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Signaling Functions of Reactive Oxygen Species†

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Abstract

We review signaling by reactive oxygen species, which is emerging as a major physiological process. However, among the reactive oxygen species, H_2O_2 best fulfills the requirements of being a second messenger. Its enzymatic production and degradation, along with the requirements for the oxidation of thiols by H_2O_2, provide the specificity for time and place that are required in signaling. Both thermodynamic and kinetic considerations suggest that among possible oxidation states of cysteine, formation of sulfenic acid derivatives or disulfides can be relevant as thiol redox switches in signaling. In this work, the general constraints that are required for protein thiol oxidation by H_2O_2 to be fast enough to be relevant for signaling are discussed in light of the mechanism of oxidation of the catalytic cysteine or selenocysteine in thiol peroxidases. While the nonenzymatic reaction between thiol and H_2O_2 is, in most cases, too slow to be relevant in signaling, the enzymatic catalysis of thiol oxidation by these peroxidases provides a potential mechanism for redox signaling.

There are multiple components of signaling by ROS[1] that are the subject of current investigations: the pathways in which ROS participate, the endogenous sources of ROS, the generation of secondary species that participate in signaling, and how ROS affect signaling and eventually gene expression. While these are all important, this article will focus on the chemical reactions through which ROS could act as signaling molecules. More emphasis will be given in this work to signaling by H_2O_2, which alone, among ROS, clearly fulfills all the chemical and biological requirements to be a “second messenger”.

Reactions of ROS similar to those occurring in signaling can be relevant as mechanisms of pathology and toxicology and in the hormetic response in which nonlethal toxicity results in adaptive responses. Cyclooxygenase requires hydroperoxides derived from ROS for its activation that leads to the production of prostaglandins (1). Also, the initiation of metal-catalyzed lipid peroxidation requires ROS and then hydroperoxides and results in production

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[1]Abbreviations: ASK1, apoptosis signaling kinase 1; Cys-GPxs, cysteine-dependent glutathione peroxidases; $HO^{\bullet}$, hydroxyl radical; C_p, peroxidatic cysteine; C_r, resolving cysteine; GPxs, glutathione peroxidases; GSTP, glutathione *S*-transferase π; NOX, NADPH oxidase; Prxs, peroxiredoxins; PTP1B, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; Sec-GPxs, selenocysteine-dependent glutathione peroxidases; Trx, thioredoxin.

of isoprostanes (2) and α,β-unsaturated aldehydes (3). The prostaglandins and isoprostanes cause signaling through G-protein-coupled receptors, while low concentrations of α,β-unsaturated aldehydes can activate several signaling pathways (4–7). However, as the production of these compounds is an indirect effect of ROS, they will not be considered further.

In the 1970s, a few studies noted that exogenously added H_2O_2 could mimic growth factor activity and that the growth factors could stimulate in cells the endogenous production of H_2O_2 (8–10). Nonetheless, it was not until studies in the late 1980s and early 1990s showed that low concentrations of exogenous H_2O_2 added to cells caused proliferation (11) and that exogenous H_2O_2 added to T-cells activated NF-κB (12) that a role of H_2O_2 in signaling became noticed by researchers outside of a small community of free radical experts. Although NF-κB activation in stimulated macrophages by endogenously generated H_2O_2 was then demonstrated (13), it was not until the existence NADPH oxidases of nonphagocytic cells was discovered (14–16) that physiological signaling by reactive oxygen species became a major topic of interest.

Concepts of how H_2O_2 participates in signaling have involved studies in which it has been proposed that the ability of enzymes to remove H_2O_2 must be overcome (the “floodgate” hypothesis that will be discussed later) while the signaling reactions by H_2O_2 occur nonenzymatically. Unfortunately for the field, these studies did not pay attention to the very important fact that H_2O_2 reacts slowly with protein thiols, the principal targets in H_2O_2-dependent signaling. Thus, a rigorous discussion about the biochemical logic of redox transitions caused by H_2O_2 is needed. The kinetics of enzymatic and nonenzymatic H_2O_2 reactions will, therefore, be the major focus of this work.

