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The Basics of Thiols and Cysteines in Redox Biology and Chemistry

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Abstract

Cysteine is one of the least abundant amino acids, yet it is frequently found as a highly conserved residue within functional (regulatory, catalytic or binding) sites in proteins. It is the unique chemistry of the thiol or thiolate group of cysteine that imparts functional sites with their specialized properties (e.g., nucleophilicity, high affinity metal binding, and/or ability to form disulfide bonds). Highlighted in this review are some of the basic biophysical and biochemical properties of cysteine groups and the equations that apply to them, particularly with respect to pKa and redox potential. Also summarized are the types of low molecular weight thiols present in high concentrations in most cells, as well as the ways in which modifications of cysteinyl residues can impart or regulate molecular functions important to cellular processes including signal transduction.

Of the twenty common amino acids, perhaps the most intriguing and functionally diverse is cysteine, one of the two sulfur-containing amino acids of the set (Fig. 1). Unlike methionine, which has its sulfur in a relatively less reactive thioether form, the thiol (or “sulfhydryl”) group of cysteine is ionizable, with a negatively-charged thiolate group being generated after deprotonation, boosting its reactivity (Fig. 1). Moreover, this thiol/thiolate group is subject to alkylation by electrophiles and oxidation by reactive oxygen and nitrogen species, leading to posttranslationally modified forms that can exhibit significantly altered functions.

In addition to cysteine, a closely related but much less common amino acid, selenocysteine (Fig. 1), also exhibits chemistry similar to cysteine, but its reactivity is distinct due to the different intrinsic chemistry of Se versus S and is enhanced at neutral pH due to its more highly stabilized deprotonated form (i.e., its lowered pK_a of ~5.2) [1, 2]. This residue is outside the 20 “standard” amino acids and is encoded by TGA, which in the absence of special selenocysteine-charged tRNAs is a stop codon. The reactivity of this residue is so similar to that of cysteine that its location within proteins has recently been used to detect catalytic, redox-active Cys residues in homologous protein family members [3].

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The unique chemical and physical properties of cysteine (Cys), selenocysteine (Sec) and thiol groups in general are critical to the biological roles these residues and chemical groups play [4, 5]; provided herein is an overview of the underlying principles that govern their diverse functions in biological systems. Before addressing protein-associated thiol chemistry and biology, this review begins with an introduction to thiol-containing small molecules encountered in biological systems.

Small molecule thiols in biology

Low molecular weight (LMW) thiols are important players in redox-mediated or regulated processes in the cell, yet the identity and levels of the various types of LMW thiols can vary widely across the biome. Most ubiquitous is the tripeptide glutathione (composed of Glu, Cys and Gly, with an unusual amide linkage between the γ-carboxylate of Glu and the amine of Cys), found in high (i.e., low mM) concentrations in many prokaryotic and eukaryotic cells. However, some species of prokaryotes, fungi and archaea rely on other sulfur compounds in addition to, or instead of, glutathione which also reach high concentrations in certain cell types [6]. Shown in Fig. 2 are some of the common (glutathione, Cys, Coenzyme A, homocysteine and lipoic acid) and organism-specific LMW thiols found in such species as Actinomycetes (mycothiol), Firmicutes (bacillithiol), halobacteria and lactic acid bacteria (γ-Glu-Cys), trypanosomes (trypanothione), fungi and mycobacteria (ergothioneine) and methanogenic archaea (Coenzyme M and Coenzyme B) [6–8]. In some organisms, several of these LMW thiols coexist in comparably high concentrations, and thus the mixed disulfides, and not just the symmetrical disulfides, must be considered as players in the complex thiol metabolism of these species [7]. Like glutathione, these other LMW thiols also participate in disulfide bonds with proteins and have a range of regulatory and metabolic functions that are only just beginning to be appreciated; much also remains to be clarified about many of their biophysical properties, as well (i.e., pK_a and redox potential) [6, 7]. With the wide diversity in the structures of LMW thiols, allowing for differential recognition by enzymes, it is not surprising that their in vivo functional roles can also vary widely.

Cysteine is a special amino acid

As one of the least abundant amino acids, Cys exhibits very distinct properties when its location and distribution among homologous proteins is analyzed using bioinformatics tools and databases of known or modeled protein structures. In an analysis of the distribution of conservation levels of each of the amino acid residues within proteins [9], four amino acids were found to be most frequently conserved, Cys, Gly, Pro and Trp. However, the conservation picture that emerges is much more complex. Cys exhibits extreme patterns of conservation at both ends of the spectrum; in areas where Cys are conserved, the degree of conservation is typically above 90%, whereas poorly conserved (highly degenerated) Cys residues were less than 10% conserved. Of the other nineteen common amino acids, only Trp also exhibits a low population of intermediate values with respect to conservation, but still not to the extent of Cys. Marino and Gladyshev suggested that these extreme patterns are likely indicative of strong selective pressure to keep Cys or Trp residues in functionally important locations and to remove them from others [9]. With Trp as the largest and most

hydrophobic sidechain and Cys as the only thiol-bearing amino acid, they are arguably irreplaceable in sites where they are functional and highly conserved. Moreover, neither residue is well tolerated on solvent-exposed surfaces of proteins, although the reasons for this are quite distinct for these two amino acids, as expanded on below.

The physical and chemical properties of Cys residues are inherently critical to their functional attributes, yet it surprising to note that there is controversy even over whether Cys residues should be considered hydrophobic residues or not. The chief reason that many hydrophobicity scales rank Cys residues as hydrophobic [10–12] is that their degree of burial (protection from solvent) is so high (Fig. 3a) [9]. However, Marino and Gladyshev argued that this was due to selective pressure during evolution to minimize the occurrence of unpaired surface Cys residues rather than the physicochemical properties of Cys [9]. In fact, calculation of the partial charge distribution on each atom of Cys and similar free amino acid sidechains using quantum mechanical approaches demonstrated that the polarity of this residue is quite similar to that of Ser, which is classified as a polar residue (Fig. 3b and [9]). Ser and Ala are typical, conservative replacements for Cys when mutagenesis studies are conducted, as the goal is ordinarily to remove the thiol functionality but introduce only minimal perturbations in structural integrity in the proteins that are mutated. Given its polarity, hydrogen bonding capabilities and size, Ser most closely fits this bill (with the sulfur replaced by a moderately smaller oxygen), although there are also strong arguments for simply removing the thiol/alcohol functional group altogether, as in an Ala replacement. It is best to consider making both substitutions where possible, as structural effects of these substituted amino acids will vary with the protein.

