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Forum Review Article

Kinetic and Thermodynamic Aspects of Cellular Thiol–Disulfide Redox Regulation

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Abstract

Regulation of intracellular thiol–disulfide redox status is an essential part of cellular homeostasis. This involves the regulation of both oxidative and reductive pathways, production of oxidant scavengers and, importantly, the ability of cells to respond to changes in the redox environment. In the cytosol, regulatory disulfide bonds are typically formed in spite of the prevailing reducing conditions and may thereby function as redox switches. Such disulfide bonds are protected from enzymatic reduction by kinetic barriers and are thus allowed to exist long enough to elicit the signal. Factors that affect the rate of thiol–disulfide exchange and stability of disulfide bonds are discussed within the framework of the underlying chemical foundations. This includes the effect of thiol acidity (pK_a), the local electrostatic environment, molecular strain, and entropy. Even though a thiol–disulfide exchange reaction is thermodynamically favorable, it will only take place if the activation energy to form the transition state complex can be overcome. This is accomplished by enzymes, such as the oxidoreductases, that direct reactions in thermodynamically favorable directions by decreasing the activation energy barrier. *Antioxid. Redox Signal.* 11, 1047–1058.

Introduction

Control of redox pathways is crucial for cells, and redox reactions are central in metabolism and in redistribution of cellular energy. While this manifests itself in the most basic aspects of cellular metabolism as seen in, for example, the mitochondrial production of energy through oxidative phosphorylation, the photosynthetic formation of glucose from carbon dioxide or the oxidation of glucose to generate energy, we will here focus on aspects of redox regulation that are specifically related to thiol–disulfide metabolism.

It is a basic premise for most organisms that the extracellular environment is oxidizing, while the intracellular environment overall is kept reducing. The intracellular redox environment is maintained by several low molecular weight substances and controlled by enzymes. Among the first reduced glutathione is the dominating low molecular weight thiol species in most organisms. In the following, the term glutathione is used when referring collectively to the oxidized (GSSG) and reduced (GSH) form of glutathione. Both in glutathione and in proteins, the amino acid cysteine is the main redox-active component. The thiol of cysteine can be oxidized reversibly by formation of a disulfide bond. Cysteine may also form adducts with molecular oxygen to form sulfenic acid, which can be further irreversibly oxidized to sulfinic acid or sulfonic acid. The redox conditions differ from one cellular compartment to another. For example, the cytosol and nucleus are considered reducing, while the ER and Golgi apparatus are considered more oxidizing (31, 51). However, several reducing and oxidizing pathways, which are not at equilibrium, are found within the individual cellular compartments and are subject to changes depending on biological activities and environmental conditions.

The formation of a disulfide bond between two cysteine residues or between a cysteine residue and a small thiol-containing molecule may have significant impact on the structure and function of the macromolecule (12). Since a disulfide bond is formed upon oxidation of the corresponding thiols, the formation and breakage of a disulfide bond is a means to regulate function in response to changes in the environment. Evidence for the involvement of thiol-based redox switches in many cellular processes is emerging (62). In some

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cases, a thiol–disulfide switch regulates cellular function by alteration of protein activity (32, 64). The general picture seems to be that cellular redox sensors do not initially sense changes in the redox environment by thiol–disulfide exchange reactions. The mechanisms rather involve detection of oxidants by radical reactions to form, for example, sulfenic acid or S-nitrosylated derivatives, depending on the type of oxidant (19, 44). To elicit a cellular response, the redox signal is typically transmitted to other proteins by thiol–disulfide exchange reactions and the signal response is in many cases associated with change of protein disulfide status. Disulfide bonds may in some cases be introduced in proteins found in environments with low glutathione redox potential. This means that the disulfide bonds exist—although possibly transiently—where they would be expected to be reduced. In the present review we wish to discuss the cellular thiol–disulfide redox regulation in the context of chemical, thermodynamic, and kinetic properties.

Physical considerations on reaction chemistry

To fully appreciate the implications of thermodynamic and kinetic aspects of redox regulation, it is necessary first to reconsider some basic features of chemical reactivity. For a chemical reaction with a single transition state, the energy landscape may be as depicted in Fig. 1. The energy barrier between the reactants and the transition state is the activation energy of the reaction. The prerequisites for a given reaction to proceed at a considerable rate is that the total energy of the products is lower than that of the reactants ($\Delta G < 0$) and that the energy barrier to the transition state is not too high (Figs. 1 and 2). The higher the activation energy, the less likely is the reaction to occur uncatalyzed, irrespective of the potential energy gain associated with the reaction. Being the biological catalysts, enzymes promote reactions by decreasing the energy barrier to the transition state. Enzymes do not affect the equilibrium of reactions, since ΔG for a reaction is independent of the reaction path. The product may not necessarily represent that of lowest energy given the available reactants (*e.g.*, energy is wasted by the hydrolysis of ATP with ubiquitous water), but rather the pathway that is kinetically favored (*e.g.*, energy is conserved by phosphorylation of glucose by hexokinase). This also means that while metabolic pathways are at the mercy of overall thermodynamic downhill energy, the control of reaction kinetics afforded by enzyme-catalyzed reactions represents a common denominator in living systems. These basic considerations are central to what needs to be considered in the following.

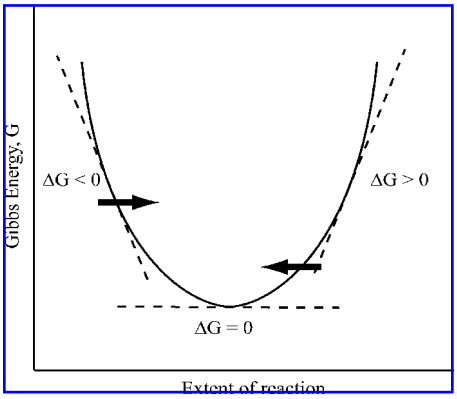


FIG. 2. Influence of Gibbs free energy on the reaction direction. The slope of Gibbs free energy function (the sign of ΔG) states whether the reaction will be thermodynamically favorable in the direction of product formation ($\Delta G < 0$) or in the direction of reactant formation ($\Delta G > 0$). Here the variation in Gibbs free energy at constant temperature is shown as a function of the mass ratio (Q) which is a measure of the extent of reaction. ΔG is given by $\Delta G = RT\ln(Q/K_{eq})$. As the reaction approaches equilibrium, ΔG approaches zero and at equilibrium $\Delta G = 0$ and Q equals K_{eq}. To the left of the equilibrium state, the slope of the Gibbs free energy function is negative ($Q < K_{eq}$), and to the right it is positive ($Q > K_{eq}$), causing the reaction to proceed in the direction of the *black arrows*. Note that even though a reaction is thermodynamically favorable, it may not take place due to activation energy barriers, see text for details.

