EVOLUTION OF THE THIOREDOXIN SYSTEM AS A STEP ENABLING ADAPTATION TO OXIDATIVE STRESS

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HIGHLIGHTS (3 to 5).

- Cysteine redox chemistry has been exploited during evolution to reflect the redox status of the cellular environment.
- The thioredoxin system is composed of a reductant, a thioredoxin reductase, and a thioredoxin; it controls the formation of disulfides in proteins under control conditions in cells.
- Thioredoxins are present in all domains and are prevalent in both anaerobes and aerobes.
- Despite the high sequence and structure conservation of thioredoxins, they show high specificity for their targets.
- Thioredoxin reductases have evolved to connect the reversible oxidative modification of enzymes to metabolism, photosynthesis and fermentation, for metabolic regulation and adaptation to changing environmental conditions.

ABSTRACT

Thioredoxins (Trxs) are low-molecular-weight proteins that participate in the reduction of target enzymes. Trxs contain a redox-active disulfide bond, in the form of a WCGPC amino acid sequence motif, that enables them to perform dithiol-disulfide exchange reactions with oxidized protein substrates. Widely distributed across the three domains of life, Trxs form an evolutionarily conserved family of ancient origin. Thioredoxin reductases (TRs) are enzymes that reduce Trxs.

emergence of variants of the enzyme that in combination with different types of Trxs meet the needs of the cell. In addition to participating in the regulation of metabolism and defense against oxidative stress, Trxs respond to environmental signals—an ability that developed early in evolution. Redox regulation of proteins targeted by Trx is accomplished with a pair of redox-active cysteines located in strategic positions on the polypeptide chain to enable reversible oxidative changes that result in structural and functional modifications target proteins. In this review, we present a

general overview of the thioredoxin system and describe recent structural studies on the diversity of its components.

Keywords.

Thioredoxin; Redox regulation; Oxidative stress; Disulfide; Thioredoxin reductase.

Abbreviations

Cys, cysteine.

DTR, deeply-rooted thioredoxin reductase.

FAD, flavin adenine dinucleotide.

FFTR, ferredoxin-dependent flavin thioredoxin reductase.

FTR, ferredoxin: thioredoxin reductase.

GSH, reduced glutathione.

HMW-NTR, high molecular weight NAD(P)H-dependent thioredoxin reductase.

LMW-NTR, low molecular weight NAD(P)H-dependent thioredoxin reductase.

NTR, NAD(P)H-dependent thioredoxin reductase.

NTRC, NAD(P)H-dependent thioredoxin reductase type-C.

Prx, peroxiredoxin.

ROS, reactive oxygen species.

Sec, selenocysteine.

TR, thioredoxin reductase.

Trx, thioredoxin.

INTRODUCTION

It is widely accepted that life originated in a reducing environment [1]. Subsequently, approximately 2.1–2.4 billion years ago, molecular oxygen increased in the atmosphere due to the activity of a group of photosynthetic bacteria known as cyanobacteria [2]. These organisms developed the ability to use light energy to extract electrons from water that were used to reduce CO_2, thereby forming nutrients and generating O_2 as a by-product. This form of photosynthesis, called oxygenic photosynthesis, evolved and enabled cyanobacteria to spread and colonize the earth [3, 4]. Cyanobacteria adapted their metabolism to enable the buildup of organic compounds for long-term energy storage. These developments sustained life and enabled multicellular organisms to evolve. The emerging antioxidant strategies developed at the site of oxygen

production made possible the protection of cellular components from the highly-reactive oxygen molecules (reactive oxygen species or ROS) that were generated.

Microorganisms had to adapt to the new scenario, and life on earth progressed to survive under oxidative conditions [5-8]. Seemingly, the adaptation of microorganisms to the aerobic atmosphere followed different avenues in families of anaerobes. A number of bacteria evolved to develop the energy-generating process of respiration while adopting strategies for protection in the presence of oxygen. Other microorganisms remained sensitive to aerobic environments, and adapted either to survive in a restricted niche or developed pathways to protect them from oxygen and oxidative damage. Other became extinct.