SUPEROXIDE, HYDROXYL RADICAL, AND SINGLET OXYGEN ARE NOT SECOND MESSENGERS

A crucial feature of a second messenger is having specificity in its interaction with effectors in signaling pathways. The specificity of signaling by ROS is defined by both kinetics and spatial relationships. Unfortunately, the kinetics of the reactions of ROS with potential targets, in competition with the enzymes that remove them, has often not been given due consideration.

Superoxide oxidizes thiols to thiyl radical, which can initiate a chain reaction, but the rate constants for this reaction are quite slow, probably no more than 10^3 M^{-1} s^{-1} at pH 7.4 (17). Indeed, the oxidation of thiols by superoxide is more likely a reaction with the protonated $HO_2^{\cdot}$ (the pK_a for $O_2^{\cdot-}$ is 4.7) than with $O_2^{\cdot-}$ itself. So at pH 7, the rate constant for oxidation of thiols by $O_2^{\cdot-}$ is insignificant in comparison to the rate constants (>10^9 M^{-1} s^{-1}) for the cytosolic and mitochondrial superoxide dismutases (18) that are abundant in their compartments. When $O_2^{\cdot-}$ is generated on the outside of cells, such as by the NADPH oxidase isoform, NOX2, in endothelial cells, it appears to be able to enter cells, resulting in signaling (19); however, once inside the cell, it would be rapidly dismuted to H_2O_2 and O_2. Therefore, although a role for $O_2^{\cdot-}$ in signaling has been proven, targets that react with $O_2^{\cdot-}$

have not been demonstrated to do so in vivo. The signaling role of $O_2^{\cdot -}$ is more likely as a precursor of H_2O_2.

Hydroxyl radical has also been thought to be involved in signaling (20); however, $HO^{\bullet}$ has no specificity as it reacts with almost any organic molecule with rate constants near the limit of diffusion. Some of the products of lipid peroxidation, initiated by different free radicals, including $HO^{\bullet}$, such as the isoprostanes can signal through receptors (4), while others, such as 4-hydroxy-2-nonenal, can participate in signaling by modifying signaling proteins, such as Keap1 (21) or SHP-1, a protein tyrosine phosphatase (22). However, this is not evidence of a direct role of $HO^{\bullet}$ as a second messenger.

Singlet oxygen is produced in cells by nonenzymatic reactions, such as photoactivation of some drugs and the reaction of hypohalous acids with H_2O_2. Studies with heme peroxidases suggest either a very low yield by prostaglandin G2 reductase (23) or a high yield in the chloroperoxidase-catalyzed oxidation of halides. The site and time specific generation of singlet oxygen in cells, which would be required for it to fit the definition of a second messenger, has not been demonstrated and seems very unlikely. Regardless, although singlet oxygen is a potent dienophile, no normal physiological process for biological oxidation by singlet oxygen has been discovered, which also precludes it as a second messenger.

HYDROGEN PEROXIDE FITS THE CRITERIA TO BE A SECOND MESSENGER

What qualifies H_2O_2 as a suitable second messenger are its enzymatic production and degradation that provide specificity for time and place and its interesting chemistry that provides specificity for oxidation of thiols. The way H_2O_2 is produced in nature is remarkably similar to the modern industrial process for the production of tons of it. In the industrial process, molecular dioxygen is reduced during autoxidation of anthrahydroquinone, which is reduced back by hydrogen in the presence of a metal catalyst. In nature, the redox moiety of the flavin in aerobic dehydrogenases transfers two electrons to oxygen and is thereafter reduced back by hydrogens extracted from the reduced substrate of the enzymatic reaction. Superoxide anion is the one-electron transfer intermediate in the reaction, and in some cases, such as in xanthine oxidase or during redox cycling of quinones reduced by microsomal electron transport systems, it can be released as the final product. Another relevant source of H_2O_2 results from a leak in the mitochondrial electron transport chain. Again, H_2O_2 comes from dismutation of $O_2^{\cdot -}$, which is primarily generated by the autoxidation of ubisemiquinone:

