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Review

The emerging roles of protein glutathionylation in chloroplasts

Mirko Zaffagnini[a,*], Mariette Bedhomme[b], Stéphane D. Lemaire[b], Paolo Trost[a,**]

[a] Laboratory of Molecular Plant Physiology, Department of Experimental Evolutionary Biology, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy
[b] Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FRE3354 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

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ABSTRACT

Reactive oxygen species play important roles in redox signaling mainly through a set of reversible post-translational modifications of cysteine thiol residues in proteins, including glutathionylation and dithiol/disulfide exchange. Protein glutathionylation has been extensively studied in mammals but emerging evidence suggests that it can play important roles in plants and in chloroplast in particular. This redox modification involves protein thiols and glutathione and is mainly controlled by glutaredoxins, oxidoreductases belonging to the thioredoxin superfamily.

In this review, we first present the possible mechanisms of protein glutathionylation and then introduce the chloroplast systems of glutaredoxins and thioredoxins, in order to pinpoint the biochemical properties that make some glutaredoxin isoforms the master enzymes in deglutathionylation. Finally, we discuss the possible roles of glutathionylation in thiol protection, protein regulation, reactive oxygen species scavenging and redox signaling in chloroplasts, with emphasis on the crosstalk between thioredoxin- and glutaredoxin-mediated signaling pathways.

Contents

1. Introduction

Light-dependent modulation of chloroplast enzyme activities is a well established mechanism of redox regulation that involves thioredoxins (Trxs) and dithiol/disulfide exchange reactions [1–3]. In virtually all photosynthetic eukaryotes, the reduction of plastid thioredoxins is accomplished by ferredoxin:thioredoxin reductase which uses photo-reduced ferredoxin as an electron donor [4]. Once reduced, thioredoxins can modulate chloroplast enzyme activities by reducing regulatory disulfide bonds. Among all chloroplast thioredoxin targets established to date, glucose-6-phosphate dehydrogenase is the only enzyme that is inhibited, rather than activated, by disulfide reduction [4,5].

Glutathionylation is a different type of post-translational modification of protein thiols that has recently emerged as an additional mechanism of redox regulation in plants, particularly in

* Corresponding author. Tel.: +39 0512091305.
** Corresponding author. Tel.: +39 0512091329.
E-mail addresses: mirko.zaffagnini3@unibo.it (M. Zaffagnini), paolo.trost@unibo.it (P. Trost).

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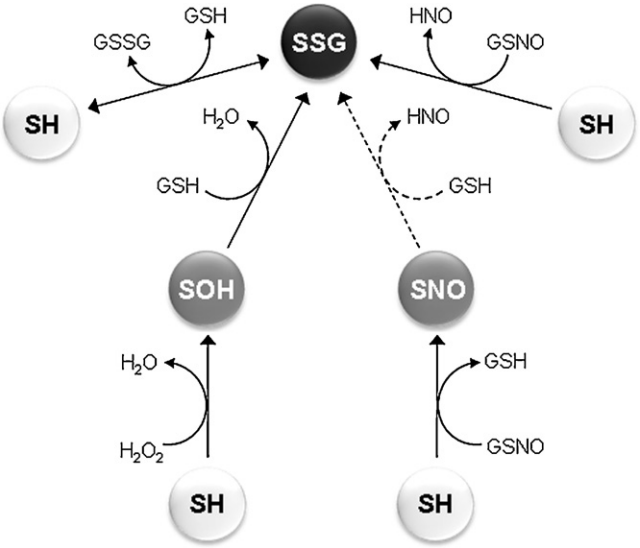

Fig. 1. Major mechanisms of protein glutathionylation. Several mechanisms can theoretically trigger protein glutathionylation. Glutathionylation can occur *via* a thiol/disulfide exchange between the target protein and oxidized glutathione (GSSG) or by reaction with nitrosoglutathione (GSNO). Glutathionylation can also occur after reaction of GSH with activated thiol derivatives such as sulfenic acids (SOH) or S-nitrosylated thiols (SNO). As discussed in the text, glutathionylation by GSSG is not expected to be a relevant process *in vivo*, except for glutaredoxins like GrxS12 that readily equilibrate with the glutathione pool. The reaction of nitrosylated thiols (SNO) with GSH is also hypothetical.

chloroplasts [6]. This reversible modification consists in the formation of a mixed disulfide between glutathione and a free accessible protein cysteine, and is generally promoted by reactive oxygen species (ROS) such as hydrogen peroxide (Fig. 1) [7]. Different proteomic studies have shown that a large number of chloroplast proteins are glutathionylated under artificially imposed oxidative stress conditions [8–13]. While glutathionylation can occur *via* non-enzymatic mechanisms *in vivo*, the reverse reaction (namely deglutathionylation) can be catalyzed by small oxidoreductases of the thioredoxin superfamily known as glutaredoxins (Grxs) [7].