Another property unique to Cys residues is their tendency to be found proximal to other Cys residues, a feature known as clustering in bioinformatics terminology. This feature of Cys distribution has been noted to increase in proteins expressed by organisms living in harsh environments [13], and is often characteristic of sites of metal binding or redox sensitivity (i.e., leading to disulfide bond formation). It should be noted, however, that, due to conformational rearrangements that can occur, not all disulfide bonds in proteins connect Cys residues that are close in structure when reduced (and thus are not considered “clustered” by these structurally guided bioinformatic analyses) [9]. Overall, taking into account the degree of burial (away from solvent and the protein surface) and tendency to be clustered, Cys residues that are exposed and isolated are the least conserved category for this amino acid. This finding can be interpreted as demonstrating an evolutionary bias away from such surface exposed, unpaired Cys residues.

Cys residues, which are highly polarizable, vary in their structural context in proteins. This has a strong influence on their reactivity, affecting such properties as their accessibility and protonation state (i.e., pK_a). For example, alpha helices exhibit a dipole moment with more positive charge at the N-terminal end, lowering the pK_a and rendering more reactive the Cys residues located in these regions (see pK_a discussion, below) [14]. Moreover, some proteins have specialized active site architectures surrounding the Cys that strongly enhance their reactivity toward specific substrates. In the peroxiredoxins, for example, H-bonding interactions not only lower the pK_a of the active site Cys, but also activate the incoming peroxide [15, 16]; the overall rate enhancement combines the pK_a effect (which accounts for

a rate of ~20 M^{-1} s^{-1} as observed with small molecule thiolates [17]) with these additional features to yield reaction rates with hydrogen peroxide as high as 10^7 – 10^8 M^{-1} s^{-1} [18, 19].

Reactions of cysteines and other thiol-containing biomolecules

Thiol groups exhibit reactivity toward electrophiles and oxidants and have high affinities for metals [20–23], making them versatile in the biological roles they can play but also potential Achilles' heels for modifications that subvert the normal biology. This delicate balance of productive versus pathogenic reactions occurring among thiol groups presents researchers with the challenge of identifying not only the specific reaction products and sites of modification occurring on thiol groups of small molecules and proteins, but also the functional effect of each type and location of modification. Fortunately, the tools and approaches for evaluating this complex array of reactivities and products have been improving over the last decade and are the subject of other reviews in this series [24] and elsewhere [25–28].

pK_a

Thiol groups, typically referring to the R-SH form of these chemical moieties, are subject to deprotonation (loss of H^+) to form charged thiolates ($R\text{-}S^-$) with distinct properties and reactivities compared with thiols, although both forms have lone pairs of electrons and are nucleophilic. The equilibrium ratio of thiol to thiolate forms varies with pH and is characteristic for specific thiol groups located within a distinct protein environment. A convenient way to track the tendency of a thiol group to be deprotonated over a range of pH values is to refer to the pK_a of a given thiol group; practically speaking, this thermodynamic parameter denotes the pH at which the populations of thiol- and thiolate-bearing molecules are equal. An approximation for this value in "unperturbed" Cys residues in proteins is ~8.5 [29] (depending on multiple factors including dielectric constant), but reactive Cys often exhibit lowered pK_a values as a result of a protein environment that stabilizes the negatively-charged thiolate anion (e.g. at the N-terminal end of a helix, as mentioned above). In proteins, specific hydrogen-bond donors and an electropositive local environment tend to lower the pK_a by stabilizing the thiolate, and a hydrophobic environment or an electronegative local environment tends to raise the pK_a by destabilizing a negatively-charged as opposed to neutral form of the side chain (for a few specific examples of these effects, see [14, 30–32]).

It is, however, an over-simplification to suggest that the pK_a lowering effects of the protein environment simply enhances Cys reactivity. While it is true that thiolates are better nucleophiles than thiols, in a given ionization state (e.g. thiolates) nucleophilicity (the affinity of a base for a carbon atom in a displacement reaction transition state [33]) actually increases with pK_a [34, 35]. It should also be noted that the protonation/deprotonation phenomenon affects the relative populations of the two species. The ratios of thiol- to thiolate anion-containing molecules change significantly with small changes in pH within ~1 pH unit around the pK_a (given that pH is related to the logarithm of the H^+ concentration: $pH = -\log [H^+]$) but a small percent of thiolate persists even several pH units lower than the pK_a (Fig. 4). The change in relative populations of thiol and thiolate molecules with pH is

readily calculated using the Henderson-Hasselbalch equation [36] which takes into account the actual pH relative to the pK_a of the species of interest:

$$pH = pK_a + log\frac{[A^-]}{[HA]} \quad (1)$$

In this equation, HA represents the protonated species (i.e., thiol) and A^- represents the deprotonated form (i.e., thiolate).

Methods for determining acid dissociation constants (pK_a values) cover a wide range of approaches; the gold standard for the measurement of pK_a is a potentiometric pH titration. Approaches for investigating pK_a values of individual Cys residues in proteins often require the use of other methods, as well, including spectroscopic detection of small differences between absorbances of thiolate and thiol around 240 nm [37, 38], NMR detection of shifting protonation state relative to solution pH [39], readouts associated with chemical reactivity [40, 41], or functional assays of the thiol/thiolate group of interest [42, 43]. Where sensitivity toward alkylation is assessed, iodoacetate or iodoacetamide are used rather than N-ethylmaleimide given the relative pH insensitivity of the Michael addition reaction occurring in the latter case [23, 44]. Because pK_a analysis can be complex and many factors can confound such determinations, the pK_a of a protein is best assessed using multiple complementary approaches [42]. The mathematics of determining pK_a with an appropriate readout are rather straightforward; however, simply dropping a “50%” line representing half of the change of the parameter being measured that is sensitive to protonation state (e.g., ε_{240} in the example of Fig. 4) to read out the pH at the midpoint is not appropriate as a pK_a “determination”. A general equation to which relevant pK_a-informative data can be fit [41, 42], as shown in Fig. 4, is:

$$y = \frac{(A \times 10^x) + (B \times 10^{pKa})}{10^x + 10^{pKa}} \quad (2)$$

In this equation, y is the value of the parameter being monitored (ε_{240} in Fig. 4), x is the pH value, and A and B are the limiting values of y (i.e., the plateaus) at high and low pH, respectively. Because the absorbance of thiolate anions at 240 nm is detectably higher than that of thiols in the example shown, A>B in Fig. 4.