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \quad (1)$$

Although dismutation can occur nonenzymatically with a rate constant of $10^5\ M^{-1}\ s^{-1}$, the rate constants for the superoxide dismutases are much faster, in the range of $10^9\ M^{-1}\ s^{-1}$ (18). Mitochondrial $O_2^{\cdot -}$ production is regulated by the energy state (24–27), O_2 concentration (26), uncoupling proteins (28), nitric oxide, and peroxynitrite (29, 30). The uncoupling proteins, which cause oxidation of the electron transport components, decrease

the level of $O_2^{\cdot-}$ production (28), while nitric oxide appears to increase the level of $O_2^{\cdot-}$ production by inhibiting cytochrome oxidase, thereby resulting in a greater reduction of the electron transport components (29). Nonetheless, the rate of mitochondrial $O_2^{\cdot-}$ production is also determined by its dismutation to H_2O_2 (31, 32). The effect of superoxide dismutase in accelerating mitochondrial H_2O_2 production occurs because dismutation pulls forward the autoxidation of semiquinones which is thermodynamically unfavorable (33).

Although all the mechanisms for producing H_2O_2 for signaling described above cannot be definitively ruled out, the major source for which there is positive evidence of a signaling function is $O_2^{\cdot-}$ generated by NADPH oxidases:

$$NADPH + 2O_2 \xrightarrow{NADPH\,\text{oxidase}} NADP^+ + H^+ + 2O_2^{\bullet-} \quad (2)$$

followed by dismutation (reaction 1).

The NADPH oxidases are regulated by either assembly of the components into a functioning enzyme complex (as for NOX1 and NOX2) or transcription of the oxidase (as for NOX4). The related DuOX (originally given this acronym because of proposed dual oxidase and peroxidase functions) enzymes appear to primarily catalyze two-electron transfer to O_2 to produce H_2O_2 (34, 35), which, as with other flavoproteins that appear to produce H_2O_2 (36), adds electrons to O_2 in two steps with the second step happening more quickly than the $O_2^{\cdot-}$ can escape from the active site.

H_2O_2 is a polar compound (dipole moment of 2.2 D) because of the dihedral angle of 120° and the H–O–O angle of 95°, in the most stable structure (37). A bond strength typical of covalent bonds, 90 kcal/mol, has been measured for the H–O bond, while the O–O bond is much weaker (51 kcal/mol) (38). Accordingly, this bond is easier to break in biochemical reactions. H_2O_2 is a strong oxidant ($E°$ = 1.776 V for the reduction to water). The oxidation to O_2 is also possible ($E°$ = 0.682 V) and takes place in the catalytic site of catalase using the energy from the reduction of the other H_2O_2:

$$2H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2 \quad (3)$$

In biological systems, the oxidizing potential of H_2O_2 is exploited by two mechanisms. By one-electron reduction (ΔG_0 = −8.8 kcal/mol), H_2O_2 is reduced usually by a transition metal ion. The hydroxyl radical ($HO^{\bullet}$) produced in the reaction is an extremely strong oxidant that reacts with practically all the biological molecules with a nearly diffusion-limited rate constant. As pointed out above, this radical cannot play any specific role in signaling.

The other mechanism is the two-electron nucleophilic substitution reaction that has been suggested for the oxidation of thiols. This reaction requires the dissociation of the thiol to a thiolate anion (RS^-) to achieve the required nucleophilicity (39):

$$H_2O_2 + RS^- \rightarrow OH^- + RSOH \quad (4)$$

While this reaction occurs spontaneously with a rate constant of 18–26 $M^{-1}\,s^{-1}$ (40), the activation energy is too high for the unassisted reaction to be fast enough to be biologically

relevant. If a proton-donating group is, however, in sufficient proximity to allow the hydroxyl ion to leave as water, then the reaction

$$H^+ + H_2O_2 + RS^- \rightarrow H_2O + RSOH \quad (5)$$

would proceed at a much faster rate. In solution, reaction 5 would not occur, as the thiolate would be protonated. Special features of active sites surrounding the peroxidatic Cys (C_p) of proteins such as Prxs and invertebrates or plant glutathione peroxidases that rely on a cysteine for catalytic activity, Cys-GPxs, allow reaction 5 apparently by favoring both the dissociation of thiol and the protonation of the leaving group. Understanding how Prxs and Cys-GPxs catalyze reaction 5 provides a general mechanism for the H_2O_2-dependent oxidation of protein thiols in a biologically relevant context. It should be noted that in most cases, any physiological hydroperoxide or peroxynitrite could substitute for H_2O_2. The exceptions would be when enzyme specificity discriminates among these compounds. Indeed, there are cases in which the alternative reactive compounds are more likely involved in signaling than H_2O_2 (41, 42).