The tripetide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the major thiol-based redox buffer of plant cells. In the cytosol and most organelles including plastids, glutathione is present at millimolar concentrations [6,14–16]. In the stroma of chloroplasts, but also in the cytosol and mitochondrial matrix, glutathione occurs largely in the reduced form (GSH) and this is particularly true under non-stress conditions, in which the levels of oxidized glutathione (GSSG) are kept to a minimum by NADPH-dependent glutathione reductase [17]. Reduced glutathione is involved in detoxification of different compounds, including ROS (through the ascorbate–glutathione cycle [18]), heavy metals (through phytochelatins) and xenobiotics (through glutathione S-transferases) [19–21]. In addition, glutathione appears to play new roles in redox regulation, protection and signaling through glutathionylation and deglutathionylation of specific target proteins [7].

The photosynthetic electron transport machinery involves chlorophylls in excited (triplet) states and electron carriers with very low redox potentials, both potentially able of reacting with oxygen and generating ROS [22,23]. Although ROS generation by chloroplasts can be considered as an unavoidable side-effect of photosynthesis [18,22,23], different types of abiotic and biotic stresses are known to stimulate the production of these potentially harmful compounds [23]. Oxidative stress occurs when ROS production overwhelms cellular detoxification systems, thereby leading to oxidative modification and potentially irreversible damage to lipids and proteins [22–24]. Under less severe conditions, however, ROS accumulation can be effectively modulated by ROS-detoxifying systems and redox signaling pathways based on reversible modifications of target proteins can finally result in adaptation responses [14,25]. Post-translational modifications of protein thiols, such as dithiol/disulfide exchange, sulfenation, glutathionylation and S-nitrosylation, are typically involved in this type of signaling [26]. Moreover ROS, and most notably H_2O_2, can also alter the redox state of the glutathione system, thereby affecting the function of glutathione as a redox buffer [17].

Although protein glutathionylation has been often considered as a mechanism of protection of protein thiols against over-oxidation, it can also work as a mechanism of regulation of protein function and is involved in the regeneration mechanisms of specific antioxidant enzymes such as peroxiredoxins and methionine sulfoxide reductases [7]. In most cases, glutathionylation has profound effects on the activity of target proteins [7] and since deglutathionylation appears to be most exclusively mediated by glutaredoxins, these redoxins constitute an essential link between ROS, glutathione and glutathionylated proteins [7].

Protein glutathionylation has been extensively studied in mammalian cells [27–30], but emerging evidence suggests that it could also be an important mechanism of protection, regulation and signaling in plants [6,7,13,31]. The reversibility of this modification further supports its significance and the few glutaredoxin isoforms in chloroplasts are probably crucial regulatory points of the whole system [7].

In this present review, we first present the possible mechanisms of protein glutathionylation and then introduce the chloroplast systems of glutaredoxins and thioredoxins, in order to pinpoint the biochemical properties that render some glutaredoxin isoforms to be master enzymes in deglutathionylation. Finally, we discuss the possible physiological roles of glutathionylation in chloroplasts, with an emphasis on the crosstalk between thioredoxin- and glutaredoxin-mediated signaling pathways.

2. Mechanisms of protein glutathionylation

Various mechanisms of protein glutathionylation have been proposed and characterized *in vitro*, but which of these may prevail *in vivo* is still a matter of debate. In general, glutathionylation has been associated with stress and proteomic studies conducted under oxidative stress conditions have led to the identification of several glutathionylated targets in different organisms [7,27–30,32]. However, the improved sensitivity of second generation proteomics has recently led to detection of hundreds of glutathionylated proteins in a single cell type, even without any exogenously applied stress [33]. This suggests a more general understanding of protein glutathionylation as a widely distributed mechanism of post-translational modification that may be involved in redox signaling events under both physiological and oxidative stress conditions.

