g. dimedone) to selectively conjugate to Cys-SOH moieties.[21, 66] A major concern with these reagents is their sluggish reactivity with Cys-SOH species, thus potentially minimizing sensitivity and limiting the ability to quantitatively assess Cys-SOH formation, and considerable efforts are ongoing to develop more reactive sulfenic acid-specific molecules.[13, 67, 68] However, quantitative analysis of Cys-SOH, even with the fastest possible reagents, may not be feasible in cases where subsequent disulfide formation is kinetically favorable (e.g. Figure 1), and attempts to quantitatively capture of Cys-SOH could also potentially perturb the cellular signaling processes and thereby introduce artifacts. Also, although these compounds are typically minimally reactive with Cys-SH, their reactivity towards other oxidized forms of cysteine is not always known and difficult to fully rule out because of the high diversity of chemical reactivity of protein cysteines and their oxidized forms.

One fate of intermediate formation of Cys-SOH is its cyclization with the protein backbone amide to form a sulfenyl amide (Figure 1), which has been identified crystallographically in e.g. oxidized PTP1B.[69, 70] Such sulfenyl amide formation not only minimizes susceptibility to more irreversible oxidation (to Cys-SO₂H and Cys-SO₃H) but may also induce or eliminate structural alterations that may be relevant in a redox-dependent response, such as redox-dependent regulation of tyrosine kinase activity [25]. The molecular factors that control formation of sulfenyl amides are still unclear, but recent computational studies suggest that a constrained cysteine conformation within a β -loop-helix fold motif favors sulfenyl amide formation from initially generated Cys-SOH.[71] Importantly, recent studies using model sulfenyl amide compounds indicated that several dimedone-based reagents used

to detect Cys-SOH are also reactive towards sulfenyl amides [26, 72], indicating that detection of dimedone-reactive proteins does not necessarily reflect the presence of Cys-SOH but may also indicate sulfenyl amides. Therefore, dimedone or chemically similar compounds are best considered molecular probes of for “two-electron oxidized cysteine species” representing either Cys-SOH or sulfenyl amides. Since both yield identical products with e.g. dimedone, additional strategies (e.g. structural analysis) would be necessary to distinguish them.

Because of the limited reactivity of H₂O₂ with most cysteine-containing proteins (see previous section), it was recently suggested that observations of dimedone reactivity within e.g. PTP1B likely reflects sulfenyl amides rather than sulfenic acids, and that such sulfenyl amide formation may involve alternative mechanisms.[26] One such mechanism was proposed to involve initial formation of Cys-SSG and elimination GSH to generate sulfenyl amide [26], which is essentially the reverse reaction of the more widely appreciated reaction of sulfenyl amides with GSH to form Cys-SSG and facilitate cellular reduction of such cysteine modification.[73] While some experimental evidence has been presented to support such a mechanism [26], it would require the absence of GSH, which is unlikely in non-stressed cellular conditions. In an attempt to explain the biological formation of Cys-SSG independent of e.g. intermediate Cys-SOH, it has been proposed that this may be mediated by GSSG [26, 73]. However, for most proteins such a mechanism would require dramatic changes in GSH:GSSG ratios to achieve significant changes in *S*-glutathionylation, which are deemed unlikely even under conditions of artificial oxidative stress [73]. For example, glutaredoxins (GRX), known for their ability to deglutathionylate proteins [18, 73], can also catalyze the reverse reaction in the presence of excess GSSG, but this would again require dramatic changes in GSH:GSSG ratios to allow for this reaction to go in the reverse direction. It has similarly been suggested [26] that glutathione *S*-transferase π 1 (GSTP1), an enzyme that was recently found to catalyze *S*-glutathionylation of various target proteins [74], might do so by utilizing GSSG. GSTP1 has a number of domains important to its function, including a G domain is important for the activation of GSH and an H-domain important in its ligandin (protein-protein interaction) function [75]. The precise mechanisms of GSTP1-catalyzed *S*-glutathionylation are not fully understood, but the catalytic activity of GSTP1 and the presence Tyr-7 within its active site are believed to be critical in promoting GSH-mediated *S*-glutathionylation of target proteins as well as GSTP1 itself [75]. It is not easy to conceive how GSSG would fit within the GSH-binding pocket of the enzyme and, to our knowledge, there is no definitive experimental evidence to indicate that GSTP1 can directly utilize GSSG to glutathionylate target proteins. Moreover, contrary to suggestions that GSTP1 and GRX can both similarly promote *S*-glutathionylation of target proteins in the presence of GSSG, experimental studies typically demonstrate opposing actions of these enzymes on protein *S*-glutathionylation in biological systems (e.g.[76, 77]). Although the importance of GSSG-driven mechanisms in promoting protein *S*-glutathionylation may be unlikely, they are difficult to rule out completely, since localized oxidative events (as highlighted in the previous section) might conceivably generate localized conditions that could allow GSSG-driven *S*-glutathionylation reactions. However, current experimental evidence overwhelmingly suggests that formation of *S*-glutathionylated proteins during physiological redox signaling (via e.g. NOX activation) proceeds via initial formation of

Cys-SOH (or related oxidized intermediates) as determined by dimedone-based probes. Indeed, NOX-dependent *S*-glutathionylation of target proteins is commonly preventable in the presence of dimedone (e.g. [25, 78, 79]), and accompanied by formation of detectable dimedone adducts (e.g. [80]). While concerns may remain regarding the selectivity of dimedone-like reagents for sulfenic acids, such observations are consistent with the notion that protein *S*-glutathionylation occurs via initial formation of Cys-SOH (or sulfenyl amides).