MECHANISMS OF CYSTEINE OXIDATION

Cysteine Oxidation Products

Cysteine can be oxidized to form several different products. These include the thiyl radical (–S$^\bullet$) by a one-electron transition, sulfenic acid (–SOH) and disulfide (–S–S–) by a two-electron transition, sulfinic acid (–SO_2H) by a four-electron transition, and eventually sulfonic acid (–SO_3H) by a six-electron transition.

In water, at neutral pH the electrochemical oxidation of a cysteine thiolate anion against a saturated calomel electrode takes place at an E_{ox} of approximately 0.1 V (43). To the best of our knowledge, there are not data available for the oxidation of a cysteinesulfenic acid derivative, but it seems reasonable assuming that the tautomerism facilitates a further oxidation to sulfinic acid, which, instead, must be more resistant to oxidation because of the presence of two electronegative atoms. A disulfide is also relatively resistant to oxidation (E_{ox} ~ 0.8 V in water at pH 7 or 1.2 V in an aprotic solvent) (44, 45). By inference, we can reasonably conclude that, when permitted, the formation of a disulfide that competes with the formation of a sulfinic acid residue prevents further oxidation of a cysteine thiol.

The sulfinic acid derivative has indeed been shown to be formed, at least in part, at the active site of Prx-2 (46, 47), but requiring a second reaction with H_2O_2, the reaction seems more likely to occur under severe oxidative stress than during physiological signaling. The same holds for the sulfonic derivatives that practically do not exist under biological conditions. It follows that the oxidation states of a Cys residue relevant for signaling are most likely to be the sulfenic acid derivative and the disulfide only.

Enzymatic Oxidation of Cysteine: The Lesson from Thiol Peroxidases

Enzymatic oxidation of substrates containing thiol groups provides a 2-fold example of the mechanism for sulfur oxidation. A Cys or a Sec residue is oxidized at the active site of a peroxidase (first mechanism), which in turn shifts the oxidation potential to a thiol-

containing substrate (second mechanism), thus regenerating the ground-state enzyme. GSH and Trx or other Trx-like proteins are the low-molecular weight substrates oxidized by the specific thiol peroxidases that use H_2O_2 as the oxidizing substrate. Trx is oxidized by Prxs 1–5 in vertebrates and by Cys-GPxs in nonparasitic invertebrates or plants.

$$H_2O_2 + Trx(SH)_2 \xrightarrow[Cys-GPxs]{Prxs\ 1-5} 2H_2O + TrxS_2 \quad (6)$$

GSH is oxidized by mammalian glutathione peroxidases, Sec-GPx, which typically contain a peroxidatic Sec residue or 1-Cys Prx-6 (48). Prx-6 appears to interact with GSTP; however, while GSTP appears to be required for Prx-6 to be active, the transferase more likely preserves activity of the peroxidase rather than participates in the peroxidase catalytic activity (A. Fisher, personal communication).

$$H_2O_2 + 2GSH \xrightarrow[Sec-GPxs]{Prx-6} 2H_2O + GSSG \quad (7)$$

In these peroxidases, the Cys or Sec at the active center is oxidized at a very fast rate by the hydroperoxide to a primary product, a SOH or Se-OH, due to an assist from amino acids in the distinct active sites, which, remarkably, are strongly conserved among the members of the two families of enzymes. Both Prx and GPx active sites promote dissociation of the thiol (or selenol), polarize the O–O bond of the hydroperoxide, and protonate the leaving OH^- group (49, 50).