It is unclear what specific features contribute to the sensitivity of a given cysteinyl residue to protein glutathionylation, whether solvent accessibility, reactivity (which depends in turn on the pK_a of the thiol), the microenvironment surrounding the target cysteine or a combination of these effects [27,34,35]. Chloroplasts contain several characterized targets of glutathionylation, including aldolase [10], triose-phosphate isomerase [10], thioredoxin-f (Trx-f) [36], A_4-glyceraldehyde-3-phosphate dehydrogenase (A_4-GAPDH) [12], 2-Cys-peroxiredoxin (2-Cys-Prx) [11], heat shock protein 70B (HSP70B) [11], peroxiredoxin IIE (Prx IIE) [37], methionine sulfoxide reductase B1 (MSRB1) [38], phosphoribulokinase [13], ribose-5-phosphate isomerase [13] and phosphoglycerate kinase [13]. Among all these targets, only for Prx IIE and MSRB1 glutathionylation was demonstrated as a mandatory step of the catalytic cycle. For all the others, glutathionylation is apparently

related to other functions, *e.g.* regulation and/or protection in the case of A_4-GAPDH [12], or signaling in the case of Trx-f [36].

In vitro at least, glutathionylation is a spontaneous process that can involve either oxidized glutathione (GSSG), GSH plus oxidants (*i.e.* H_2O_2), nitrosoglutathione (GSNO) or even NO-donors in combination with GSH (Fig. 1).

The simplest, basic mechanism of glutathionylation is a spontaneous thiol/disulfide exchange between a protein cysteine thiol (Prot-SH) and GSSG, as described by the following reaction:

$$\text{Prot-SH} + \text{GSSG} \Leftrightarrow \text{Prot-SSG} + \text{GSH}$$

The equilibrium constant of this reaction is defined as the K_{ox}, a useful thermodynamic parameter that corresponds to the GSH/GSSG ratio at which 50% of the protein target is in the glutathionylated form:

$$K_{ox} = [\text{Prot-SSG}] \times [\text{GSH}]/[\text{Prot-SH}] \times [\text{GSSG}]$$
$$= [\text{Prot-SSG}]/[\text{Prot-SH}] \times [\text{GSH}]/[\text{GSSG}]$$

Although the K_{ox} of only few glutathionylated proteins have been directly determined [39,40, authors unpublished observations], protein cysteines that are susceptible to glutathionylation are generally believed to have K_{ox} values around or below 1 [39,40], implying that glutathionylation would only occur at equimolar levels of GSH and GSSG. This condition is apparently very far from the physiological redox state of glutathione in chloroplasts and other sub-cellular compartments. However, as discussed below, the glutathione redox state of plant tissues does change upon stress.

Normal GSH/GSSG ratios in whole plant tissues range from 10 to 20 [17], but these values represent an average of different cell compartments having different redox states. For instance, the glutathione redox state of chloroplasts, mitochondria and the cytosol is much higher than in vacuoles or in the lumen of the endoplasmic reticulum [16,17]. Estimates of GSH/GSSG ratios by genetically coded, redox-sensitive fluorescent probes (roGFP2) gave very high values, around 10^5, for the cytosol of *Arabidopsis* [41,42]. Provided that the NADPH/NADP ratio of the cytosol may be close to one [14,25], a glutathione redox state of 10^5 would imply that the activity of glutathione reductase is sufficient to keep the equilibrium between the two redox pools [17,25]. In principle, this condition might apply also to chloroplasts, but still it is not completely clear whether roGFP reliably reflects only the glutathione redox state *in vivo* [17,25].

On the other hand, several studies reported that the glutathione redox state is perturbed and subjected to oxidation (*i.e.* GSSG accumulation) in plants exposed to different types of stress, such as ozone [43], pathogen attack [44,45], chilling [46], drought [42] as well as in catalase deficient mutants [16,47,48]. Indeed, Noctor and co-workers have demonstrated that in *Arabidopsis* catalase-deficient mutant *cat2*, the redox state of the glutathione pool is a sensitive indicator of H_2O_2 levels in leaves [16,48]. Hydrogen peroxide accumulation could cause a decrease of the GSH/GSSG ratio without affecting the NADP(H) redox state, thus implying that GR, at least under stress conditions, may not be active enough to keep the equilibrium between glutathione and NADP(H) redox couples [48]. These and previous studies [47] strongly suggest that, under conditions of increased H_2O_2 availability, GSSG accumulates in chloroplasts up to concentrations that may exceed 0.5 mM, corresponding to a GSH/GSSG ratio between 3 and 4 [16].