Overall, the production and consumption of O_2 in places with energetic electron fluxes favored the production of ROS as inevitable by-products [9-11]. These had the potential for non-specific reactions with components of the cell that may cause irreversible damage—for example, lipids, nucleic acids, proteins, metals, chlorophylls or iron-sulfur centers. Molecular antioxidant machineries evolved for protection against these reactive species thereby opening the door for their use in signaling and regulation.

Among biological systems, certain protein amino acids proved to be sensitive to molecular oxygen or its derivatives [12]. Histidine, methionine, cysteine (Cys), tryptophan and tyrosine were particularly vulnerable to oxidative modification. The oxidation of Cys in proteins turned out to be of particular significance as the thiol group can pass through reversible oxidative modifications which impact protein structure and function. By controlling the oxidation status of selected protein thiols, cells found a way to modulate particular pathways. The Trx system evolved as a key redox component in the biological kingdoms: it functions in dithiol-disulfide exchange reactions with a variety of proteins, thus controlling major pathways under different physiological conditions. Phylogenetic studies have concluded that the Trx system is of ancient origin with roles that include serving as an electron donor to drive antioxidant systems and regulate proteins in response to a changing redox environment.

CYSTEINE OXIDATION IN PROTEINS: DISULFIDES AS MOLECULAR SWITCHES

Cys is characterized by the presence of a sulfur atom that provides proteins with a variety of functions, including catalysis as a nucleophile in enzyme active sites, a site for metal binding or covalent cross-link formation for stabilizing protein arrangement. The side chains with Cys residues display different susceptibilities to oxidation due to the physical-chemical properties of the Cys thiol group [13, 14]. These, in turn, are influenced by the microenvironment surrounding the amino acids in a particular tertiary structure.

At physiological pH, the sulfhydryl group of reactive Cys can undergo deprotonation and assume a broad range of reversible or irreversible oxidative modifications in response to changes

in the intracellular redox environment [7, 15] (Figure 1). The primary oxidation product of the thiolate form is sulfenic acid (Cys-SOH) [16, 17], that is usually unstable and either reacts with a sulfhydryl group of other Cys residues to form intra- or inter-molecular disulfides. Alternatively, the Cys reacts with reduced glutathione (GSH), generating glutathione-protein mixed disulfides (termed glutathionylation; Cys-SSG) [18], or is oxidized to sulfinic acid (Cys-SO_2H). Sulfinic acid can be either reduced back to the sulfhydryl form by a sulfiredoxin [19-21], or undergo further oxidation to sulfonic acid (Cys-SO_3H)—an irreversible step leading to protein inactivation. The thiolate may also react with reactive nitrogen species (RNS) and covalently attach a nitrogen monoxide group in a reaction termed S-nitrosylation (Cys-SNO) [22, 23] (not shown in Figure 1).

Among the variety of oxidized forms, disulfides are of particular interest as they are stable, reversible and largely resistant to further oxidation [24]. The formation and breaking of disulfide bonds had been evolutionarily exploited to alter protein function, rapidly adjusting protein activity to the prevailing redox environment. This reaction is key to regulating metabolic processes in coordination with environmental conditions and needs of the cell. One example initially developed by cyanobacteria centered on the light activation of enzymes during the day, and their deactivation in the night. This mechanism was expanded with plants, giving a regulatory network connecting metabolism to light availability [25, 26].

In addition to Cys, selenocysteine (Sec) may also be susceptible to redox modification [27, 28]. Sec is a trace element present in selected enzymes. Structurally, Sec is very similar to Cys except that selenium replaces a sulfur atom. Due to the chemical properties of selenium, Sec in proteins is usually present as selenolate at physiological pH. Selenolate is much more reactive than thiolates, thus providing selenoenzymes with high catalytic efficiency. Sec participates in dithiol/disulfide-like exchange reactions forming a selenosulfide bond.

THE THIOREDOXIN SYSTEM

In biological systems, protein disulfide bonds are reduced to the sulhydryl level primarily by the thioredoxin system, that is composed of a reduced substrate, a thioredoxin reductase (TR) and thioredoxin (Trx), a low-molecular-weight disulfide protein (Figure 2). Upon transfer of reducing equivalents from the reduced substrate to Trx catalyzed by TRs, Trx distributes the reductant to selected targets via a dithiol-disulfide exchange resulting in the structural and functional modification of the protein (Figure 2).