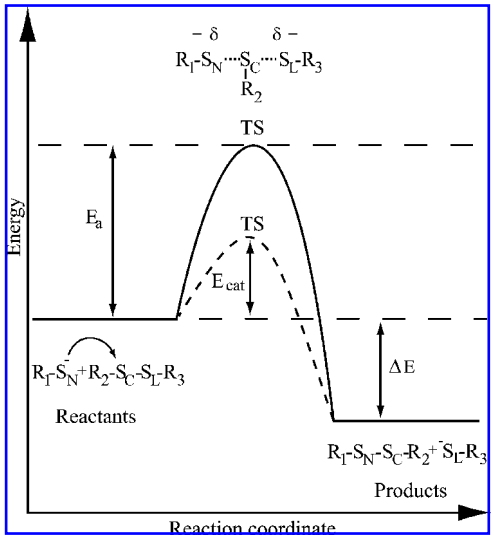


FIG. 1. Schematic energy diagram of a thiol–disulfide exchange reaction. The reaction proceeds through a nucleophilic attack by a thiolate anion on a disulfide to form a transition state (TS) between the nucleophilic thiol (S_N), the central thiol (S_C), and the leaving thiol (S_L). The energy barrier between the reactants and the transition state (TS) is the activation energy (E_a) of the reaction. E_a is decreased by the action of catalysts (E_{cat}). The potential energy difference between reactants and products are denoted ΔE.

Another aspect of the thiol group is the relative ease with which it engages in reactions with other thiols to form disulfide bonds in the presence of an oxidant (*e.g.*, molecular oxygen) and the relatively low activation energy required for thiols and disulfides to equilibrate in thiol–disulfide exchange reactions. Quantum mechanical calculations made by Fernandes and Ramos give an activation energy of 62.0 kJ/mol for the reaction between methylthiolate and oxidized dithiothreitol (DTT) (17). In comparison, the activation energy for ATP hydrolysis in water is much higher and was calculated to be 147 kJ/mol (1). When catalyzed by myosin, it is reduced to a maximum of 61.1 kJ/mol (25). In the interpretation of biological data and in design of experiments, it is important to

consider the interplay between catalyzed and spontaneous reactions.

The Thiol–Disulfide Exchange Reaction

The thiol–disulfide exchange reaction is a bimolecular nucleophilic substitution reaction (S_N2) where the attacking nucleophile is the thiolate anion (Fig. 1). The reaction proceeds with one linear transition state (Note 1) and this geometry will in many practical instances impact the kinetics of the reaction (17). If a cysteine residue, for example, is placed in a protein at a position where it is difficult to attain a linear transition state, it will be less reactive.

Equilibrium conditions

The equilibrium constant (K_{eq}) for a thiol–disulfide exchange reaction is normally referred to as K_{ox} (for oxidation constant), see Box 1 for a definition. K_{ox} can be calculated for any reaction given that K_{ox} for each reactant with the same reference thiol is known (Box 1) and may also be expressed as the ratio between the forward and reverse rate constants. Thus, a connection between rates and thermodynamics for a reaction is established. The relationship implies that only factors that affect the forward and reverse rate constants of a reaction differently will affect the reaction equilibrium. K_{ox} is directly related to other thermodynamic constants such as the standard redox potential of the disulfide bonds involved and the standard Gibbs free energy for the reaction (Box 1 and Box 2).

The redox potential is a measure of the electron affinity of chemical species. The more positive their redox potential is, the higher their tendency is to acquire electrons from other chemical species and thus the more oxidizing they are. For disulfide bonds, the stability and redox potential are equivalent properties and are usually expressed as a standard redox potential (Box 2). Glutathione is often chosen as the standard to which other thiol–disulfides are compared thermodynamically. Importantly, the equilibrium constants and standard redox potentials do not give information about whether a reaction will occur spontaneously under nonstandard conditions (Box 1 and Fig. 2).

Nonequilibrium conditions

From a purely thermodynamic point of view, the transfer of electrons from a protein with low affinity for electrons to another protein with higher affinity will be associated with a negative change in Gibbs free energy and therefore be thermodynamically favored (Fig. 2). However, due to kinetic barriers, this does not always happen in living systems, as seen for the thiol oxidoreductases of the *Escherichia coli* periplasm, DsbA and DsbC (Box 3). In this system, thiol oxidation of one set of substrates is promoted though the efficient oxidation of the soluble thiol-disulfide redox enzyme DsbA by the membrane protein DsbB. At the same time disulfide reduction takes place for a different set of substrates through the continuous reduction of DsbC by the membrane protein DsbD. DsbB and DsbD are in turn themselves oxidized and reduced, respectively, by ubiquinone in the bacterial respiratory system and thioredoxin in the cytosol (21, 34). It may seem paradoxical that oxidation and reduction of cysteine residues can occur side by side in the same compartment. Nevertheless, as the substrates for DsbA and DsbC are different, this system is just a manifestation of a specific kinetic channelling by enzymes. The specific chemical guidance through biochemical pathways in combination with the separation of chemical species by kinetic barriers is generally accepted in other biological systems. Living cells are at steady-state, while equilibrium in a cell is only reached after death. Interestingly, the redox potentials of DsbA and DsbC are fairly similar (Box 2 and Box 3) (58, 70), so while the function of enzymes *in vivo* as either reductases or oxidases is frequently argued on the basis of the standard redox potential of the enzyme, such a correlation cannot be taken for granted.

BOX 1. Thiol–Disulfide Equilibrium

Thiol–disulfide exchange reactions involve a nucleophilic attack of a thiolate anion on an existing disulfide bond, resulting in an exchange of the covalent disulfide bond (Fig. 1). This means that the deprotonation reaction of a thiol to the corresponding thiolate anion (Eq. 1) and the equilibrium constant of this reaction (Eq. 2) hold information about reactivity. The equilibrium constant for a deprotonation is directly related to the pK_a value of the thiol (Eq. 3). The net reaction for the thiol–disulfide exchange between a protein dithiol and glutathione disulfide (GSSG) is shown in Equation 4. For simplicity, all thiol groups are depicted in their protonated form. The corresponding equilibrium constant is referred to as K_{ox} (Eq. 5).

Equilibrium is a dynamic state where forward and reverse reactions take place with equal rates so that equilibrium concentrations are maintained. Thus, as given in Equation 5, the rate constants of the reaction (k_1 and k_2) is related to the equilibrium constant K_{ox}.

$$PSH \rightleftarrows PS^- + H^+ \qquad \text{(eq. 1)}$$

$$K_{eq} = K_a = \frac{[PS^-]_{eq}[H^+]_{eq}}{[PSH]_{eq}} \qquad \text{(eq. 2)}$$

$$pK_a = -\log K_a \qquad \text{(eq. 3)}$$

$$P_{SH}^{SH} + GSSG \underset{k_2}{\overset{k_1}{\rightleftarrows}} P_S^S] + 2GSH \qquad \text{(eq. 4)}$$

$$K_{eq} = K_{ox} = \frac{[P_S^S]]_{eq}[GSH]_{eq}^2}{[P_{SH}^{SH}]_{eq}[GSSG]_{eq}} = \frac{k_1}{k_2} \qquad \text{(eq. 5)}$$

The equilibrium constant is related to the change in standard Gibbs free energy of the reaction (Eq. 6). When the reaction is out of equilibrium, the change in Gibbs free energy associated with the reaction is altered from the standard free energy with a correction term (Eq. 7). K_{ox} and $\Delta G^{o'}$ can only be used to predict the direction of the thermodynamically favorable reaction if the concentrations of all involved species are 1 *M* (standard state). In all other cases, the mass ratio *Q* must be calculated, and the ratio between K_{ox} and *Q* will give the sign of $\Delta G'$ (Eq. 8) and hence reveal the direction of the thermodynamically favored reaction (Fig. 2).

$$\Delta G^{o'} = -RT \ln K_{eq} \qquad \text{(eq. 6)}$$

$$\Delta G' = \Delta G^{o'} + RT \ln Q \qquad \text{(eq. 7)}$$

$$\text{where } Q = \frac{[P_S^S]][GSH]^2}{[P_{SH}^{SH}][GSSG]} \text{ for the reaction in Equation 4}$$

$$\Delta G' = RT \ln\left(\frac{Q}{K_{eq}}\right) \qquad \text{(eq. 8)}$$

R is the gas constant (8.31 J K^{-1} mol^{-1}) and *T* is the temperature (K). $\Delta G'$ is the Gibbs free energy (J mol^{-1}) at pH 7, $\Delta G^{o'}$ is the standard Gibbs free energy (J mol^{-1}) at 1 atm, 298 K and pH 7.