In principle, such a low GSH/GSSG ratio might cause spontaneous glutathionylation (*via* thiol disulfide exchange) of protein targets characterized by K_{ox} values in the same range of the GSH/GSSG ratio, and probably several proteins fulfill these requisites [39]. However, additional experiments aimed at determining the K_{ox} of the different chloroplast proteins sensitive to glutathionylation are required to understand the relevance of this mechanism *in vivo*. Moreover, glutathionylation by GSSG is generally a slow process and *in vitro* experiments often require long incubations with millimolar concentrations of GSSG in order to obtain complete glutathionylation of target proteins [9,49]. Therefore, even if thermodynamically feasible (a condition which might occur under stress), GSSG-mediated glutathionylation is likely to be kinetically limited in most conditions.

Interestingly, a chloroplast glutaredoxin from poplar (GrxS12) is a remarkable exception to this rule. This protein reacts rapidly (in seconds or less) with both GSSG and GSH and has a K_{ox} of 309 at pH 7.0 [50]. This implies that GrxS12 can readily equilibrate with the glutathione pool *in vivo* and is predicted to be, for example, 50% glutathionylated in the presence of a GSH/GSSG ratio of 300. As explained below, the physiological implications of this property may be relevant as GrxS12 controls the glutathionylated state of its targets (which may be numerous) and GrxS12 is only active in this sense when it is not glutathionylated itself. In addition, it should be noted that K_{ox} values of GrxS12 decrease with pH, and the K_{ox} is 69 at pH 7.9 (a typical pH value of the chloroplast stroma in the light) indicating that GrxS12 should be more prone to glutathionylation in darkened than illuminated chloroplasts [50]. No other plant proteins, including other plant glutaredoxins, are now known so far to share this feature.

Most probably, the most general mechanism of protein glutathionylation *in vivo* involves activated thiol derivatives, mainly sulfenic acids (—SOH) (Fig. 1) [7,9,51]. The two-electron oxidation of protein thiols to sulfenic acids can be rapidly performed by non-radical oxygen-derived species such as hydrogen peroxide (H_2O_2) or peroxynitrite ($ONOO^-$) [51,35]. Protein cysteines that are susceptible to glutathionylation are generally acidic (pK_a below 6) and the reaction of thiolates ($—S^-$) with H_2O_2 first leads to sulfenic acid formation (primary oxidation), and sulfenates ($—SO^-$) can be further oxidized to sulfinates ($—SO_2^-$) and sulfonates ($—SO_3^-$) [51]. The spontaneous reaction of GSH with specific thiol sulfenates in proteins leads to the formation of mixed disulfides (Prot-SSG) [7,51]. In principle, GSH itself could also undergo sulfenation (GSOH) and then react with protein cysteines, but the low reactivity of the cysteine moiety of GSH (pK_a between 8.6 and 9, [52] probably limits this reaction *in vivo* [51]. Sulfenates are typically unstable and the conversion to more oxidized forms (sulfinates and sulfonates) is thought to be irreversible [51]. For this reason, the reaction of GSH with a thiol sulfenate could constitute a mechanism of protection of protein cysteine residues from irreversible oxidation, as demonstrated *in vitro* for chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and GrxS12 [12,50].

Glutathionylation can also result from the reaction of nitrosoglutathione (GSNO) with a protein thiol (Fig. 1). GSNO is a relatively stable molecule that can act as an NO reservoir but can also trigger either glutathionylation or nitrosylation (—SNO) [53], depending on the target. GSNO triggers glutathionylation *in vitro* of several plant proteins including glycine decarboxylase [54] and GrxS12 [50]. However, the protein requisites and/or conditions that favor glutathionylation *versus* nitrosylation upon reaction of GSNO with protein thiols remain unclear [55].

It is also theoretically possible for a protein thiol to be nitrosylated (by GSNO or other NO-donors) before being glutathionylated by GSH. In this sense, nitrosylation would work similarly to sulfenation in activating the protein. However, in the absence of solid experimental support, this mechanism is still speculative.