The targets of the Trx system are diverse, including participants in processes such as: (i) general metabolism—*e.g.*, as a substrate for ribonucleotide reductase in DNA synthesis and 3′-phosphoadenylylsulfate reductase in sulfur assimilation [29-31]; (ii) antioxidant systems—the regeneration of antioxidant enzymes such as 2-Cys peroxiredoxins [32, 33] and in oxidative

damage repair recycling enzymes such as methionine sulfoxide reductase [30, 34]; (iii) cell signaling pathways where Trx serves to regulate enzymes in response to environmental signals [35, 36]; (iv) functions not directly related to a redox reaction—for example as a redox-powered chaperone interacting with unfolded and denatured proteins dependent on the redox state of Trx [37].

The Trx system is widely distributed and likely diversified during evolution, thereby resulting in variation in the nature and composition of its components among organisms and enabling adaptation to diverse environmental conditions and functional needs.

DIVERSITY THIOREDOXIN REDUCTASES

All organisms have a TR for maintaining Trxs in the reduced state. TRs catalyze the transfer of reducing equivalents from reduced substrates to oxidized Trxs that then fulfill numerous functions. Our knowledge of the TR family has dramatically expanded in recent years due to advancement in our understanding of structure and the identification of new substrates for reducing Trx. Members of the TR family differentiated during evolution and are currently divided into two types according to the attached cofactor: (i) ferredoxin:thioredoxin reductases (FTRs), which are iron-sulfur enzymes; and (ii) flavin-thioredoxin reductases, that are flavoenzymes with a non-covalently bound flavin adenine dinucleotide (FAD).

(i) FTRs are heterodimeric enzymes composed of a catalytic subunit (FTRc), which contains a 4Fe-4S center and a redox-active disulfide bond, and a variable subunit (FTRv) with a structural role [38]. During the catalytic reaction FTRc interacts with Fdx and Trx to form a ternary complex for delivering electrons from Fdx to Trx [39] (Figure 3A). FTRs are present in most cyanobacteria and plastids [40]. In autotrophic cells, FTR receives electrons from photosynthetically reduced Fdx, thereby connecting Trx-based regulation to light. Homologue genes of FTRc have been detected in the genomes of bacteria with deep phylogenetic roots [41], though a functional association with Fdx and Trx awaits further investigation. Current results suggest that FTRc originated in microaerophilic bacteria that used Trx to regulate CO_2 fixation [41, 42]. When incorporated into oxygen-evolving bacteria, a variable subunit (FTRv) was acquired for protecting the labile 4Fe–4S center from oxidative inactivation [41, 43]. The development and evolution of FTR likely enabled cyanobacteria to expand during evolution.

(ii) Flavin-thioredoxin reductases are a heterogeneous group of enzymes with a non-covalently bound FAD that can be classified according to the reduced donor substrate. The most common and largest group accepts reducing equivalents from NADPH, and less frequently from NADH. NAD(P)H-dependent TRs (NTRs) are structurally and mechanistically divided into two main groups that have followed different evolutionary paths [44, 45]. The two groups are named

according to their molecular weight: low molecular weight (LMW)-NTRs and high molecular weight (HMW)-NTRs that, respectively, contain subunits of about 35 kDa and 55 kDa.

- LMW-NTRs are homodimeric enzymes with an FAD and a redox-active CxxC motif per monomer [46] (Figure 3B). They are present in plants, prokaryotes and lower eukaryotes, such as yeast. The enzyme has a bimodular structure that provides it with two stable conformations that correlate with the catalytic cycle [47]. During the cycle, FAD is not exposed to the solvent. The isoalloxazine ring is covered either by NAD(P)H or oxidized disulfide within the monomer. The enzyme thus oscillates between two conformations that include the reduction of FAD by NAD(P)H followed by the transfer of the reduction equivalents to its oxidized disulfide. Reduction of the CxxC motif in TR induces a conformational change that exposes the dithiol to the solvent for reducing Trx [47]. A special type of enzyme belonging to this group is found in most photosynthetic organisms that evolve oxygen. The protein, called NTRC (from NTR type-C), is formed by a LMW-NTR fused to its own Trx in a single polypeptide chain [48].