Sulfenic acid as the reactive primary product of the oxidation by the peroxide can also be formed at the active site of some other non-peroxidase proteins. Using a specific antibody against the protein adduct formed by reaction with dimedone, a reagent that reacts rather specifically with sulfenic acid, this epitope was found in normal cells, with even greater amounts in cancer cells (51). The actual physiological or pathological relevance of this oxidation needs to be clarified; however, although nonspecific oxidations are possible, the proteins undergoing a physiological redox switch must fulfill the mandatory criteria for lowering the activation energy of the nucleophilic displacement reaction described above. In Prxs or Cys-GPxs, this primary oxidation product evolves into other species, depending on the specific environment. When there is a suitable Cys in the same subunit, i.e., the resolving Cys (C_r) as in atypical 2-cysteine Prxs (Prx-5 of mammals) and in the majority of Cys-GPxs, an intrachain disulfide is formed as the oxidized intermediate of the peroxidase. This is usually associated with some unfolding of the protein (52). In the case of typical 2-cysteine Prxs, such as Prxs 1–4 of mammals, a Cys residue of a second subunit is the thiol reacting with the –SOH group thereby producing an intermolecular disulfide as the oxidized intermediate. The fast reduction of both these types of disulfides by Trx or Trx-like proteins accounts for the reductive step of the peroxidatic cycle in atypical/typical 2-cysteine Prxs and plant/invertebrate Cys-GPxs. Notably, fast kinetic analysis of the peroxidatic reaction of thioredoxin peroxidase of *Mycobacterium tuberculosis* showed that the C_p is directly reduced by Trx, although a C_r also exists (53). The formation of the disulfide between C_p and C_r, observed in the absence of Trx, was interpreted not as a necessary intermediate of the peroxidatic cycle but as a kind of “parking of the oxidized form”. The reaction is seen as

"protective" for the enzyme, since the formation of an irreversibly overoxidized form in the absence of the reducing substrate was prevented. Lacking fast kinetic data for other peroxidases, we do not know how often this interesting mechanism occurs in nature.

When there is not a suitable C_r, such as in Sec-GPx or mammalian Prx-6, the reaction of the primary oxidation product occurs with GSH, yielding a mixed disulfide with the reducing substrate GSH as the oxidized intermediate of the enzymatic reaction. The overall specificity for the enzymatic oxidation of the target emerges from the specificity of the interaction of the enzyme first with the hydroperoxide and then with the thiol-containing substrate. Accordingly, the kinetics of the enzymatic reaction follows a ping-pong mechanism in which ternary complexes are not formed (54, 55).

The steady-state kinetic analysis of different Prx subtypes indicates that the rate constants for the oxidation of the Cys residue at the active site range from 2×10^3 to 4×10^7 M^{-1} s^{-1}, depending on the hydroperoxide and the individual Prx (55–58). These values are comparable with those obtained for the Cys-GPxs (from 4×10^3 to 1.3×10^6 M^{-1} s^{-1}), while values lower than 3.4×10^5 M^{-1} s^{-1} have never been obtained for the Sec-GPxs (54). From these measurements, we can conclude that the structure of the active site fits the requirements for a physiological oxidation of a thiol to sulfenic acid, where the nucleophilic displacement reaction is accelerated by the donation of a proton to the leaving HO^- group.

The formation of a disulfide by reaction of the primary oxidation product with a C_r that may be in the same protein or on another subunit, or the formation of a mixed disulfide with GSH, followed by thiol–disulfide exchange, provides an example of the second mechanism for the oxidation of a thiol in a biological environment. Understanding of the mechanism of Prxs and GPxs indeed highlights the general mechanisms required for the functional redox switch of a suitable Cys residue by a hydroperoxide:

(a) direct oxidation of a thiol to sulfenic acid, when the suitable Cys is assisted in the active site as in reaction 5

$$\mathrm{H^+ + H_2O_2 + protein - S^- \rightarrow H_2O + protein - SOH} \quad (8)$$

(b) formation of a disulfide

$$\mathrm{protein - SOH + RSH \rightarrow protein - SSR + H_2O} \quad (9)$$

(c) thiol–disulfide exchange

$$\mathrm{protein - SSR + R'SH \rightleftarrows protein - SSR' + RSH} \quad (10)$$

or

$$\mathrm{protein - SSR + R'SH \rightleftarrows protein - SH + RSSR'} \quad (11)$$

where R(R')SH is any accessible thiol.