Redox potential

Another thermodynamic parameter of relevance to the chemistry of thiol groups is the reduction potential, often less formally referred to as the redox potential. While a summary of the principles of this topic are provided here, the reader is referred to textbooks with more extensive coverage of this subject [36, 44, 45]. Any pair of molecules that can be interconverted by the addition or loss of electrons are referred to as a redox pair, and their interconversion can be written in the form of a reductive or oxidative half reaction. A full reaction involves the coordinated reduction (gain of electrons) of one species and the oxidation (loss of electrons) of another. For analyzing their energetics, such simple bimolecular electron transfer reactions are broken down into two half reactions, with the

electrons being gained or lost explicitly noted in each. For example, the reduction of pyruvate to lactate, as catalyzed by lactate dehydrogenase, is accompanied by the oxidation of NADH to NAD^+, as shown in the following two half-reactions:

$$\text{Pyruvate} + 2\,e^- + 2\,H^+ \iff \text{Lactate} \quad (3)$$

$$\text{NADH} \iff NAD^+ + 2\,e^- + H^+ \quad (4)$$

$$\text{Net reaction: Pyruvate} + \text{NADH} + H^+ \iff \text{Lactate} + NAD^+ \quad (5)$$

To evaluate the driving force for the full reaction to occur, one would consider the standard reduction potential, E°, associated with each of the individual half reactions. This quantity refers to the electrode potential that would be associated with each half reaction, compared to the standard hydrogen electrode, under "standard" conditions defined as 1 M concentrations of each component, a temperature of 298.15 K, and a pressure of 1 atmosphere. By convention, biochemical standard conditions use a pH value of 7 (rather than pH of 0 associated with 1 M H^+ ions) and this is noted by the inclusion of a "prime" symbol so that E°′ designates the biochemical standard reduction potential. Table 1 provides a small sampling of such values which can be used to calculate the reduction potential associated with a given redox pair under any defined set of conditions (such as physiologically relevant concentrations of each component) according to the Nernst equation:

$$E' = E^{\circ\prime} - \frac{RT}{n\mathfrak{F}} \ln \frac{[\,reduced\ form\,]}{[\,oxidized\ form\,]} \quad (6)$$

In this equation T is the absolute temperature (in K), n is the number of electrons transferred in the reaction, and R and $\mathfrak{F}$ are the universal gas and Faraday constants, respectively (8.314 J K^{-1} mol^{-1} and 96,485 J V^{-1} mol^{-1}). Referring to Table 1, the E°′ values associated with the redox pairs shown in reactions 3 and 4 (written in the table as reductive half reactions, unlike reaction 4) are −0.19 and −0.32 volts, respectively. The change in E°′ (ΔE°′) for the net reaction (5) is calculated from the difference between the standard biochemical reduction potentials of the component half reactions:

$$\Delta E^{\circ\prime} = [E^{\circ\prime}\ \text{of the half-reaction containing the oxidizing agent (pyruvate in this case)}] - [E^{\circ\prime}\ \text{of the half-reaction containing the reducing agent (NADH in this case)}] \quad (6)$$

If the resulting value of ΔE°′ is positive, the reaction under standard conditions and pH 7 is spontaneous; ΔE°′ for reaction (5) is +0.13 V, and is therefore spontaneous under standard conditions. Importantly, the standard free energy change associated with this reaction can also be calculated from ΔE°′ according to the following relationship:

$$\Delta G^{\circ'} = -n\,\mathfrak{F}\Delta E^{\circ'} \text{ (or equivalently } \Delta G = -n\,\mathfrak{F}\Delta E \text{ under any conditions)} \quad (7)$$

The ΔG°′ associated with reaction (5) is thus highly favorable, at −25.1 kJ/mol.

Like with other processes governed by equilibrium constants, differences in redox potentials associated with electron transfer reactions affect the populations of reduced and oxidized molecules. For example, if a population of reduced, dithiol-containing molecules is mixed with a population of oxidized, disulfide-containing molecules AND there is a kinetic pathway operable to allow for electron transfer between the two, then the reduced population will begin to become oxidized and *vice versa* (at a rate governed by the kinetics) until equilibrium is reached (i.e., the rate of oxidized molecules becoming reduced is the same as the rate of reduced molecules becoming oxidized, so that there is no net change in populations). We typically think of redox pairs with very low E°′ values as being electron donors in reactions with redox pairs of higher E°′ value, although biological systems are complex and rarely at equilibrium. It is also possible for the redox potential of the electron-donating redox pair in a pathway to exhibit a redox potential that is higher than the acceptor pair. For example, poplar PrxQ (a peroxiredoxin which reacts with hydroperoxides in its reduced form) and its physiological reductant, thioredoxin (Trx), exhibit redox potentials (E°′) for their reductive half reactions (i.e. converting the disulfide to dithiol forms) of −325 and approximately −290 mV, respectively (Table 1) [46]. Given these values, fully reduced Trx mixed with fully oxidized PrxQ at T=298 K in an isolated system would reach an equilibrium state of ~20% reduced PrxQ and ~80% reduced Trx [47]. This calculation makes use of a derivative of the Nernst equation:

$$E^{\circ'}(Prot1) - E^{\circ'}(Prot2) = \left(\frac{RT}{n\mathfrak{F}}\right) ln \left(\frac{[Prot2_{ox}] \times [Prot1_{red}]}{[Prot1_{ox}] \times [Prot2_{red}]}\right) \quad (8)$$

While these thermodynamic considerations govern equilibrium “set points”, electron transfer in living systems is very dynamic and involves many players. The re-reduction of Trx by NADPH-dependent thioredoxin reductase, as well as electron transfer from PrxQ to peroxides, ensures that the forward reaction continues in spite of their “inverted” redox potentials. Thus, linked equilibria and flux through the various redox systems of organisms can promote reactions that are, in isolation, moderately unfavorable. Furthermore, emphasis must be placed on the kinetic pathways and rates associated with given reactions, as these considerations more than the thermodynamic driving forces tend to dominate in determining which of many competing reactions occur in the dynamic and complex environment of a living cell [48, 49].

Thiol-disulfide exchange

With these discussions of electron transfer between dithiols and disulfides, it is also important to note that disulfide bonds, when formed in the complex thiol-containing mixtures of living systems, are not “locked in.” Rather, there can be one or a series of thiol-disulfide exchange reactions (Fig. 5, top) that can occur in mixtures of redox-sensitive

molecules as long as donors and acceptors can interact in the appropriate orientations for attacking and leaving groups, as emphasized by Thorpe and Winther [23, 50]. For this reason, procedures to analyze disulfide-bonded proteins and small molecules in complex mixtures must typically include a thiol-blocking step (e.g., alkylation), especially before denaturants are added, so that “scrambling” of the native pattern of disulfide and thiol groups can be avoided [23–25, 51–53]. An additional consideration to retain and analyze only “native” disulfides in complex, cell-derived samples is the blocking of Cys sulfenic acids; denaturation without sulfenic acid blocking is likely to lead to the creation of new disulfide bonds with other thiols to yield non-physiological complexes (see below). It should be noted that typically-used thiol-alkylating agents (e.g., iodoacetamide, N-ethymaleimide and methyl methanethiolsulfonate) cross-react with sulfenic acids. While this unhelpfully interferes with labeling approaches to detect sulfenic acids, it may also helpfully contribute somewhat to quenching of the further formation of non-native disulfide bonds in biological samples [53].