BOX 2. Redox Potential

Any redox reaction can be described by the two corresponding half reactions, each consisting of a conjugated redox pair (Eq. 9 a–c). The tendency for redox reactions to take place depends on the affinity for electrons of the involved species. By definition, electrons will tend to flow from the conjugated redox pair of lower reduction potential to the pair of higher reduction potential. The Nernst equation relates the redox potential of a half reaction to the standard redox potential and the present concentrations of the electron donor and acceptor (Eq. 10 a–c). The change in redox potential ($\Delta E'$) associated with the reaction is defined in Equation 11.

$$P_{SH}^{SH} + GSSG \rightleftarrows P_{S}^{S}] + 2GSH \quad \text{(eq. 9a)}$$

$$\text{Half reaction 1: } P_{SH}^{SH} \rightleftarrows P_{S}^{S}] + 2e^- + 2H^+ \quad \text{(eq. 9b)}$$

$$\text{Half reaction 2: } GSSG + 2e^- + 2H^+ \rightleftarrows 2GSH \quad \text{(eq. 9c)}$$

$$E' = E^{o'} + \frac{RT}{nF} \ln \frac{[\text{electron acceptor}]}{[\text{electron donor}]} \quad \text{(eq. 10a)}$$

$$\text{Half reaction 1: } E_1' = E^{o'}_{P_{SH}^{SH}/P_{S}^{S}]} + \frac{RT}{nF} \ln \frac{[P_{S}^{S}]]}{[P_{SH}^{SH}]} \quad \text{(eq. 10b)}$$

$$\text{Half reaction 2: } E_2' = E^{o'}_{GSH/GSSG} + \frac{RT}{nF} \ln \frac{[GSSG]}{[GSH]^2} \quad \text{(eq. 10c)}$$

$$\Delta E' = E'_{acceptor} - E'_{donor} = E_2' - E_1' \quad \text{(eq. 11)}$$

The redox potential is proportional to the Gibbs free energy of a reaction (Eq.12). From this a relation between the equilibrium constant of the reaction and the standard redox potential may be deduced (Eq. 13 and 14). $\Delta E^{o'}$ and $\Delta G^{o'}$ are constants of the reaction and will only predict in which way a reaction is thermodynamically favorable when the concentrations of all involved species are 1 *M*.

$$\Delta G' = -nF\Delta E' \quad \text{(eq. 12)}$$

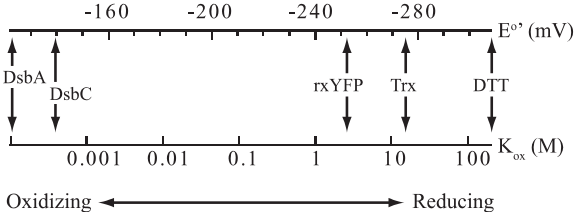
$$\Delta G^{o'} = -nF\Delta E^{o'} \quad \text{(eq. 13)}$$

$$RT \ln K_{eq} = -nF\Delta E^{o'} \quad \text{(eq. 14)}$$

From this, the standard redox potential of a conjugated redox pair in equilibrium with another conjugated redox pair of known standard redox potential can be calculated (Eq. 15).

$$E^{o'}_{P_{SH}^{SH}/P_{S}^{S}]} = E^{o'}_{GSH/GSSG} + \frac{RT}{nF} \ln K_{ox} \quad \text{(eq. 15)}$$

K_{ox} and $E^{o'}$ of different dithiols relative to glutathione are shown below (48, 51, 57, 70, 74). The values are calculated using Equation 15 and a standard redox potential of -240 mV for the glutathione conjugated redox pair (56). Note that small differences in $E^{o'}$ correspond to large differences in K_{ox}.



n is the number of electrons transferred, *F* is the Faraday constant ($9.65*10^4$ C mol^{-1}), E' is the redox potential (V) at pH 7, and $E^{o'}$ is the standard redox potential (V) at 1 atm, 298 K, pH 7.

BOX 3. Kinetic Barriers—Separation of Oxidative and Reductive Pathways in the *E. coli* Periplasma

An illustrative example of kinetic separation is the barrier between the DsbA/DsbB pathway and the DsbC/DsbD pathway. These pathways are found in the bacterial periplasma and are the system responsible for protein disulfide bond formation (34). DsbA has an oxidase function in oxidation of cysteine residues to disulfides in the folding process. The oxidation equivalents are obtained from the periplasma membrane spanning protein DsbB. In parallel, the DsbC/DsbD pathway function in disulfide isomerase activity. DsbC reduces incorrect protein disulfide bonds and is in turn reduced by DsbD, another periplasmic membrane spanning protein. This means that DsbA needs to be in its oxidized form to function in catalysis and DsbC needs to be in the reduced form. A study by Rozhkova *et al.* (58) showed that the reaction between off-pathway redox pairs is in the order of 1,000 times slower than the reaction rates between DsbA/DsbB and between DsbC/DsbD.

DsbA and DsbC do not mutually equilibrate in vivo

The significance of the kinetic separation between the DsbA and DsbC pathways can be seen by the following calculations. The redox reaction of DsbA with glutathione is given by Equation 16 and K_{ox} for the reaction is 0.12 m*M* [Eq. 17; (70)]. A similar reaction scheme for DsbC yields Equation 18 and K_{ox} for DsbC with glutathione is 0.45 m*M* [Eq. 19; (58)].

$$DsbA_{red} + GSSG \rightleftarrows DsbA_{ox} + 2GSH \quad \text{(eq. 16)}$$

$$K_{ox(A)} = \frac{[DsbA_{ox}][GSH]^2}{[DsbA_{red}][GSSG]} = 0.12 \text{ mM} \quad \text{(eq. 17)}$$

$$DsbC_{red} + GSSG \rightleftarrows DsbC_{ox} + 2GSH \quad \text{(eq. 18)}$$

$$K_{ox(C)} = \frac{[DsbC_{ox}][GSH]^2}{[DsbC_{red}][GSSG]} = 0.45 \text{ mM} \quad \text{(eq. 19)}$$

Dividing Equation 17 with Equation 19 yields Equation 20:

$$\frac{K_{ox(A)}}{K_{ox(C)}} = \frac{[DsbA_{ox}][GSH]^2[DsbC_{red}][GSSG]}{[DsbA_{red}][GSSG][DsbC_{ox}][GSH]^2} \quad \text{(eq. 20)}$$

At equilibrium, the terms for [GSH] and [GSSG] cancel out and Equation 21 is obtained.

$$\frac{K_{ox(A)}}{K_{ox(C)}} = \frac{[DsbA_{ox}][DsbC_{red}]}{[DsbA_{red}][DsbC_{ox}]} = 0.27 <=>$$
$$\frac{[DsbA_{ox}]}{[DsbA_{red}]} = 0.27 \frac{[DsbC_{ox}]}{[DsbC_{red}]} \quad \text{(eq. 21)}$$

This means that at equilibrium the $[DsbC_{ox}]/[DsbC_{red}]$ ratio is approximately a factor of 4 greater than that of the $[DsbA_{ox}]/[DsbA_{red}]$ ratio. What is in fact observed *in vivo* is that while the reduced form of DsbA is hardly detectable in the *E. coli* periplasm (40), it is the oxidized form of DsbC that is absent (37, 55). If we conservatively assume that this corresponds to a $[DsbA_{ox}]/[DsbA_{red}]$ ratio of at least 20 and a $[DsbC_{ox}]/[DsbC_{red}]$ ratio of $<1/20$ it means that the actual *in vivo* ratios are greater than a factor 1,600 (20*20*4) from equilibrium.