Only reaction 8 requires an oxidant, H_2O_2, while in reactions 9–11, scrambling takes place among cysteine residues but the overall redox status of the thiols is maintained. It should be noted, however, that in reaction 9, either the protein–SOH or RSH species would need to be in the anionic form for the reaction to proceed, while in reactions 10 and 11 the R'SH species would need to lose its proton as well.

SIGNALING BY CYSTEINE OXIDATION

The cysteinesulfenic acid derivative could be stable when in an apolar environment not accessible to solvent, and the approach by reactive molecules is sterically inhibited (59). Oxidation of protein cysteine to sulfenic acid can be detected using dimedone, a compound that reacts quite specifically with sulfenic acid. Several proteins containing the reactive group have been detected using a specific antibody (51) or by using dimedone linked to a fluorescent probe (60). More specifically, besides the active site of some oxidoreductases (61), the formation of a cysteinesulfenic acid has been reported or suggested for proteins involved in cell signaling. OxyR is a nuclear factor that in bacteria stimulates, through interaction with RNA polymerase, transcription of genes enhancing resistance to oxidants (62). The activation of the nuclear factor takes place through the formation of a cysteinesulfenic acid in the presence of submicromolar concentrations of H_2O_2 with a rate constant for reaction in the range of 10^5 M^{-1} s^{-1} (63). This cysteinesulfenic acid then reacts relatively slowly with a cysteine that is not in proximity (17 Å), resulting in the formation of a disulfide (64). This implies that, although the overall mechanism is still a matter of debate (65), a major conformational shift has to take place following oxidation, driving the modification of the interaction with DNA. The reversal of the reaction is accomplished by glutaredoxin by a thiol–disulfide exchange reaction (59).

Less conclusive evidence has been obtained for the actual role of a cysteinesulfenic acid in the redox regulation of Fos and Jun, an AP-1 transcription factor complex (66), while definitive evidence supports the role of Trx and HAP/Ref1 in reduction of cysteine in AP-1 that is required for its transcriptional activity and binding to DNA (67–69).

In *Saccharomyces cerevisiae*, exposure to an oxidizing environment activates adaptive gene expression through the transcription factor, Yap-1, that is activated by oxidation. This does not take place by direct interaction with H_2O_2 but requires a Cys-GPx subtype, yeast GPx-3. The oxidation of the C_p, Cys-36, of GPx-3 results in the formation of a mixed disulfide with Cys-598 of Yap-1. Cys-303 of Yap-1 than displaces it, and an intramolecular disulfide between Cys-598 and Cys-303 of the transcription factor is formed, eventually accounting for the shift to its active form. Trx turns off the signaling pathway, by reducing both the sensor and the regulator (70). The emerging function of yeast GPx-3 as a sensor of oxidants and redox translator underscores the peculiar role of the active site of GPxs in reacting specifically and at a high rate with H_2O_2. The outcome of the reaction also suggests the role of a cysteinesulfenic acid intermediate, although its involvement has been only indirectly assumed.

There is an increasing number of proteins that appear to form a protein–glutathione mixed disulfide under physiological conditions, including PTP1B (71), cytosolic $NADP^+$-

dependent isocitrate dehydrogenase (72), the yeast 20S proteasome (73), ryanodine receptor type 1 (74), signal transducer and activator of transcription 3 (also called STAT 3), (75), caspase-3 (76), and the phosphatase and tensin homologue deleted from chromosome 10 (also called PTEN) (77). When considering glutathionylation in a biological environment, the most reasonable mechanism must fulfill the constraints described above for the formation of a sulfenic acid, in turn reacting with GSH.

$$\text{protein} - SO^- + GSH \rightarrow \text{protein} - SSG + OH^- \quad (12)$$