Apart from the GSSG-dependent mechanism, which might be excluded for most proteins, all other mechanisms of glutathionylation depicted in Fig. 1 may occur *in vivo*, resulting in the same final product, *i.e.* the glutathionylated form of the target protein (Prot-SSG). The stability of this product would in principle depend on the glutathione redox state because of the possible interchange reaction between the glutathionylated protein (Prot-SSG)

and GSH (Fig. 1). However, although this reaction would be thermodynamically favored under all conditions (K_{ox} around 1), we should consider again its kinetic limitation. The spontaneous reaction between most glutathionylated targets and GSH is extremely slow and often undetectable (glutaredoxins being remarkable exceptions) [50, author unpublished observations]. As a rule, glutathionylated proteins do not equilibrate with the glutathione pool *in vitro*, but this condition might be different *in vivo* as long as the reaction could be enzymatically accelerated. Glutaredoxins are effective deglutathionylating agents *in vitro* and the major candidates for a role in protein deglutathionylation *in vivo* [7].

3. The glutaredoxin and thioredoxin systems of chloroplasts

3.1. Glutaredoxins

Glutaredoxins are ubiquitous in aerobic organisms and share with thioredoxins the overall tridimensional structure (thioredoxin fold). This consists of a four stranded β-sheet surrounded by at least three α-helices. The active site contains a characteristic four-amino acids motif with one or two reactive cysteines (CXXC/S) [56,57]. The plant glutaredoxin family contains more than 30 members localized in different cell compartments (for recent reviews on the topic, see [7,58]. Based on phylogenetic analyses and the active site structure, glutaredoxins were divided in three classes, each class containing a variant of the active site motif and peculiar functional properties [56,57,59]. Additional classes of glutaredoxins were also identified more recently [58,60], but these are restricted to specific organisms or are distantly related to classical glutaredoxins and will not be reviewed here.

Most of our knowledge about glutaredoxins in photosynthetic organisms derives from *Arabidopsis*, poplar and the green alga *Chlamydomonas reinhardtii*. Among these organisms, *Arabidopsis* contains the larger set of chloroplast glutaredoxins, consisting of four glutaredoxins that belong to class I (GrxS12 and GrxC5; active site: CSYS and CSYC, respectively), and class II (GrxS14 and GrxS16; active site: CGFS) (Table 1). In the nomenclature commonly used for glutaredoxins, the letter following Grx indicates whether a cysteine (C) or a serine (S) occupies the fourth position of the active site sequence. Chloroplast localization of all plant glutaredoxins was experimentally demonstrated [61–63].

Glutaredoxins are generally unable to reduce protein disulfide bonds, but can often reduce protein-glutathione mixed disulfides. Indeed, some glutaredoxins, having a conserved GSH binding site in the active site [62,63], are highly selective for Prot-SSG substrates and catalyze deglutathionylation reactions much more efficiently than other disulfide oxidoreductases such as thioredoxins [64]. The capability of both GrxS12 and GrxC5 to regenerate (*i.e.* deglutathionylate) specific substrates such as chloroplast A_4-GAPDH, Prx IIE and MSRB1 was experimentally demonstrated [38,62,63,65]. It is not known whether other plastid Grxs of higher plants (*i.e.* GrxS14 and GrxS16) are also active in deglutathionylation, but Grx3 from *C. reinhardtii*, ortholog of GrxS14, does catalyze the deglutathionylation of A_4-GAPDH [64].

Recently, Rouhier and co-workers demonstrated that some plastid glutaredoxins can assemble iron–sulfur (Fe–S) clusters by means of their active site cysteines, implying that when the Fe–S cluster is bound to the glutaredoxin, the protein cannot be active in deglutathionylation reactions [60,61,63]. Class II GrxS14 and GrxS16 can incorporate transferable (oxygen(air)-labile) Fe–S clusters and appear to have a role in Fe–S cluster assembly/biogenesis [61]. By contrast, class I GrxC5, which bind Fe–S clusters in a more stable and not transferable conformation [63], may have a different function (see below for further discussion).