Molecular basis for the kinetic barrier between DsbB and DsbC

Determination of the definite basis for kinetic separations of pathways is complicated, since many different alterations of a protein may lead to the same change in function. However the molecular basis of the barriers in the Dsb systems is relatively well understood. Investigation of DsbC mutants capable of complementing DsbA null mutant cells gave the first clue to the molecular basis for the kinetic separation. The study showed that interference with residues in the DsbC dimerization interface leading to loss of dimerization of DsbC resulted in complementation of the DsbA null mutants (5). Other experiments suggest that it was not the dimerization *per se* that prevents the oxidation of DsbC by DsbB but rather the geometry of the active site in DsbC (59). This was concluded based on the behavior of mutant variants of DsbC that differed from the wild-type enzyme by deletion of residues in the α-helical linker between the thioredoxin domain and the dimerization domain. Recently the crystal structure of DsbA in complex with DsbB was determined (33). By superposition of one DsbC protomer with the DsbA molecule it became visible that there was not room for the other DsbC protomer since this would be colliding with the membrane.

Parameters Affecting the Kinetic and Thermodynamic Conditions of Thiol–Disulfide Exchange

The rate of protein thiol–disulfide exchange reactions and the stability of disulfide bonds are affected by many factors. These include the pK_a of the involved thiols, the charges of nearby amino acid residues, and steric and mechanical strain. In general, it is difficult to completely distinguish the impact of these parameters on the rate of the reaction and on the thermodynamic properties of reactants and products. With this in mind, we will, in the following, discuss some of the different factors that influence the reactivity and stability.

Implication of cysteine pK_a *values on reaction rate and equilibrium*

The reaction rate of thiol–disulfide exchange is affected by the pK_a values of all thiols involved in the reaction. The attacking species in thiol–disulfide exchange reactions is the thiolate anion and accordingly, the observed rate constant depends on the fraction present as deprotonated thiol. Therefore, the pK_a value of the thiol and the pH of the solution directly influence the rate of the reaction. The thiol pK_a value of most cysteine residues is in the range of 8–9. Thus, cysteine thiols are predominantly in the neutral protonated state under physiological conditions. A decrease of pK_a of the nucleophilic thiol will increase the fraction of deprotonated thiol and therefore enhance the rate. However, the relationship between the pK_a value of the attacking thiolate and the rate are not always straightforward. Features that act to decrease pK_a may also decrease the nucleophilic character of the thiol and hence make it less reactive (61, 63, 67). The effect of lowering pK_a on rate enhancement will in general be most significant when pK_a values are close to solution pH. A small increase in the concentration of the nucleophile resulting from a further reduction of pK_a will be undermined by the corresponding decreased nucleophilicity resulting from electron withdrawal. In other words, a decrease of the pK_a value from, for example, 8.5 to 6 will have a significant effect on the fraction present as deprotonated thiol at neutral pH, while the sometimes observed very low pK_a values, in the range of 3–4, will not have an additional effect on the concentration of thiolate anion.

The reaction rate is also affected by the pK_a values of the central sulfur atom and the leaving group thiol. Systematic studies on small molecules by Wilson *et al.* (67) have shown that the leaving group character is improved when pK_a of the thiol is lowered. Thus, as the pK_a of the leaving thiol is decreased, the rate constant for the reaction will increase (67). Experimental data obtained from small molecule, peptide, and protein studies have revealed that the reaction rate may be described as a function of the sum of the contribution from each individual thiol pK_a value (10, 61, 63, 67). The function is referred to as the Brønsted equation (Note 2). From this relationship it appears that the best leaving group, when a thiolate anion attacks an asymmetrical disulfide (R_1SSR_2), will be the thiol with lowest pK_a. In particular for proteins, the geometrical restrictions will also be very important in directing the reaction in ways that will ultimately determine the leaving group.

pK_a values of protein thiols are influenced by charges on adjacent side chains, the orientation and strength of nearby dipoles, through-bond inductive effects, and solvent accessibility, as previously excellently reviewed by Gilbert (20). The pK_a can be decreased by stabilization of the negative charge of the thiolate anion, for example, by introducing electron-withdrawing groups, or positive charges in proximity of the thiol (10, 45). In small molecules, the pK_a of thiols may also be decreased by resonance stabilization of the negative charge on the thiolate anion (26). Since thiol–disulfide exchange reactions involve negatively charged species both in the ground states and in the transition state, their rates are particularly sensitive to electrostatic factors that alter the electron distribution around the sulfur atom (10, 30). Negative charges near the thiol tend to destabilize the thiolate anion and decrease the observed reaction rate, while positive charges often increase the rate. Again, specific interactions between the reactants may introduce overlaying effects that can become quite significant.

As is the case for the rate constant, the equilibrium constant of a thiol–disulfide exchange reaction is also affected by the pK_a values of the involved thiols. By substituting the Brønsted equation into Equation 5, Box 1, a correlation between K_{ox} and pK_a is obtained (Note 3). This shows that pK_a of the nucleophilic thiol and pK_a of the leaving group thiol both come into play. A study of the effect on kinetics and K_{ox} of introducing positive charges near a protein dithiol underpins this notion (30). Whereas the introduced positive charges resulted in a general increase of rate of disulfide bond formation, both decreased and increased values of the equilibrium constant were observed. This illustrates the experimental difficulty in varying pK_a of one of the involved thiols isolated without affecting other parameters. The criterion for K_{ox} to be affected is that the reactants are either stabilized or destabilized relative to the products by the collective alteration of pK_a values.

Because of the importance of the thiol protonation state, the pH of the aqueous solvent may also affect the kinetics and thermodynamics of the reaction. The collective effect of pK_a of the thiols and pH may result in different stabilization of reactant and product thiolate anions and hereby make K_{ox} pH dependent. In general, an increase in pH corresponds to the effect of a decrease in pK_a. Therefore pH-sensitive equilibrium constants will decrease with increasing pH (Note 3). This relation was shown for the reaction between redox sensitive yellow fluorescent protein (rxYFP) and glutathione and also for the reaction between *E. coli* DsbA and glutathione (51, 70).