- HMW-NTRs are homodimeric enzymes; each monomer contains three active sites: an FAD, a disulfide in the form of CxxxxC, and a third redox-active group with a Sec amino acid in most of the cases (GCUG motif, with U representing Sec), or a Cys residue, that is capable of selenosulfide bond formation [14, 49, 50] (Figure 3C). Evolutionary studies suggest that HMW-NTRs and glutathione reductases have a common origin, but differ in the Sec active-site that is not present in GRs [51]. The GCUG motif is positioned in a flexible C-terminal tail [52] which gives the enzyme broader substrate specificity—a feature not possible in LMW-NTR. This arrangement allows the reduction of other substrates such as selenite, lipid hydroperoxides, lipoic acid, cytochrome c and H_2O_2, in addition to Trxs [50, 53]. The enzyme is present in most eukaryotes, except yeast and plants.

Apart from the two NAD(P)H-dependent TRs, three groups of flavin-TRs function independently of pyridine nucleotides (Figure 4). The classification is based on the identity of the electron donor, namely ferredoxin, coenzyme F_{420} or an unknown cofactor.

- FFTR (from ferredoxin-dependent flavin thioredoxin reductase) was discovered more than 35 years ago in the nitrogen-fixing anaerobe *Clostridium pasteurianum* [54]. Surprisingly, the enzyme showed the molecular properties of NTRs but, similar to photosynthetic FTRs, was found to be reduced by ferredoxin. Recent crystallography studies have given an understanding of its properties [55]. The structures revealed a modular organization similar to LMW-NTR, and captured a conformation in which the FAD is completely exposed where it may interact with Fdx. Representatives from this enzyme group are mostly present in fermentative bacteria of the Clostridium type.

- DTR (from deeply-rooted TR) was recently recognized and characterized in the ancient cyanobacterium *Gloeobacter violaceus* [56]. The enzyme was found to be inactive with pyridine nucleotides. Its functional electron donor is currently unknown. DTR consists of modular structures similar to LMW-NTRs and FFTR. However, the X-ray structure shows that the isoalloxazine group of FAD is solvent excluded by a highly conserved structural element that seems to play a regulatory role. The enzyme is found in several types of bacteria, including Aquificace, Chloroflexi, Bacillus, Firmicutes, Chlorobi, Nitrospirae, and Cyanobacteria as well as a few marine algae.

- F420-thioredoxin reductase (from F420-dependent TR) has been detected in *Methanocaldococcus jannaschii* and other anaerobic methanogens. Biochemical studies demonstrated that the TR from *M. jannaschii* enzyme receives reducing equivalents from coenzyme F_{420} instead of NAD(P)H [57]. Previous work demonstrated a functional Trx system for oxidative stress recovery in *M. jannaschii* [58].

Our knowledge of the diversity of the TR family has expanded in the recent years. Studies have revealed structural and mechanistic similarities as well as differences among the enzymes [55, 56, 59]. They suggest that flavin TRs evolved from a common gene ancestor by duplication events (Figure 4). The use of different cofactors in TRs may be related to the fact that Fe–S clusters are very sensitive to oxygen, while flavins are versatile organic cofactors functional in a broad spectrum of cells, including strict anaerobes. The structural variation in the functional modules and regulatory elements in TRs relates to the variation in the ability of the reductases to deliver electrons to Trx that is connected to metabolism, photosynthesis and fermentation, and for adaptation to specific metabolic and environmental situations. In some organisms, the co-occurrence of different types of TRs raises questions related to their functional redundancy and putative crosstalk between relevant pathways [55-57].