In addition to the migration of protein disulfide bonds to other protein sites, it should be noted that sulfenic acids and potentially other labile thiol derivatives can react with LMW thiols to form mixed disulfides through a process referred to as S-thiolation [6, 7]. Again, such disulfides are susceptible to exchange upon attack by other free thiol groups, which can either be a pathway for reductive recycling (see below) or another manner in which disulfide bonds can migrate.

Oxidative products of thiol groups in addition to disulfides

Thiol oxidation and adduction in biological systems leads to the formation of various reversible and irreversible products that, in the former case, can be recovered biologically through the action of cellular reductants like glutathione and thioredoxin. Among the products of Cys oxidation, sulfenic acids, S-nitrosothiols and disulfides are of particular interest given the roles that they play in redox cycling and/or regulation of enzymes and transcription factors involved in cell signaling processes [26, 54–56]. Electrophile adduction, generally an irreversible process, is also an important physiological signal, e.g. in triggering Nrf2 transcriptional activation upon modification of Cys residues in Keap1 [22, 57].

The two electron oxidation of thiol(ate) groups, e.g. by hydrogen peroxide, peroxynitrite and other hydroperoxides, generates the simplest oxyacid of sulfur, the sulfenic acid (R-SOH) (Fig. 5). Rates for this reaction can vary from vanishingly slow up to as high as 10^8 M^{-1} s^{-1} in peroxidase active sites [18, 49]. This species reacts readily with proximal thiol groups to form disulfide bonds, but in the absence of such thiol groups, are subject to further, irreversible oxidation to sulfinic (R-SO_2H) and sulfonic acid (R-SO_3H) species, although typically at lower rates than the initial oxidation of the thiolate [58–60]. One exception to the irreversibility of sulfinic acid formation has been discovered; the sulfinic acid of hyperoxidized Prxs can in some cases be reduced by a repair enzyme known as sulfiredoxin [61–63]. Sulfenic acids can also condense with one another (with a loss of water) to form thiosulfinates, or with amine or amide groups to form sulfenamides (also known as sulfenyl amides) [64, 65]. Other products of oxidative reactions include S-nitrosothiols and

persulfides, which are undoubtedly critical as signaling intermediates, although much remains to be discovered regarding the details of the chemistry generating these species in biological systems [66–68]. One electron oxidation of thiol groups in the presence of radicals can generate the thiyl radical, another reactive species that can go on to form a diverse range of products once formed [69].

Oxidative and reductive processes in biological systems

Oxidation of proteins in cells can be (i) part of a normal enzymatic mechanism, (ii) caused by reactive oxygen species (ROS) elicited as a side product through "leakage" of electrons from the electron transport chain during normal metabolism, (iii) a result of ROS generated from toxins or damaging agents, or (iv) evoked following the regulated generation of cellular oxidants for signaling purposes (Fig. 6) [28, 54]. The now famous "mitochondrial leak" of electrons through oxidative phosphorylation occurring in the electron transport chain of the inner mitochondrial membrane, while not as pronounced as once estimated (with an earlier estimate of ~5% of the total O_2 consumed) [70, 71], is nonetheless an unavoidable consequence of metabolism responsible for the generation of superoxide ($O_2^{\cdot-}$) and other oxidizing byproducts. $O_2^{\cdot-}$ and/or H_2O_2 can also be produced by reduced flavoprotein oxidases or monooxygenases with physiological or pathophysiological reactivity with O_2 (e.g., xanthine oxidase, aldehyde oxidase, glucose oxidase, D-amino acid oxidase and cytochrome P450 reductases).

Activated phagocytes like neutrophils and macrophages are a major source of $O_2^{\cdot-}$ and other potent oxidants such as those shown in Fig. 6. In these and other non-phagocytic cell types, as well, the NADPH oxidase (Nox) family of enzymes (Nox1 – Nox5 plus Duox1 and Duox2) generate $O_2^{\cdot-}$ or in some cases H_2O_2 directly [72, 73]. Special enzymes within phagocytes and eosinophils (myeloperoxidase, lactoperoxidase and eosinophil peroxidase) produce hypohalous (HOCl and HOBr) and pseudohypohalous (HOSCN) acids from H_2O_2 and halides (Cl^- and Br^-) or thiocyanate (SCN^-) [74, 75]. These species are potent oxidants and halogenating agents with microbicidal activities and thus are critical for proper immune function. The gaseous signaling mediator nitric oxide ($NO^{\cdot}$) produced by activated or constitutive nitric oxide synthases is also a generator of peroxynitrite if produced in proximity to short-lived $O_2^{\cdot-}$ [76]. Further, hydroperoxides and H_2O_2 are generated through action of proinflammatory enzymes involved in prostaglandin, prostacyclin and leukotriene synthesis, lipoxygenases and cyclooxygenases [77]. Were it not for the powerful antioxidant enzymes that protect cells from this pool of oxidants, such species would rapidly rise to toxic levels within every cell type.

Protective antioxidant enzymes like superoxide dismutase (which dismutates one oxidant into another, generating H_2O_2 and O_2), catalase, peroxiredoxins (Prxs) and glutathione peroxidases (Gpxs) catalyze oxidant removal by metal-, selenium- or thiol-dependent processes that are sustained, in Prxs and Gpxs, by the cellular reductase systems fueled by NADPH or NADH (Fig. 7).

Enzymes and transcription factors which operate through thiol-based oxidative processes must be regenerated in each catalytic or activation cycle to allow progression through another cycle of activity (Fig. 7). In general, this involves the reductive recycling of

oxidized effector proteins by cellular reductants like GSH, glutaredoxin (Grx) and Trx. As alluded to above, these reductase systems ultimately derive their electrons from NADPH (or in some special cases NADH), which shuttle electrons into the reductant pool via flavin groups and intermediary redox-active disulfide centers [78–80]. While thioredoxin reductase (TrxR) regenerates the protein-based reductant Trx, glutathione reductase regenerates the LMW thiol glutathione. This small thiol can act as a reductant directly (e.g., for glutathione peroxidases) or can mediate reduction in cooperation with Grx proteins (see below). The trypanosomal reductase system of trypanothione reductase, trypanothione and tryparedoxin (a Trx homologue) operates very similarly [81]. An apparently unusual redox relay system in *Clostridium pasteurianum* that recycles a peroxiredoxin (Cp20) in that organism (Fig. 7) consists of a thioredoxin reductase homologue with specificity for NADH (Cp34) and a Grx homologue (Cp9) that requires no glutathione (a LMW thiol that is not present in *C. pasteurianum*), nor any other LMW thiol, for its Cp20 reducing activity [82]. There can be considerable overlap in contributions of various reductants to reductive recycling of a given redox protein (e.g. [47, 83, 84], although this overlap is apparently not sufficient to drive equilibration of the different reductase systems. The redox “poise” (and direct targets of reductase activity) of Trx (oxidized and reduced forms) and GSH/GSSG redox pairs is different in many or all biological systems [85–87]; redox poise of these systems also varies greatly between different compartments of bacterial and eukaryotic cells [88].