Effects of entropy and strain on rate and equilibrium

Disulfide bonds come in two variants: intramolecular and intermolecular. While there is no chemical distinction between the covalent bond once formed, there are considerable differences in the contribution from different factors on their formation. In this context, it is helpful to think of the entropy as a measure of the randomness and disorder in a system, where a gain of entropy helps to drive a reaction. Formation of intramolecular disulfide bonds are frequently associated with less entropy loss than the formation of intermolecular disulfide bonds. This is because thiols in the same molecule are already linked, whereas formation of an intermolecular disulfide bond will link two molecules together, which will be associated with loss of translational and rotational degrees of freedom. The effective concentrations of the thiols may be very high when intramolecular disulfide bonds are formed. Any factor that places two cysteine residues close together with the right geometry will decrease their spatial degrees of

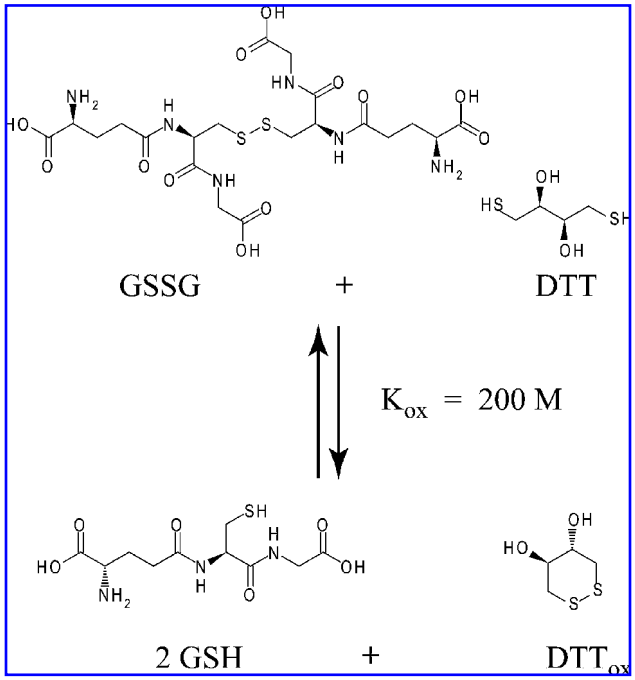


FIG. 3. Reaction between DTT and glutathione. The ring structure formed in the dithiol DTT upon oxidation is very stable and the equilibrium constant (K_{ox}) of the reaction is 200 *M* at pH 7 (11, 57).

freedom and increase the effective molarity, factors that together result in an increase in the reaction rate and the equilibrium constants in favor of disulfide bond formation. Therefore formation of intramolecular disulfide bonds will typically be more efficient than formation of intermolecular disulfides. This is illustrated by the reaction between the two small molecules glutathione and DTT shown in Fig. 3. While the oxidation of the dithiol DTT leads to the formation of an intramolecular sterically favorable disulfide, the oxidation of the monothiol GSH results in the formation of an intermolecular disulfide (GSSG). Accordingly, the equilibrium for this reaction is strongly shifted towards the oxidized form of DTT (11, 57).

On the contrary, formation of a disulfide bond might result in strain of the molecule. The introduced strain is directly connected to the stability of the bond. If only little or no strain is introduced upon formation, the bond will be stable (50). Very high K_{ox} values have been observed for structural disulfides, for example, a K_{ox} of bovine pancreatic trypsin inhibitor is found to 10^5 *M* (13). In contrast, if the formation of the disulfide bond imposes strain on the protein, by making it less flexible, this will make the disulfide bond less stable (the breakage of the bond will be associated with a larger gain in entropy compared to the situation without strain on the molecule) (69). Such disulfides will have low K_{ox} values, as exemplified by DsbA, where the overall structure of the oxidized protein is less stable than the reduced (71).

Thiol–disulfide exchange reactions were found to be promoted by force using single molecule force-clamp spectroscopy (66). This means that if the formation of a disulfide bond is associated with stretching of the molecule, resulting in strain on the formed bond, it will affect the kinetics, making it more reactive.

In the folding pathways of secretory disulfide-containing proteins there is interplay between the folding and the disulfide bond formation. On the one hand, the formation of a disulfide bond in an unfolded protein leads to a significant loss of translational, rotational, and vibrational degrees of freedom. If such disulfides represent a structurally incorrect disulfide, it will remain unstable and will not be supported by an overall stabilization of the structure on the folding pathway. On the other hand, noncovalent interactions that impose organization on the unfolded state will make disulfide bond formation more favorable due to a decrease of entropy-loss associated with its formation (9). Thus, the formation of protein structure will drive the formation of the correct disulfide bonds. It is important to recognize that formation of correct structural disulfide bonds may indeed proceed more efficiently under reducing conditions, because isomerization is favored and because formation of correct disulfides are ultimately driven by formation of the correct fold (22). Once the proteins are secreted into a more oxidizing environment, reduction of disulfide bonds will be thermodynamically unfavorable and this will effectively lock the structure in a folded conformation.

Catalysis of Thiol–Disulfide Exchange Reactions

Enzymes that catalyze thiol–disulfide exchange are classified as oxidoreductases and in general share the thioredoxin fold (Fig. 4). The active site contains at least one but often two cysteine residues arranged in a CXXC motif. A general feature is that the N-proximal cysteine residue acts as the nucleophile

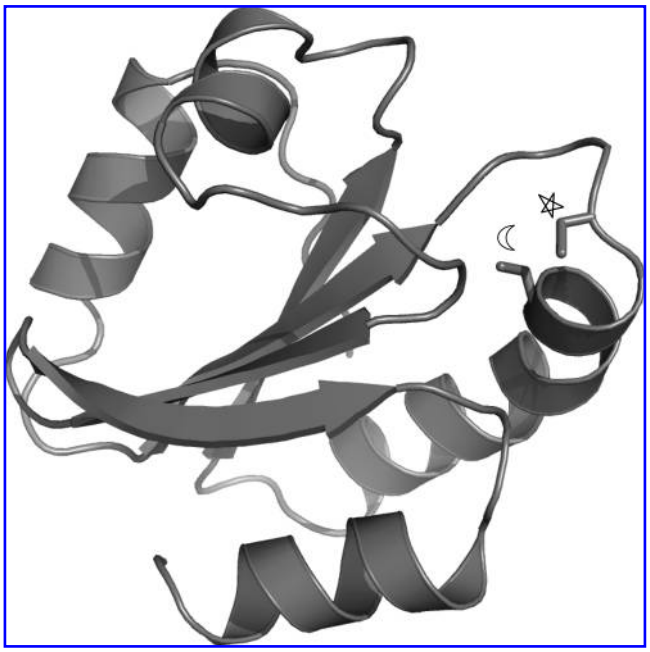


FIG. 4. The thioredoxin fold. As an example of the thioredoxin fold (a central β-sheet sandwiched by four helices), the human enzyme is shown. The enzyme is in the reduced state and the reactive thiol (indicated by a *star*) and the buried thiol (indicated by a *moon*) of the CGPC active site motif are shown in stick model (Model drawn in Pymol based on PDB entry 1AIU) (2, 14).