A disulfide exchange reaction between a cysteine residue in a protein and GSSG is also possible and is catalyzed by the Trx-like protein glutaredoxin (Grx):

$$RSH + GSSG \underset{\rightleftarrows}{^{Grx}} RSSG + GSH \quad (13)$$

The oxidizing substrate GSSG is formed by oxidation of GSH in reaction 7. It is well established that formation of protein–glutathione mixed disulfides occurs under severe oxidative stress and in the endoplasmic reticulum (78, 79). In the cytosol, under physiological signaling conditions, however, the low concentration of GSSG is unlikely to push this reaction toward formation of the mixed disulfide. Moreover, it should be pointed out that for thiol–disulfide exchange the actual barrier that limits the oxidation of proteins by GSSG is thermodynamic. Indeed, the mixed disulfide formed must have a redox potential higher than that of the $[GSH]^2/[GSSG]$ couple, which although theoretically possible in some extreme cases, is extremely unlikely. In addition, we also must consider that the kinetics of this reaction is expected to be slow unless it is catalyzed.

Although Trx and GSH can react with H_2O_2 nonenzymatically, the Prx-catalyzed rate of reaction to form $TrxS_2$ is several orders of magnitude faster (reaction 6) (56). Oxidized Trx is known to be involved in at least one signaling pathway, the activation of ASK1, an upstream protein kinase kinase kinase in the $p38^{MAPK}$ and Jun N-terminal kinase (JNK) pathways (80). ASK1 is normally inhibited by reduced Trx; however, when the Trx is oxidized, it dissociates from ASK1, allowing it to dimerize and self-activate and then activate downstream protein kinase kinases (80). This has been shown to occur with endogenous generation of H_2O_2 produced in macrophages through stimulation of NOX2 (81).

Another example is in TRP14, a Trx-related protein, in which its active site cysteine appears to form a specific disulfide link with the LC8 cytoplasmic dynein light chain (82).

The Case of PTP1B

Possibly the most studied signaling protein proposed to form a sulfenic acid intermediate is PTP1B. The rate constant for the oxidation of the PTP1B active site cysteine by H_2O_2 is, however, only in the range of 9–43 M^{-1} s^{-1} (71, 83, 84). This is approximately the same rate constant as for the thiolate moieties of low-molecular weight compounds, which are in the range of 18–26 M^{-1} s^{-1} (40). Thus, while it has been demonstrated that PTP1B can react in vitro with H_2O_2 to form a sulfenic acid that goes on to form a sulfenyl–amide intermediate (85, 86), this is rather unlikely to occur in a cell where it would be in competition with Prxs

and Sec-GPxs, which are abundant in the cytosol and react with H_2O_2 1 million times more rapidly. The intracellular concentration of GSH in its thiolate form would be 12.4 μM, calculated from an intracellular pH of 7, the GSH thiol pK_a of 8.9 (87), and 1 mM GSH, which is near the low end of the intracellular GSH concentration range (88). With a similar rate constant for reaction with H_2O_2 as the PTP1B active site cysteine, 12 μM GS^- would outcompete PTP1B [estimated to be 8.3 nM in HepG2 cells (89)] and prevent its oxidation even in the absence of Prx and Sec-GPx activity. In theory, a very high local concentration of H_2O_2 could allow a reaction with a slow rate constant to occur in competition with the hydroperoxide reducing enzymes; however, in the one study where the endogenous source of the H_2O_2 was identified, it was produced on the other side of the plasma membrane from PTP1B (90). Nonetheless, reversible oxidation of the PTP1B active site cysteine does occur (83), forming a mixed disulfide with GSH during cell signaling (90). Assuming that the thrice measured rate constant for the oxidation is correct, mechanisms other than direct oxidation of the enzyme followed by reaction with GSH must be involved. The use of the active site thiolate in place of the resolving cysteine or GSH by a peroxidase would be consistent with the principles for thiol oxidation described above.