THIOREDOXINS

Thioredoxins (Trxs) are low-molecular-weight proteins (10-12 kDa) with a redox-active disulfide that typically transfer reducing equivalents to oxidized proteins via a dithiol-disulfide exchange reaction [60]. The amino acid sequence of Trxs contains an invariant redox-active motif in the form of WCGPC [61]. The protein adopts a so called Trx-fold, consisting of a four-stranded β-sheet sandwiched by three α-helices [62] (Figure 5). The active site is located on a surface loop at the beginning of an α-helix with the N-terminal Cys (or catalytic Cys) exposed to the solvent, and the C-terminal Cys (or resolving Cys) in a buried location. The structural environment together with the local amino acid composition confers the redox-active CxxC motif with singular physico-chemical properties. Under physiological conditions, the catalytic Cys can be deprotonated and, upon recognition and docking of an oxidized target, can perform a nucleophilic

attack on the disulfide bond of the target protein to form a mixed disulfide [63, 64] (Figure 6). The structural disturbances caused in the microenvironment result in the deprotonation of the resolving Cys in Trx that attacks the mixed disulfide, delivering oxidized Trx and the reduced target (Figure 6).

Evolutionary studies have concluded that Trxs appeared at early stage in evolution [65, 66]. The identification of a regulatory system based on Trxs in obligate anaerobes that were likely present prior to the advent of oxygen indicates that the protein played a fundamental role in cells on the anaerobic earth [42, 57, 67-69]. An expansion of the system occurred in response to oxygen, and new capabilities appeared throughout the biosphere.

Organisms often contain multiple forms of Trxs that are located in different part of the cell. For example, two Trxs are functional in the cytosol of *Escherichia coli* [70]; in humans, one Trx is distributed between the cytosol and the nucleus, and another is located in mitochondria [71]; and as many as 20 forms of Trxs distributed in the cytosol, mitochondria and chloroplasts are present in the model plant *Arabidopsis thaliana* [72]. It has been proposed that the expanded Trx system in the anaerobe *Bacteroides fragilis*, which is composed of at least four Trxs, is an important component in combatting oxidative stress *in vivo* and *in vitro* [73].

The different forms of Trx show little structural variation, and the specificity for target recognition seems to be derived from electrostatic patches at the molecular surface [43, 74, 75]. No common principle has been found for the molecular mechanisms by which redox modulates the activity of particular targets. Thus, the reversible modification of regulatory Cys can alter the catalytic properties of the enzyme or lead to a conformational change with subsequent modulation of activity or molecular interactions [25]). The oxidation of Cys under physiological conditions can protect proteins from further oxidation. It should be noted that Trx-targets can be regulated at multiple levels. Enzymes can respond, for example, to the binding of substrates or allosteric effectors, leading to metabolic changes [25]. Trxs themselves may be subjected of regulation by posttranslational modifications, such as nitrosylation [76] or glutathionylation [77], that alter activity.

Plants, and chloroplasts in particular represent good models for studying the diversity and evolution of the Trx systems that display regulatory and antioxidant functions. Due to the spatial and temporal concurrence of electron and energy transfer reactions in photosynthetic oxygen evolution, significant amounts of ROS are produced within the thylakoid membrane [78]. Plastids are well equipped with an antioxidant protection system composed of low-molecular weight components (*e.g.,* reduced glutathione, ascorbate and carotenoids) as well as enzymatic scavenging mechanisms (*e.g*., catalase, superoxide dismutase, ascorbate peroxidase) to regulate ROS homeostasis in response to developmental and environmental changes [79]. In this

antioxidant system, Trxs participate in the recycling of oxidized enzymes such as 2-Cys peroxiredoxins (Prxs), widely distributed proteins that reduce various peroxide substrates [80].

Further, chloroplasts make extensive use of thiol-based redox regulation and signaling associated with diurnal regulation, demonstrating the prominent role that this type of regulation acquired during evolution. Plants inherited the system from cyanobacteria that had developed a form of regulation to convert a light signal derived from the photosynthetic electron transport chain to a thiol signal [25, 41]. The system expanded by incorporating newly developed Trx forms and target enzymes. Classical examples of light/dark regulation through the reversible oxidation of Cys include selected enzymes of the Calvin-Benson cycle, the first committed enzyme of the oxidative pentose phosphate pathway, NADP-dependent malate dehydrogenase, and the CF1 gamma subunit of the ATP-synthase. These changes to coordinate carbon assimilation with the availability of CO_2 and light and prevent futile ATP hydrolysis in the dark [43]. The chloroplast thioredoxin system is not only associated with diurnal regulation, but it also functions in an oxidative type of regulation chloroplasts display in response to ROS [41, 81, 82]. A model for a role for the Trx system in balancing antioxidant activities and redox regulation has been recently proposed for chloroplasts [83] (Figure 7).