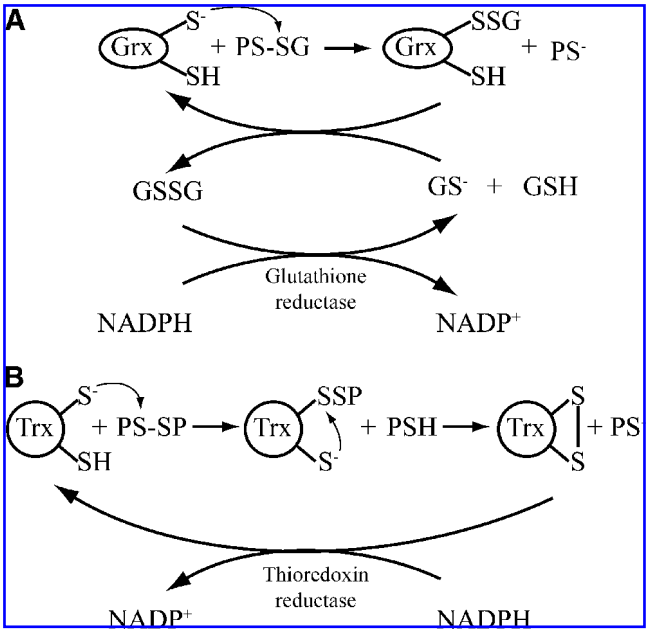


FIG. 5. Reaction pathways for disulfide bond reduction in the cytosol. (**A**) Glutaredoxin (Grx) has a high specificity for glutathione and therefore has a preference for reduction of glutathionylated proteins (PSSG). Grx catalyze the reduction of PSSG through a thiol–disulfide exchange mechanism, which results in the formation of glutathionylated Grx. This intermediate is reduced by GSH, resulting in the regeneration of reduced Grx and the formation of GSSG. In turn, GSSG is reduced by NADPH catalyzed by the glutathione reductase. (**B**) Thioredoxin (Trx) functions in the direct reduction of protein–protein disulfide bonds (PSSP). In contrast to Grx, Trx reduces PSSP through a dithiol mechanism resulting in the formation of an intramolecular disulfide bond between the two thiols of the active site. The reduction of oxidized thioredoxin is catalyzed by thioredoxin reductase and the reducing equivalents are provided by NADPH.

in the substitution reaction with the substrate (47). This means that substrate thiol–disulfide exchange is catalyzed by thiol–disulfide exchange with the enzyme.

Among the thioredoxin-fold oxidoreductases the glutaredoxins stand out. The glutaredoxins either have one or two cysteines in the active site, but are found to catalyze primarily by a monothiol mechanism (Fig. 5A) (24). Glutaredoxins have a specific binding site for glutathione that gives them a high specificity for glutathionylation/deglutathionylation reactions (73). In the reaction mechanism, a mixed disulfide between glutaredoxin and glutathione is formed. By a subsequent thiol–disulfide exchange with reduced glutathione the enzyme is regenerated. Other oxidoreductases utilize a dithiol mechanism (Fig. 5B). In this reaction a mixed disulfide between the enzyme and the protein substrate is formed. This intermediate is released by a nucleophilic attack by the second cysteine residue in the active site (72).

How do these enzymes catalyze thiol exchange? A key factor is that the pK_a value of the N-proximal cysteine residue in general is low, whereas the pK_a value of the C-proximal cysteine is close to what would be expected for a generic cysteine thiol. As discussed above, the main effect of this is that the effective concentration of the nucleophile is high, but it also enhances the leaving group character of the thiol in the second thiol–disulfide exchange reaction.

An extreme example is the N-proximal cysteine in the CXXC motif, Cys30, in the *E. coli* protein DsbA which has a pK_a value of 3.5 (23, 49). Stabilization of the thiolate anion of Cys30 is obtained by hydrogen bonding to backbone amides of nearby residues which is favored in the N-terminal of α-helices together with a minor contribution from positive charge of the α-helix dipole (18, 27, 41, 52, 53). The low pK_a value might be an advantage in increasing the nucleophilic and leaving group character, but will also preserve the catalytic activity at low pH, enabling disulfide bond formation even under such intrinsically unfavorable conditions in the *E. coli* periplasm. Similar stabilizations are seen in other oxidoreductases and in glutaredoxins a conserved positive charge (Arg or Lys) are also found to interact with the N-proximal cysteine residue (35).

Different oxidoreductases have different substrate specificities. Since the overall fold of this class of enzymes is identical, this must be achieved by differences in the local environment around the active sites. Investigations of the human glutaredoxin 1 and glutaredoxin 2 have revealed that there are interesting differences in their substrate interaction (36). Two glutathionylated protein substrates were compared, and glutaredoxin 1 was found to have a higher K_m but also a higher k_{cat} for both substrates compared to glutaredoxin 2. Although both enzymes catalyze the reactions the differences indicates that their physiological function might be different.

The Cellular Thiol–Disulfide Redox Status

There are several aspects of cellular metabolism which require a reducing environment. For example, it is a prerequisite for proper protein synthesis that the amino acid cysteine is kept in its reduced state in order to be incorporated into proteins. Likewise, another central metabolite, coenzyme A, requires a free reduced thiol in order to be functional in acyl thioester transfer reactions. In addition to this, reduced cysteine thiols are involved in the function of numerous transcription factors and enzymes. Whereas redox conditions can be controlled within the boundaries of the cell-enclosing lipid bilayer, the control that can be exerted on the extracellular space is naturally much less stringent. Thus, in the environment of most living organisms, molecular oxygen is a factor that needs to be counted in. This explains the tendency of cysteine residues to form disulfide bonds in proteins found in the extracellular space. However, the presence of a cysteine residue in the sequence of a secretory protein does not necessarily entail that it participates in a disulfide bond. Examples of unpaired cysteines in secretory proteins are carboxypeptidase Y and invertase from *Saccharomyces cerevisiae* (6, 54).

The cell contains several redox pairs, each defining a redox potential, and individual pathways that are not in equilibrium with one another can coexist. This is possible only because they are separated by kinetic barriers. Therefore, in the definition of compartments as reducing or oxidizing, it is necessary to keep in mind that it does not imply that all molecules in a reducing compartment are reduced or vice versa (Box 3).

Chemistry of Redox Switches—Disulfides in Reducing Compartments

Whereas oxidoreductases and disulfide isomerases use disulfide bond formation and breakage as part of their catalytic mechanism, other proteins use reversible disulfide bonds as a redox-switch to regulate their function. There are also examples of redox-active proteins that are regulated by nonactive-site disulfide redox-switches (60, 65).

Proteins that sense the redox state of the surroundings can be divided into different groups based on their chemistry of action. Mechanisms include formation of internal disulfide bonds, change of metal ion status, and reversible modification of free thiols (44). Some proteins are regulated by more than one of these mechanisms. Many of the proteins involved in redox sensing, regulation, and signaling are found in the cytosol and nucleus. These cellular compartments are regarded as reduced; the reduced state of protein thiols is largely favored, and the steady state concentrations of glutathionylated proteins are very low (REH and JRW, unpublished data). There are two cytosolic pathways that are responsible for keeping protein thiols reduced—the thioredoxin/thioredoxin reductase pathway and the glutaredoxin/glutathione reductase pathway, which both use NADPH as electron donor (Fig. 5).

Increasing evidence for an involvement of glutathionylation in redox signaling and regulation are found, for a recent review see Shelton and Mieyal (62). Protein glutathionylation is reversible and catalyzed by glutaredoxin and when considering its involvement in regulation of specific proteins, it is necessary to keep the half-life of the glutathionylated species in mind. To be present in significant amount under nonextreme cellular oxidative conditions, there must be kinetic barriers to glutaredoxin to prevent rapid deglutathionylation.