Generally, Trx-like systems serve as protein disulfide (or sulfenic acid or S-nitrosothiol) reductase systems, while Grx, which typically relies on GSH and glutathione reductase-mediated regeneration, acts on glutathionylated proteins or small molecules [89, 90]. Nonetheless, in special cases Grx can serve direct dithiol-dependent protein reductase functions as well [47, 82, 83, 91]. It should be noted that in plants, reducing power is derived from photosynthesis through the light reactions of photosystems II and I and the small redox protein ferredoxin, which in turn reduces ferredoxin:$NADP^+$ reductase and replenishes NADPH [92]. In nonphotosynthetic organisms, the NADPH pool is replenished by the pentose phosphate shunt [93].

Cysteine redox modifications relevant to enzyme catalysis and regulation of biological processes

With their special and “tunable” chemical reactivity and ability in most cases to be regenerated, the thiol groups of Cys residues (as well as the less common selenol groups of Sec) play essential, if not exclusive, roles in redox sensing in addition to their catalytic or other functional roles in proteins. Oxidant sensing to trigger the upregulation of cellular defenses is relatively well understood in a number of microbial systems. In a wide variety of bacteria, oxidants (specifically H_2O_2 and $O_2^{\cdot-}$) are sensed directly by transcription regulators using thiol- (e.g., OxyR, OhrR and SarZ) or metal-based (e.g., SoxR and PerR) mechanisms [57, 94–97]. With yeast systems, the situation is considerably more complex, involving both peroxide defense enzymes as well as responsive transcriptional regulators, many or most of which operate through thiol redox modifications [98, 99]. Beyond these simple organisms, the use of redox mechanisms to sense and respond to various types of stimuli is both widespread and multi-faceted; we are at an early point in recognizing these

redox-regulated proteins and pathways in multicellular organisms, but the excitement is building as we gain more and more details about which systems and proteins respond to their redox environment and how their responses are orchestrated [100–102]. Add to that our increasingly fine-tuned understanding of some of the regulated ROS generating systems that produce a significant fraction of the oxidants to be sensed, and it is clear that we CAN begin to construct models for these complex biological networks, but that continued research will be required in order to better understand the redox chemistry underlying vital cell processes.

We present next just a few examples of redox-sensitive, regulated proteins to illustrate some of the mechanisms involved, referring the reader to reviews and landmark proteomics papers that provide a broader view of the developing detailed knowledge of redox sensing and regulation across many biological systems [54, 86, 87, 102–109].

It can hardly be missed that Cys modifications will influence thiol-based functional sites since any modified Cys by definition lacks the thiol/thiolate group. But thiol group modifications can not only switch off reactivity at such sites (e.g., as in protein tyrosine phosphatases, peroxiredoxins and ribonucleotide reductases), but also switch on an alternative activity or function, as with the acquired acyl phosphatase activity of glyceraldehyde-3-phosphate dehydrogenase in its sulfenic acid form [110, 111] or the oxidation-mediated transcriptional activation of disulfide-bonded OxyR [94, 112] and Yap1 [113, 114]. Importantly, it is clear that redox regulation affects other posttranslational modification-altering molecules; the regulation of phosphatases (typically inhibited by oxidation) as well as kinases (with wide ranging effects) point to well established examples of this redox modulation of phosphorylation status. Crosslinking of subunits of the same or different proteins can have a big effect on signaling proteins. For example, the signaling kinase PKGIα is activated upon dimerization which is enforced when a disulfide bond is formed between the same Cys residue (Cys42 and Cys42′) on two different subunits (implying the oxidation of Cys42 in one subunit to sulfenic acid, then condensation with another subunit as the other Cys42′ thiol group approaches) [115]. Other cellular signals are also affected by redox modulation. For example, the sarcoplasmic reticulum/endoplasmic reticulum-associated calcium efflux pump SERCA, which regulates intracellular calcium concentration by mediating uptake of calcium into endoplasmic reticulum stores, becomes activated when its critical Cys (Cys674) is oxidized to S-nitrosocysteine or Cys sulfenic acid, then to the mixed disulfide with glutathione [116, 117]. Extracellular matrix integrity and composition is also under redox control. For some if not all matrix metalloproteinases (enzymes involved in the degradation of extracellular matrix components), activation occurs through sulfinic acid generation at Cys100 in the propeptide, also via initial oxidation to S-nitrosocysteine and/or Cys sulfenic acid (e.g., in rodent and human MMP-9 and MMP-7) [118–120]. In another enzyme family exemplified by nitrile hydratase, cofactor binding is redox regulated. In this case, two cysteines must undergo oxidative modification, one to sulfinic acid and another to sulfenic acid (γ-Cys131 and γ-Cys133, respectively, in bacterial thiocyanate hydrolase), in order to create the nonheme iron binding site critical to its enzymatic function [121, 122]. Thus, the details and effects of redox modification, like the more widely appreciated and better understood effects of phosphorylation, are unique to each protein and critical to understand at a detailed molecular level.

In both chemical and biological terms, Cys sulfenic acid is central to the oxidative pathways of redox regulation. In particular, it is the initial product formed upon reaction of Cys thiols with the small, neutral and highly diffusible oxidant H_2O_2, which has become recognized as a major player in the redox pathways that promote growth factor signaling [72]. It is important to note that H_2O_2 will be present at some level wherever superoxide is being generated due to the rapid nonenzymatic as well as enzymatically-catalyzed dismutation of $O_2^{\cdot-}$. Because Cys sulfenic acid has multiple potential fates once formed, it can even play multiple roles within a single protein; in peroxiredoxins, SOH acts both as a catalytic intermediate and as a point of oxidative regulation, becoming inactivated by sulfinic acid formation. Reactivity of peroxiredoxins toward H_2O_2 is high and formation of the SOH is quite rapid; progression to disulfide bond formation occurs at more variable rates and is affected by many features of these proteins, including oligomeric state [19, 60]. This and the concentration of H_2O_2 appear to be the major factors in whether the SOH will partition toward disulfide bond formation or sulfinic acid generation. It has been hypothesized that this sensitivity toward inactivation by H_2O_2, which is generally higher in peroxiredoxins from multicellular organisms, co-evolved with the widespread use of H_2O_2 as a signaling molecule to enhance its levels and thus contributions to signaling [123]. There are some proteins in which sulfenic acid is a highly stable species, shielded from reactivity with other thiol groups and oxidants; NADH peroxidase from *Enterococcus faecalis* is an excellent example of this, where the SOH form of the single Cys in the protein is the natural oxidized product and is highly stabilized until its direct reduction by the reduced form of the FAD cofactor in this enzyme [58, 124]. Features that stabilize SOH are beginning to be recognized as more examples are found, including those in the protein databank where some detectable fraction of SOH on specific Cys residues is reported [52, 125, 126].