Much is known about the mechanism of disulfide reduction via the Trx system, but details on thiol re-oxidation have been scanty. It was not until this year that three independent groups described a mechanism of disulfide formation in the dark based on Prx and H_2O_2 [84-86] (Figure 8).

FINAL CONSIDERATIONS

Evolutionary studies suggest that thiol-based redox signaling is of ancient origin. Nature has selected Cys as a critical amino acid for catalysis, metal binding, structure, signaling and regulation in proteins. Though highly conserved, it is one of the less abundant amino acids. Its sensitivity to oxygen together with the stability of its derivatives gives Cys the capacity to function as a molecular redox switch to alter the properties of a protein in response to change in redox state. The reversible oxidation of Cys under physiological conditions also serves to protect proteins from over-oxidation while modulating their function. The development of a Trx system with both regulatory and antioxidant functions in response to environmental changes took advantage of the physiochemical properties of thiol groups that enable Cys residues to pass through different oxidative states reversibly. Cells have devised multiple ways to modulate Trx-linked processes during evolution, with the acquisition and combination of different types of TRs and Trxs functioning under diverse conditions. These developments increased the number of enzymes that adopted new modes of regulation. However, the molecular mechanisms responsible for Trx target recognition and docking have been only partially elucidated [87, 88]. In a protein,

not all Cys are equally susceptible to redox modification, and susceptibility to oxidation depends on the structural microenvironment [89]. There are no structural features common to Trx targets, except the conservation of two Cys susceptible to reversible oxidation. This property makes it very difficult to predict the reactivity of a Trx and its specificity in interacting with a target enzyme.

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FIGURE LEGENDS

Figure 1. Oxidative modifications of cysteine. Oxidation of cysteine thiol/thiolate (SH/S^-) in a protein (P) by an increased concentration of ROS (peroxides) leads to the formation of sulfenic acid (SOH), which can react either with another thiol to form an intra- or intermolecular disulfide bond (S-S) or with reduced glutathione (GSH) to become glutathionylated (S-SG). These oxidative modifications can be reversed by the Trx or the glutaredoxin (Grx) systems, respectively. Further oxidation of sulfenic acid to sulfinic acid (SO_2H) may be repaired by sulfiredoxins (Srx); oxidation to sulfonic acid (SO_3H) is thought to be generally irreversible *in vivo*.

Figure 2. Hierarchical structure of the redox chain from reduced substrates to target proteins in a reaction catalyzed by the thioredoxin system. TRs catalyzed the transfer of electrons from a reduced substrate to the disulfide bridge of Trx which, in turn, reduces target proteins via a dithiol/disulfide exchange reaction.

Figure 3. Physiological reduction of thioredoxins. (A) Heterodimeric ferredoxin:thioredoxin reductase (FTR), composed of a catalytic subunit (FTRc) and a variable subunit (FTRv), contains an unique 4Fe-4S center and a disulfide bridge (S-S) in FTRc. With the participation of the two redox centers, FTR transfers electrons from Fdx to the disulfide bridge of Trx; (B) Each monomer of the low-molecular-weight NADPH-dependent thioredoxin reductase (LMW-NTR) homodimers contains one flavin (FAD) and a disulfide bridge. The transfer of electrons from NADPH occurs between the two redox centers (FAD and S-S) of the same subunit to Trx; (C) High-molecular-weight NADPH-dependent thioredoxin reductases (HMW-NTRs) homodimers contain three redox centers per monomer: one FAD, a disulfide bridge, and a third motif that contains a Cys-Sec pair (S-U) in most cases. FAD and the disulfide of one monomer participate in the electron transfer reaction together the Sec motif of the second monomer, and finally Trx. Upon reduction, Trx distributes the electrons to the final oxidized targets.