An example of a system that responds to increased oxidant concentration in the cytosol is the yeast transcription factor Yap1. Yap1 is a leucine zipper transcription factor that is activated by the oxidant receptor peroxidase 1 (Orp1) following Orp1 reaction with H_2O_2 (Fig. 6) (16). The mechanism by which the Yap1/Orp1 system senses and channels the signal of increasing H_2O_2 levels to a transcriptional response is relatively well understood. Orp1 is specifically modified by H_2O_2 at one cysteine residue, which has a pK_a value of 5.1, by formation of a sulfenic acid derivative (46). Yap1 is inactive in its reduced form and gets activated in response to elevated H_2O_2 upon reaction with the Orp1 sulfenic acid derivative (15). This results in the active form of Yap1 containing a specific disulfide bond (16). The formation of the disulfide bond in Yap1 alters the structure of the protein and results in a nuclear accumulation by masking of a nuclear export signal (68). Yap1 activates the transcription of genes that encode for antioxidants including thioredoxins (43). Activated Yap1 is inactivated by Yap1 specific thioredoxin by reduction of the disulfide bond, which leads to unmasking of the nuclear export signal and export of Yap1 to the cytosol (15, 68).

The *E. coli* transcription factor OxyR is another interesting example of a cellular redox switch. Similar to Yap1, the function of OxyR is regulated by the cellular redox environment. The C-terminal domain of OxyR contains a redox sensing loop with two conserved cysteine residues (Cys199 and Cys208; (12)). A very small elevation of the H_2O_2 concentration is sensed by OxyR by reaction with Cys199 presumably forming a sulfenic acid derivative (3, 75). In turn, a disulfide bond between Cys199 and Cys208 is formed (75). Activation of OxyR alters its DNA binding site, allowing interaction with DNA and at the same time results in exposure of RNA polymerase binding sites (42). OxyR induces, among other genes, the transcription of glutathione reductase and glutaredoxin 1. Glutaredoxin 1 has been shown to specifically reduce OxyR and hereby deactivate the transcription factor. Crucially, this reduction is slow (*i.e.*, on a time scale of hours) (75). Investigation of the crystal structure of the reduced form (OxyR C199S) and the oxidized form of OxyR showed that the two cysteines are in the order of 17 Å apart in the reduced

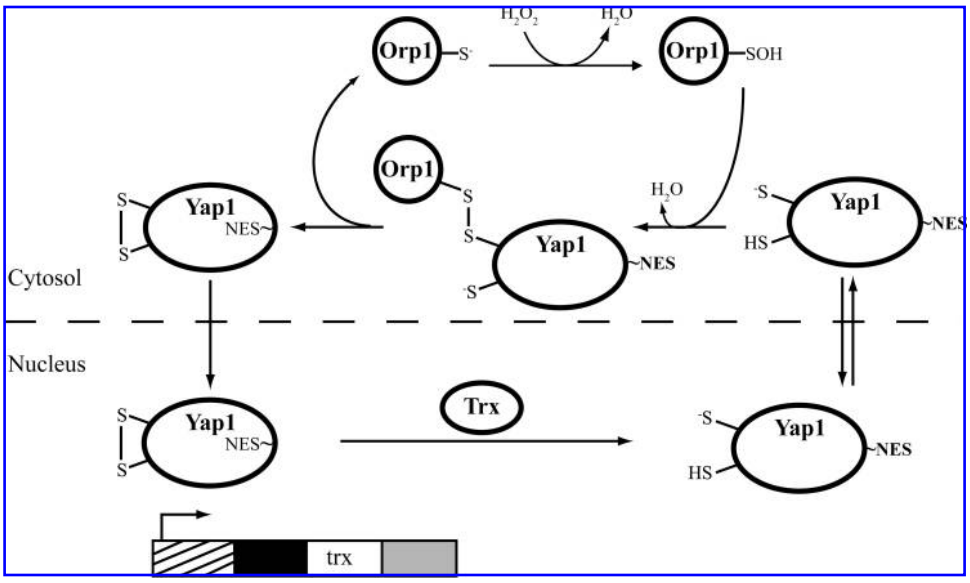


FIG. 6. Reaction pathway for the H_2O_2 redox sensing system Orp1–Yap1. Abundance of reduced Yap1 in the nucleus is low due to the presence of a nuclear export signal (NES). During oxidative stress, a cysteine residue in the redox sensor Orp1 reacts with cytosolic H_2O_2 to form a sulfenic acid, which in turn oxidizes reduced Yap1 involving a thiol–disulfide exchange reaction. The disulfide bond formation alters the structure of Yap1, resulting in masking of the NES. Oxidized Yap1 accumulates in the nucleus where it activates the transcription of genes that encode antioxidants including enzymes of the thioredoxin family. Finally, in a slow feed-back cycle, Yap1 is inactivated by thioredoxin-mediated reduction, leading to an unmasking of NES and export to the cytosol.

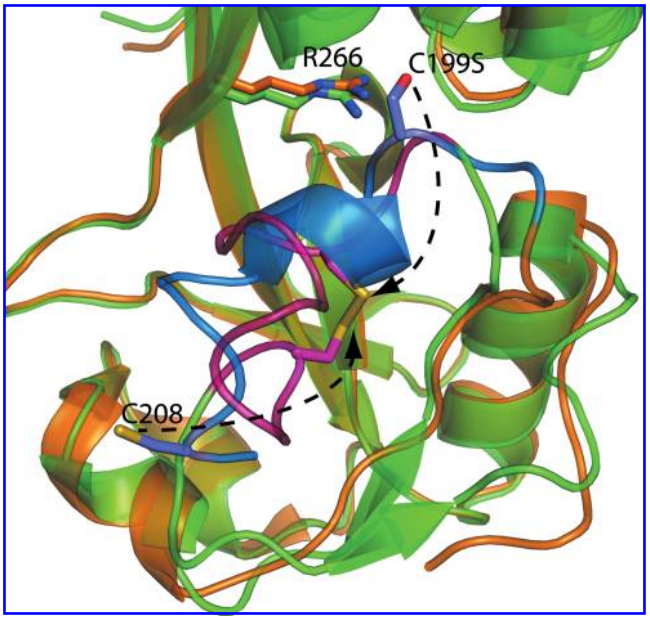


FIG. 7. Structural differences between oxidized and reduced OxyR. The structure of reduced OxyR C199S (*orange*; PDB entry 1I69) is aligned with the structure of the oxidized OxyR (*green*; PDB entry 1I6A). The loop containing the redox active cysteine residues are colored *blue* (reduced) and *pink* (oxidized). The two cysteine residues (C199 and C208) are 17 Å apart in the reduced structure. When the disulfide bond is formed, the nuclophilic C199 is moved out of its environment in the reduced structure and is no longer stabilized by an arginine residue (R266) (12). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

structure. The formation of the disulfide bond leads to significant alteration of the structure, moving Cys199 out of its local environment in the reduced form (Fig. 7) (12). In the reduced form, Cys199 interacts with the side chain of Arg266, which must result in stabilization of the thiolate anion of Cys199, making it more reactive in the reaction with H_2O_2. Moving of Cys199 away from Arg266 upon formation of the disulfide bond presumably results in stabilization of the disulfide bond, since the leaving group character of Cys199 must be decreased when the thiolate anion is no longer stabilized by Arg266 (12).