Given their central position with respect to biologically-important oxidation pathways and their unique reactivity relative to other Cys forms, sulfenic acids have become important targets for analysis by proteomic approaches in order to reveal reactive Cys sites in proteins [52, 127]. These efforts are complementary to other Cys-based proteomics approaches which target other modifications or chemical attributes [108, 128–132]. The detection of SOH-containing proteins within cells is also becoming an important approach for learning about protein oxidation involved in cell signaling processes [133–135]. In an evaluation of protein oxidation detected using a fluorescent probe targeted to sulfenic acid (DCP-Rho1) applied to cells activated with the lipid growth signal lysophosphatidic acid (LPA), our group recently found evidence for enhanced protein oxidation around “redoxosomes” (ROS-generating, signaling endosomes) which were also enriched in internalized LPA receptors [134]. This suggests to us that the ability of sulfenic acid-directed probes to report on the spatial distribution of newly oxidized proteins, some but not all of which are tethered in signaling complexes at membranes near where ROS are being produced, is also related to the transience of the SOH modification (typically being converted to disulfide bonds of some sort as they mature). With the enhancement in chemical probes and methodologies for detecting not only protein oxidation, but also the small molecule oxidants that cause the oxidation of proteins [26, 27], the stage is set for rapid expansion of our knowledge of detailed mechanisms involved in redox regulation and sensing in biological systems.

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Highlights

- Many cellular redox processes rely on low MW thiols and cysteines in proteins
- Cysteine is unusual as a polar amino acid that is typically buried in proteins
- Cysteine reactions are affected by pK_a, redox potential and kinetic properties
- Numerous biological pathways require or are modulated by redox-sensitive cysteines

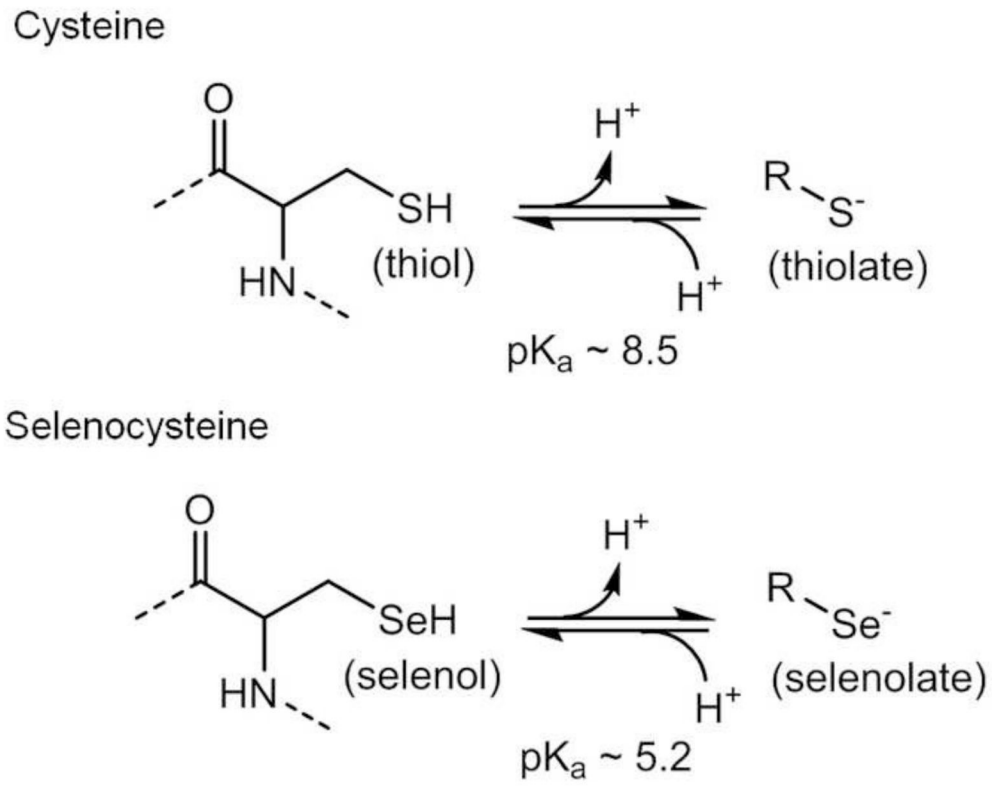


Figure 1.

Structures of cysteinyl and selenocysteinyl residues within proteins. The aminoacyl groups are shown to the left, with dotted lines representing peptide bonds to the next residue on either side. Both protonated (left) and deprotonated (right) forms of these amino acids are depicted with average pK_a values (that can vary in particular protein microenvironments).

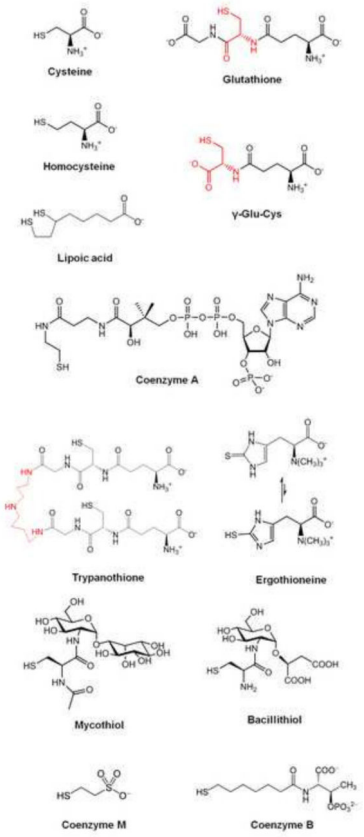


Figure 2.

Biologically relevant thiol-containing small molecules. Red highlights the cysteinyl moiety in glutathione and the spermidine linker in trypanothione. Not shown is glutathione amide (found in some γ-proteobacteria), which includes an amide rather than carboxylate group on the Gly of glutathione.