FLOODGATE HYPOTHESIS

In this hypothesis, inactivation of Prx occurs by overoxidation of the active site cysteine to sulfinic acid and this permits H_2O_2 to then react with its targets. Prx, which reacts very rapidly with H_2O_2, can react a second time to produce the sulfinic acid:

$$Prx-SOH+\mathrm{H_2O_2} \rightarrow Prx-SO_2\mathrm{H}+\mathrm{H_2O} \quad (14)$$

This reaction, which can occur during oxidative stress, has also been proposed to occur during physiological signaling and to act as a floodgate that regulates redox signaling (46). An energy-dependent restoration of the thiolate in the Prx active site is catalyzed by the enzyme, sulfiredoxin (91, 92), which allows Prx to once again inhibit H_2O_2-dependent signaling. It has been demonstrated that exogenous H_2O_2 can cause the overoxidation of Prx-2 and cause cell cycle arrest (93); however, this has yet to be shown under physiological conditions. Mutation of Prx-2 has been shown to enhance signaling in response to platelet-derived growth factor, which is consistent with the floodgate hypothesis (94); however, Prxs interact directly with several signaling proteins, consistent with a role different from the removal of H_2O_2. A yeast form of Prx-1, Tpx1, forms a peroxide-induced disulfide complex between itself and Sty1, a yeast JNK (95). Mammalian Prx-1 (also called PAG) binds to the GSTP–JNK complex, thereby preventing the release of JNK from the complex and inhibiting its activation (96). Prx-1 also specifically binds and inhibits c-Abl, a nonreceptor tyrosine kinase (97) and binds to and alters the activity of c-Myc (98).

There are three major problems with the floodgate hypothesis. First, the role of Sec-GPxs has been overlooked. Cells, if not supplemented, are severely depleted of selenium and express the cytosolic GPx at a very low level (99). Thus, in many cell studies that showed Prxs to be the major enzyme eliminating H_2O_2, GPx deficiency actually existed. Nonetheless, in vivo, where selenium depletion is rare, inactivation of Prxs by overoxidation would still leave a significant GPx activity that would prevent the flood. The second major

problem is the requirement of the hypothesis for a second hit on the same Prx molecule to eliminate a significant amount of activity before H_2O_2-dependent signaling occurred. The rate of oxidation of Prx-1 was thought to result in the inactivation of 0.072% of Prx-1 during each round of catalysis (94). Whether this relatively slow rate of inactivation is sufficient to allow the floodgate hypothesis to account for H_2O_2-dependent signaling is, therefore, questionable. Data suggesting a more rapid rate of reversal of the oxidation of Prx isoforms to sulfinic acid (100) and questioning of the extent of Prx overoxidation (101) cast further doubt on the hypothesis. The third major problem is that, even in the absence of both Prx and GPx activities, the cytosolic 12.4 μM GS^- (see the section on PTP1B above) would outcompete the nanomolar concentration of any target protein for nonenzymatic reaction with H_2O_2. Finally, even if the floodgate hypothesis were to be shown to be correct, it does not address the mechanism by which H_2O_2 actually signals.

CONCLUSIONS

From a review of the chemistry and biochemistry of ROS, it has become clear that H_2O_2 is the only one that clearly has the characteristics of a second messenger. Superoxide is a major precursor of H_2O_2 rather than a direct participant in signaling, while hydroxyl radical lacks specificity.

The specificity of H_2O_2 as a second messenger comes from its reactions with specific, “oxidation prone” protein Cys residues in local environments that lower the pK_a. Nonetheless, having a Cys in the thiolate form is not sufficient for the definition of an oxidation prone Cys. The reaction must be fast enough to compete with peroxide-removing enzymes and can occur only with assistance in breaking the O–O bond of H_2O_2, resulting in the formation of a cysteinesulfenic acid and water.

The protein cysteinesulfenic acid formed either is stable or reacts with (a) another Cys in the same or another protein, giving rise to a conformational shift; (b) GSH, producing a glutathionylated protein; (c) an amide in the backbone of the protein; or (d) another hydroperoxide. Options a–c are readily reversed, while option d requires more complex reactions to restore the original cysteine thiolate. Glutathionylated proteins in cytosol are produced by either reaction b or exchange of GSH with a protein disulfide catalyzed by glutaredoxin.

The formation of glutathionylated signaling proteins, such as PTP1B, or intramolecular disulfides, such as in the thioredoxin that modulates ASK1, is the posttranslational modification that is essential to redox signaling. The kinetics of thiol oxidation suggests that only enzymatically catalyzed oxidations are likely to be part of signaling and that it is the enzymatic use of H_2O_2 rather than its overcoming of antioxidant defense as in the floodgate hypothesis that defines signaling by ROS.

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