4. Concluding remarks and challenges for future research

The diverse landscape of intermediates formed by the oxidation of protein cysteine residues indicates the involvement of elaborate molecular mechanisms that control their formation, stability, and influence on protein structure and function. In spite of major technical challenges to quantitatively detect them, current evidence strongly supports the ability of H₂O₂ (or related oxidants) to promote cysteine oxidation by initial formation of Cys-SOH species (and/or sulfenyl amides), especially in the context of highly localized biological redox signaling pathways that control cell differentiation, proliferation, motility, etc. Nevertheless, the precise functional consequences of Cys-SOH or other cysteine modifications remain incompletely understood, and because of their wide-ranging stability and reactivity, quantitative detection of Cys-SOH will presumably require unique detection approaches depending on the protein in question, as well as complementary approaches (e.g. mass spectrometry, molecular dynamics) to gain better insight into the functional significance of this cysteine modification. Analysis of reaction kinetics with purified recombinant proteins in combination with enzymology and structural biology will remain to be highly informative, but we recommend extreme caution in interpreting such findings in the context of cell biology without a firm knowledge local physicochemical properties in specific cellular areas that are distinct from aqueous solutions, and where chemical reactions are largely dictated by proximity-based mechanisms rather than passive diffusivity. When conclusions and predictions are made regarding biological relevance, it is imperative that these are based upon the use of probes or reagents applicable in these settings. Thoughtful integration of experimental findings from various research disciplines (chemical kinetics, biophysics, biology etc.) will be essential to gain a fuller appreciation and understanding of biological redox signaling and to target these redox-based events for therapeutic purposes. Based upon the progress made in this area in recent years, including the development of new probes and the increasing appreciation of redox perturbations in chronic inflammatory diseases, cancers, etc., addressing these challenges will represent a worthwhile quest.

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Figure 1. Oxidized cysteine thiol intermediates involved in ROS-mediated redox signaling

Biological oxidants [O], such as H₂O₂, oxidize cysteine thiols (Cys-SH) to sulfenic acids (Cys-SOH). Cys-SOH can react with reduced thiols (RSH) to form disulfide bonds (promoted by GST when R = glutathione) or with adjacent amide bonds to form sulfenyl amides, which can also react with thiols to produce disulfides, can be reduced back to Cys-SH by thioredoxin/glutaredoxin systems (TRX, GRX). In the presence of excess oxidant, Cys-SOH can be oxidized further to irreversible sulfinic (Cys-SO₂H) and sulfonic (Cys-SO₃H) acids.

Figure 2. Proposed model for protein cysteine oxidation by NOX-derived H_2O_2

Activation of NOX within an assembled signaling complex will first result in oxidation of the most proximal and reactive protein Cys-SH (e.g. Prx) by the first H_2O_2 equivalent, after which subsequent H_2O_2 equivalent(s) can oxidize additional protein target(s) within the signaling complex or accumulate or reach more distant targets. The sphere of redox signaling will be controlled in part by regulating the strength and duration of NOX activation as well as reducing mechanisms that reverse cysteine oxidation.

Table 1

Differences between aqueous solutions and intracellular environments that impact on kinetics of H₂O₂-mediated protein Cys-SH oxidation.

Factor Influencing Rate	Aqueous Solution	Intracellular Environment
H₂O₂ Production	Fixed concentrations based on steady-state concentrations	Highly dynamic and localized (depending on e.g. NOX activation); non-steady state
H₂O₂ Localization	Homogenous distribution; random generation (e.g. chemical or enzymatic systems)	Highly localized in distinct cell regions (e.g. near plasma membrane or within endosomes) in case of NOX activation
Target Protein	Homogenously distributed	Uneven distribution due to interactions with membrane lipids or other proteins; enriched in signaling complexes by active recruitment
Solvent properties	Aqueous solution; diffusion coefficient (diffusivity) depends on temperature, viscosity, molecule size	Gel-like properties of cells or cell regions affect viscosity and may minimize diffusivity
Collision Frequency	Determined primarily by passive diffusion	Molecular collisions may be enhanced in signaling scaffolds, due to high local concentrations and interactions with carrier proteins that affect diffusion
Steric effects	Minimal and well-known in homogenous solution	Less well defined in protein complexes, may be altered due to protein-protein interactions
Activation Energy	Determined by protein structure in solution	Structural alterations in a signaling complex, due to e.g. protein-protein interactions or interactions with membranes, may affect transition states