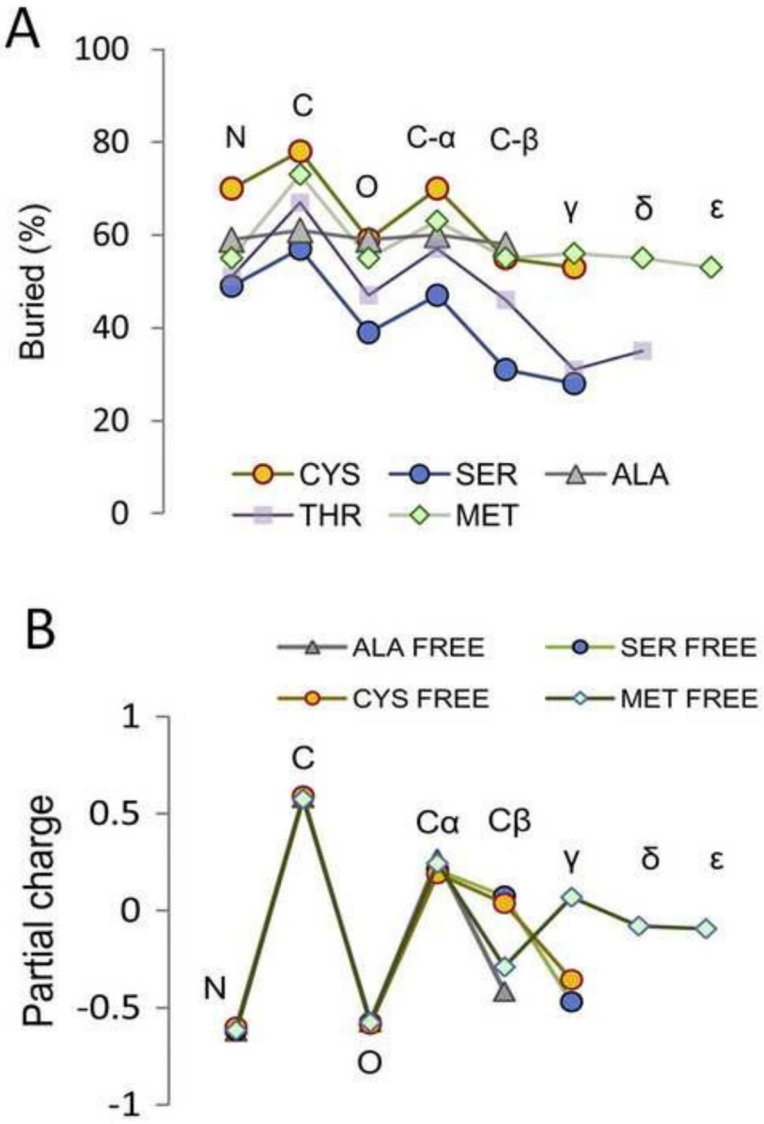
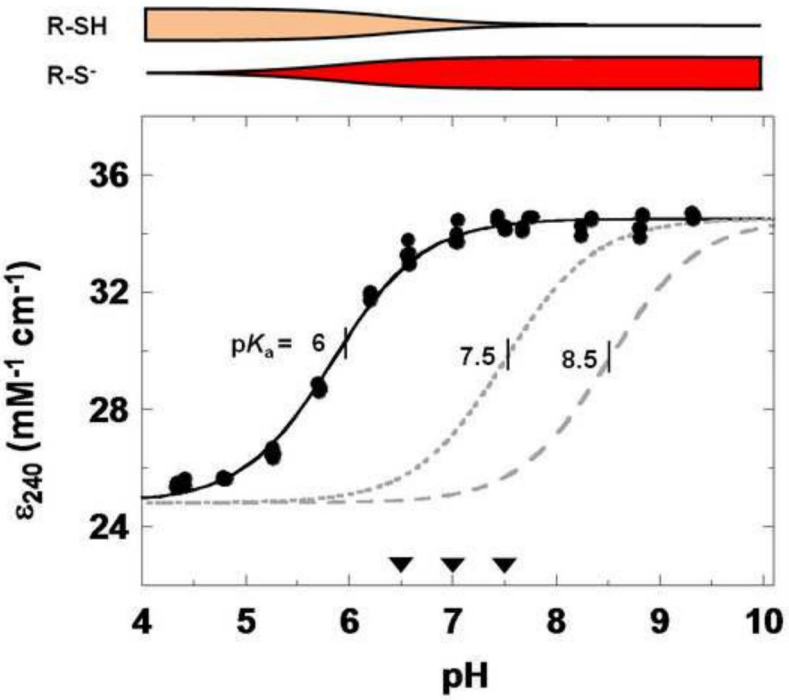


Figure 3.

Cysteine is the least exposed residue in proteins, yet its chemical–physical properties are those of a polar residue. (a) Cys exposure was calculated for approximately 15,000 nonredundant proteins from PDB as described in Marino & Gladyshev, 2010. The percentage of burial is shown for each composing atom of Cys and, for comparison, for each atom in Ser, Ala, Thr, and Met. Above each point, the positions along the side chain are reported (i.e., Cα, Cβ, Cγ, Cδ, and Cε). Note that Thr is Cβ branched (i.e., has two γ atoms and no δ atom). (b) Calculations with a QM approach are plotted as a function of atoms composing an amino acid residue. For comparison, residues with charge distributions similar to that of Cys are shown (data provided by Dr. Annick Thomas and Dr. Robert Brasseur). Reprinted with permission from Marino & Gladyshev (2010). Copyright 2010, Elsevier Ltd.



pK_a	% RS^- pH 6.5	% RS^- pH 7.0	% RS^- pH 7.5
6.0	76	91	97
7.5	9	24	50
8.5	1	3	9

Figure 4.

pH titrations to determine thiol pK_a, and population shifts over biological pH ranges relative to thiol pK_a. Absorbance data collected at 240 nm can in some cases be used to track thiolate formation at various pH values (with fits conducted using the equation given in the text). Using the Henderson-Hasselbalch equation (also in the text), ratios of the populations of protonated and deprotonated species can be calculated given the pK_a of the thiol of interest and the pH of the system (shown in table).

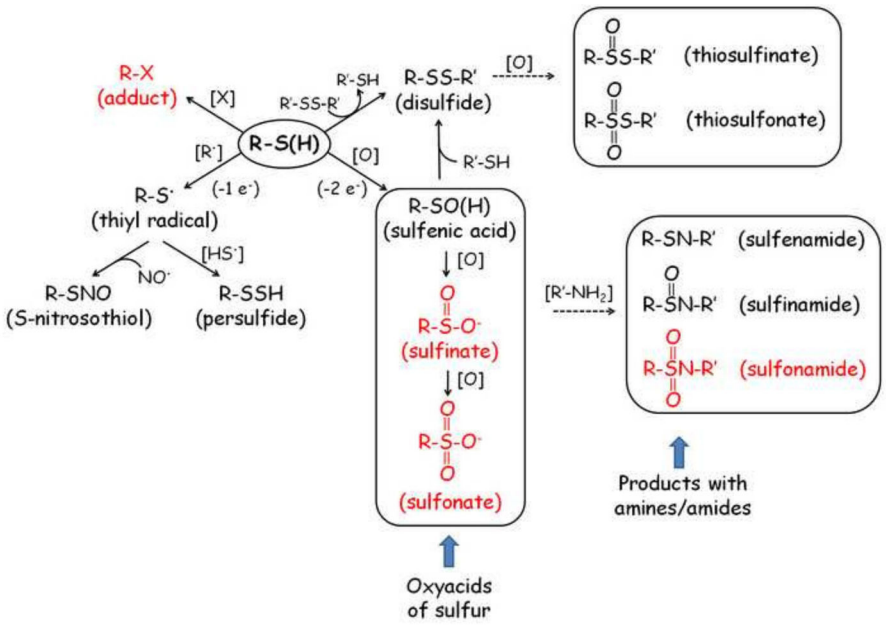


Figure 5.