Figure 4. Diversity and evolution of thioredoxin reductases (TRs). The function of TRs relies on the ability of its cofactors to accomplished the required enzymatic activity. During evolution, one group of enzymes incorporated an iron-sulfur (Fe-S) center that, in coordination with a disulfide (S-S), catalyzes the transfer of reducing equivalents from ferredoxin (Fdx) to thioredoxins (Trx). A second group is formed by FAD-dependent enzymes, that is further subdivided into two groups according to structural and mechanistic features. A first subgroup includes the high-molecular-weight NADPH-dependent TRs (HMW-NTRs) that, in addition to FAD, have two cysteine residues (S-S) and a cysteine-selenocysteine (S-U) pair and employs NADPH as substrate donor; HMW-NTRs belong to the glutathionine reductase (GR) superfamily. A second subgroup is constituted by enzymes that contain an FAD and a disulfide bond; these subgroup is composed by a variety of enzymes that have evolved diverse structural and catalytic properties and react with different substrates, namely NADPH, Fdx, the deazaflavin F420 and unknown (X). NTRC, which reacts with NADPH and likely resulted from the fusion of LMW-NTR and Trx domains [90], is represented in the figure by the LMW-NTR enzyme.

Figure 5. Ribbon diagram showing the structural elements of reduced thioredoxin of *Escherichia coli* (Protein Data Bank code 1fb0, [91]). The structure is shown in rainbow color with blue for N-terminus (Nt) and red for C-terminus (Ct). The protein is folded into a central β-sheet surrounded by 4 α-helices. The catalytic and resolving Cys (Cys_{cat} and Cys_{res}, respectively) forming part of the WCGPC motif at the beginning of the second α-helix (α2) are displayed in stick representation. The figure was created with The PyMOL Molecular Graphics System, v 1.8, Schrödinger, LLC.

Figure 6. Mechanisms for reaction of thioredoxin (Trx) with target proteins. Trxs are reduced by thioredoxin reductases (TRs). Under physiological conditions, the thiolate of the CxxC active site initiates a nucleophilic attack on the disulfide bond in a target protein. Due to local conformational perturbations, the transient intermolecular disulfide formed is resolved by the second Cys of the Trx, resulting in the formation of an intramolecular disulfide in Trx and the release of reduced target.

Figure 7. Interaction of the redox regulatory and antioxidant systems in chloroplasts. (A) In chloroplasts, ferredoxin (Fdx) acts as the central distributor of photosynthetically derived-electrons to different partner proteins, such as ferredoxin:thioredoxin reductase (FTR) for redox regulation and ferredoxin–NADP reductase (FNR) for NADPH production. As noted by the plus symbol (+) several enzymes of the Calvin-Benson cycle become activated on reduction by the Trx system. Chloroplasts contain a special type of NADPH-dependent thioredoxin reductase (NTR) with a fused Trx domain at its C-terminus that receives reducing equivalents from NADPH generated either via FNR in the light (or by oxidative pentose phosphate pathway in the dark; not

shown). The enzyme, named NTRC, is active in the regeneration of the 2-Cys peroxiredoxin (2CP) enzyme of the antioxidant system; (B) Under an oxidative event with accumulation of oxidized 2CP, such as a lack of NTRC activity, a recent mechanistic model has proposed that Trxs would provide the reducing equivalents for the antioxidant enzyme, deviating them from the regulatory proteins of the CB cycle with consequences in plant growth [83].

Figure 8. Current view of the redox control of regulatory proteins in chloroplasts. In illuminated chloroplasts, ferredoxins (Fdxs) distribute photosynthetic electrons to oxidized thioredoxins (Trxs) in a reaction catalyzed by (ferredoxin:thioredoxin reductase) FTR. In turn, Trxs reduce target proteins in a dithiol/disulfide exchange reaction that modify its structure or activity. At night, redox-regulated proteins are oxidized via the Trx-like protein 2 (TrxL2)/2-Cys peroxiredoxin (2-Cys Prx) pathway, in a reaction dependent on hydrogen peroxide [85].

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