Cytosolic redox switches illustrate that the disulfide bond status of the cytosol is important for cellular functions. During exposure to reactive oxygen species, redox switches may form a disulfide bond in an environment with low glutathione redox potential to become activated. In order for this disulfide bond to elicit a signal it is required to be sufficiently long lived. This is only possible if there are kinetic barriers between the regulatory disulfide bonds and the reductive pathways. Regulation by dynamic redox equilibrium states may represent an alternative mechanism. When, for example, the cellular redox potential of glutathione is changed by environmental factors, thiol–disulfide switches may respond purely to thermodynamic changes. This requires that the equilibrium with glutathione is rapid and the relevant protein disulfide switch has a sufficiently low redox potential (51). Large changes in total cellular glutathione redox status have been observed in many cell types during changes in growth conditions or oxidative stress (38). For example, the glutathione redox potential of whole-cell extracts are found to range from −260 to −200 mV when proliferating and differentiating cells are compared (39). A change specifically in cytosolic glutathione redox potential in response to changes of growth conditions is similarly seen in mammalian cell cultures containing a GFP-based redox sensor, however, the values here are in a more reducing regime (−320 to −280 mV) (28, 51). The contribution of this mechanism to cellular redox regulation remains to be investigated since redox potentials of switches are largely unknown and comprehensive determination of compartmental changes of, for example, the redox potential of the glutathione couple are challenging.

Implications of Kinetic Barriers in Analytical Redox Sensing

When analyzing redox conditions using, for example, GFP-based sensors, it is important to keep kinetic barriers between different redox pools in mind [for review, see Björnberg *et al.* (7)]. As demonstrated by Østergaard *et al.* (51), the redox sensitive yellow fluorescent protein (rxYFP) can be used to measure the yeast cytosolic levels of reduced and oxidized glutathione. Interestingly, the observation that the rxYFP redox-sensor is partly disulfide bonded *in vivo* illustrates that if a disulfide bond is sufficiently stable, there are no inherent barriers to its formation in the cytosol. In this particular instance, oxidation is catalyzed by glutaredoxins that are normally associated with reduction of disulfide bonds. However, basic chemistry of glutaredoxins dictates that they catalyze the equilibration between the glutathione redox pair and substrate protein thiols (Fig. 8A). For most disulfide bonds, one might expect that the cytosolic glutathione redox potential is sufficiently low to react and form the corresponding glutathionylated protein in an uncatalyzed reaction. This in turn will be efficiently reduced by glutaredoxin.

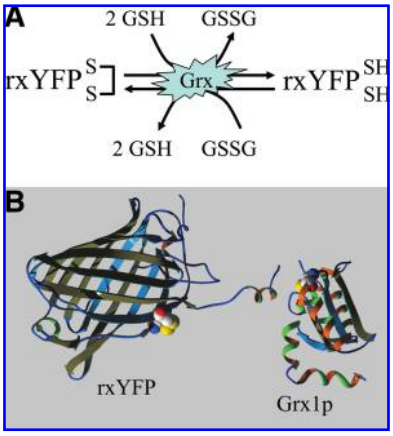


FIG. 8. Glutaredoxin is required for rxYFP to equilibrate with the glutathione pool. (**A**) Glutaredoxin (Grx) catalyze the equilibration of rxYFP with the cellular glutathione pool. Reduced glutathione (GSH) and oxidized glutathione (GSSG). (**B**) The crystal structure of rxYFP fused to *S. cerevisiae* glutaredoxin 1 C30S (PDB entry 2JAD, (29)). Disulfide bonds are shown in space-filling rendering. The linker between rxYFP and Grx is partly absent from the model, due to flexibility. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Thus, the use of rxYFP as cellular glutathione redox sensor hinges on two prerequisites: a) that it changes redox state at a relevant redox potential, and b) that it equilibrates fast and selectively with the glutathione pool catalyzed by glutaredoxin (Fig. 8). To compensate the need for the catalytic action of endogenous glutaredoxin, a fusion protein between rxYFP and glutaredoxin was constructed (Fig. 8B) (8). Recently, based on this principle, a fusion between the redox sensitive green fluorescent protein and glutaredoxin has been developed and demonstrated to function in measurements of the cellular redox potential of glutathione in mammalian cells (28).

In general, when a specific sensor is used in experiments to answer questions about cellular redox conditions, it is very important to evaluate with which redox pool the sensor equilibrates. In other words, it is not possible to develop a sensor for "*the redox potential*" of a given cellular compartment, since more than a single redox potential must be expected to exist. Most likely, all cellular compartments contain redox couples that are not in mutual equilibrium and therefore hold different redox potentials.

Perspectives on Redox Regulation

We hope with this review to have highlighted some theoretical implications of thiol–disulfide redox chemistry on cellular redox control and the function of thiol–disulfide redox enzymes. The basic message is that even though a reaction is thermodynamically favorable, it will only take place if there is not a significant kinetic barrier. In the cytosol, regulatory disulfide bonds may well be thermodynamically out of equilibrium with glutathione and regulation must then take place at the level of kinetic control of reduction. With this in mind, understanding which factors that affect the stability and rate of reduction might shed light on which cysteine residues can be expected to participate in regulatory disulfide bonds. In addition, better understanding of the substrate specificity of the different oxidoreductases that are expressed in the cytosol and nucleus will be important for further understanding of the premises for formation of regulatory disulfide bonds.

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Abbreviations

DTT, dithiothreitol; GFP, green fluorescent protein; GSH, reduced glutathione; GSSG, oxidize glutathione; K_{eq}, equilibrium constant; K_{ox}, oxidation equilibrium constant; Orp1, oxidant receptor peroxidase 1; rxYFP, redox sensitive yellow fluorescent protein.

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Appendix

Note 1

A recent theoretical study involving a model peptide implies that the thiol–disulfide exchange takes place by an addition-elimination reaction with a trisulfur anionic intermediate with a geometry very similar to the geometry of the transition state in the S_N2 reaction (4).

Note 2

The Brønsted equation is given by: $\log k = \beta_N pK_a^N + \beta_C pK_a^C + \beta_L pK_a^L + D$, where k is the rate constant, β are the Brønsted coefficients for the nucleophilic thiol (N), the central thiol (C) and the leaving group thiol (L), and D is a constant. The value of β_N in thiol–disulfide exchange reactions has been determined with relatively high accuracy to +0.5 (63, 67). The values of β_C and β_L have not been measured directly, but calculated from indirect measurements. β_C is in the order of (−0.3) and β_L is in the range of (−0.5) to (−0.7). These values are not determined with an accuracy that allows interpretation of the transition state (63). Recent work suggests that the charge is shared by the nucleophilic and leaving group sulfur atoms, while the central sulfur is neutral in the transition state (17).

Note 3

The equilibrium constant for a reaction (K_{eq}) is defined as the ratio between the forward (k_1) and reverse (k_2) rate constants (Box 1). Substituting the Brønsted equation into this expression for a single thiol-disulfide exchange reaction $R_1S_1^- + R_2S_2S_3R_3 \rightleftarrows R_1S_1S_2R_2 + R_3S_3^-$ gives: $\log K_{eq} = (\beta_N - \beta_L)(pK_a^1 - pK_a^3)$. Note that if there is a large difference between pK_a^1 and pK_a^3 then K_{eq} will be strongly pH dependent (23, 63, 71). A corresponding relation may be derived for a reaction consisting of two successive thiol–disulfide exchange reactions as Equation 4, Box 1.

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