Modified and oxidized products of thiol groups. Shown are some of the biologically significant modifications occurring on small molecule and protein thiols, including 1 and 2 electron oxidized forms as well as adducts formed with electrophiles (X). Species in red may not be recoverable biologically; one exception is $R\text{-}SO_2^-$ in peroxiredoxins which is recycled by a dedicated repair protein, sulfiredoxin. Chemical pathways shown are representative mechanisms and are not meant to imply that these are the only or most important biological reactions. Dotted lines and brackets are used to indicate overall chemical relationships between groups, but not necessarily specific chemical pathways.

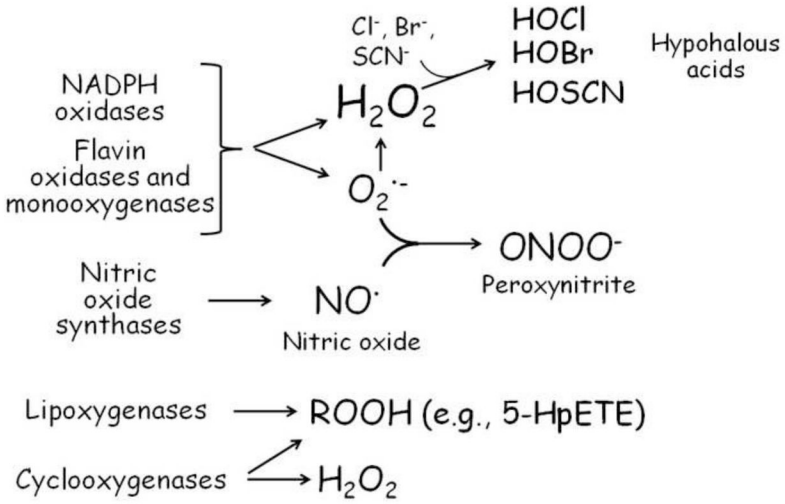


Figure 6.

Biological oxidants of thiol groups. Although neutrophils and macrophages were the first cell types recognized to generate cellular oxidants as an essential part of the body's immune defense, it has subsequently been recognized that there are many sources of oxidants present in many other cell types, as well (see text for details).

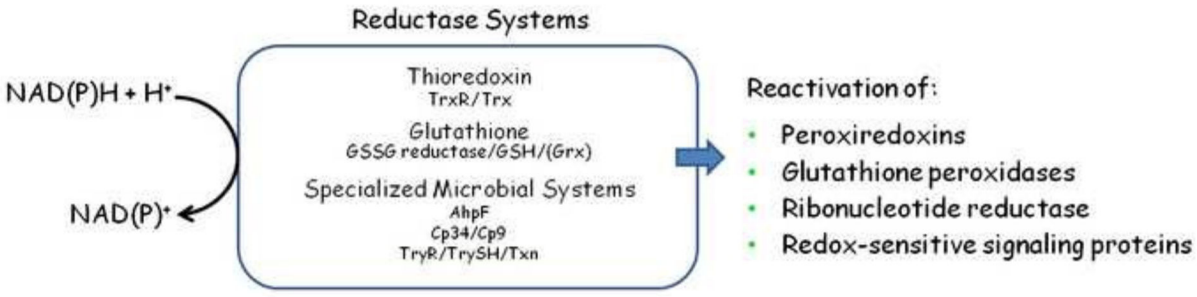


Figure 7.

Electron flow from reduced pyridine nucleotides (NADPH and NADH) and flavin-based reductase systems to reductively recycle key oxidized proteins in cells. Among the reductase systems are the thioredoxin (Trx) system, including thioredoxin reductase (TrxR), the glutathione (GSH) system including glutathione reductase which recycles oxidized glutathione (GSSG), in some cases with the help of glutaredoxins (Grx), and sets of specialized microbial systems with a more restricted distribution in biology. This includes the bacterial peroxiredoxin (AhpC) regenerator AhpF, the clostridial system Cp34 (a Trx reductase homologue) and Cp9 (a Grx homologue), and the trypanothione (TrySH) system, including its reductase (TryR) and the Trx-like small protein tryparedoxin (Txn).

Table 1

$E^{\circ\prime}$ values of Biochemical Redox Couples[a]

Redox Couple	$E^{\circ\prime}$ (V)
½ O_2 + 2 H^+ + 2 e^- ⇔ H_2O	+0.816
O_2 + 2 H^+ + 2 e^- ⇔ H_2O_2	+0.295
Fumarate + 2 H^+ + 2 e^- ⇔ Succinate	+0.03
St AhpC(SS) [b,c] + 2 H^+ + 2 e^- ⇔ *St* AhpC$(SH)_2$ [c]	− 0.178 [d]
Pyruvate + 2 H^+ + 2 e^- ⇔ Lactate	−0.19
GSSG + 2 H^+ + 2 e^- ⇔ 2 GSH	−0.23
*Ec*Grx1(SS)[e] + 2 H^+ + 2 e^- ⇔ *Ec*Grx1$(SH)_2$	−0.233 [f]
Poplar Trx(SS) [g] + 2 H^+ + 2 e^- ⇔ Poplar Trx$(SH)_2$	~−0.29 [h]
NAD^+ + H^+ + 2 e^- ⇔ NADH	−0.32
Poplar PrxQ(SS) + 2 H^+ + 2 e^- ⇔ Poplar PrxQ$(SH)_2$	−0.325 [h]

[a] If not otherwise referenced, values are from Segel, I. H. *Biochemical Calculations.* New York: John Wiley & Sons, Inc.; 1976: 414–415, and Loach, P. A. In: Fasman, G. D., ed. *Handbook of Biochemistry and Molecular Biology*: CRC Press; 1976: 123–130.

[b] *St* AhpC = *Salmonella typhimurium* AhpC, also known as alkyl hydroperoxide reductase.

[c] SS refers to the disulfide form; $(SH)_2$ refers to the dithiol form

[d] From Parsonage, D.; Karplus, P. A.; Poole, L. B. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc Natl Acad Sci U S A* 105:8209–8214; 2008.

[e] *Ec*Grx1=*Escherichia coli* glutaredoxin 1

[f] Åslund, F.; Berndt, K. D.; Holmgren, A. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. *J Biol Chem* 272:30780–30786; 1997

[g] Trx = thioredoxin

[h] Rouhier, N.; Gelhaye, E.; Gualberto, J. M.; Jordy, M. N.; De Fay, E.; Hirasawa, M.; Duplessis, S.; Lemaire, S. D.; Frey, P.; Martin, F.; Manieri, W.; Knaff, D. B.; Jacquot, J. P. Poplar peroxiredoxin Q. A thioredoxin-linked chloroplast antioxidant functional in pathogen defense. *Plant Physiol* 134:1027–1038; 2004.