The reduction of chloroplast glutaredoxins can occur *via* two different systems. Some glutaredoxin isoforms [62,63] are specifically reduced by GSH while others [64] use the same reducing system used by chloroplast thioredoxins (ferredoxin:thioredoxin reductase). Whether any given chloroplast glutaredoxin is linked to GSH or ferredoxin:thioredoxin reductase as reducing systems mainly depends on the mechanism of the deglutathionylation reaction. As explained below, GSH-reducible glutaredoxins typically follow a monothiol mechanism, whereas the mechanism of FTR-linked glutaredoxins is dithiol.

3.2. Thioredoxins

Thioredoxins (Trx) share with glutaredoxins the overall tridimensional structure and a similar conserved sequence motif in the active. The two invariant cysteines of the WC[G/P]PC motif are involved in dithiol/disulfide exchange reactions. Similar to glutaredoxins, plant thioredoxins constitute a multigene family and are classified into different groups depending on primary sequence and sub-cellular localization. In chloroplasts of higher plants, five major types of thioredoxins (f, m, x, y and z) probably account for about half of the total thioredoxin pool in this compartment [4,66,67].

The reduction of thioredoxin-f, -m and -z by ferredoxin:thioredoxin reductase, and thus their physiological link with light reactions of photosynthesis, was experimentally demonstrated [4,68]. Whether other Trx isoforms could also be reduced by the same system is currently unknown. Chloroplasts do not contain a classical NADPH-Trx reductase like the cytosol, but contain NTRc, a hybrid protein constituted by a Trx module fused to an NTR module that uses NADPH as a source of reducing power [69]. Thioredoxin-x, -y, -z, and NTRc preferentially function as electron donors to several antioxidant enzymes such as peroxiredoxins, methionine sulfoxide reductases or glutathione peroxidases [68,70–73]. By contrast, Trx-m and Trx-f principally act as regulators of metabolism, including the Calvin–Benson cycle [2,4], thereby potentially contributing to the fundamental modulation of carbon fixation during natural light/dark regimes. In spite of the general acceptance of the role of thioredoxins in the light-regulation of photosynthesis, clear *in vivo* evidence for this function is still lacking.

3.3. Glutaredoxins and thioredoxins have different reactivity

The reactivity of a protein cysteine is influenced by the acidity of its thiol (pK_a). The thiolate form ($-S^-$) is much more reactive than its protonated counterpart (−SH). In both thioredoxins and glutaredoxins, the N-terminal cysteine of the active site sequence initiates the reaction by nucleophilic attack of the target disulfide [7]. The reactivity of the N-terminal cysteine depends on the biochemical environment of the active site. Moreover, the cysteine is invariably located at the N-terminal end of α-helix and the orientation of the intrinsic dipole of the α-helix increases the acidic character of the cysteine. As compared with the pK_a of 8.5 for free cysteines, both thioredoxins and glutaredoxins are generally believed to contain more acidic and therefore more reactive N-terminal cysteines in their active site.

Different glutaredoxin isoforms from various organisms have very acidic catalytic cysteines, with pK_a values ranging from 3.5 to 4.5 [74–76]. The pK_a of poplar GrxS12 was recently determined as 3.9 [50], a value that guarantees deprotonation of the cysteine to the thiolate form ($-S^-$) at any physiological pH of the stroma. Stroma pH oscillates between 7 in the dark and 7.9 in the light [77]. No other chloroplast glutaredoxin has been yet characterized in this respect.

The exact pK_a values of plastid thioredoxins are also unknown, but are demonstrably much higher than in the case of

Table 1
Chloroplast glutaredoxins (Grxs) from *Arabidopsis thaliana* (At), poplar (*Populus tremula* × *tremuloides*, Ptt) and *Chlamydomonas reinhardtii* (Cr).

Grx class	Name in *Arabidopsis*	Accession number[a]	Poplar homolog	*Chlamydomonas* homolog	Active site sequence	Physiological reductant	Disulfide reduction[b]	P-SSG reduction	Catalytic mechanism	Established and potential functions
Class I	AtGrxS12	At2g20270	PttGrxS12	–	CSYS	GSH [62]	Yes [63]	Yes [62]	Monothiol [63]	Deglutathionylation (A_4-GAPDH) [62]; stress response (Prx IIE, MSRB1) [37,38]
	AtGrxC5	At4g28730	–	–	CSYC	GSH [63]	Yes [63]	Yes [63]	Monothiol [63]	Stress response (Prx IIE, MSRB1) [63]; Fe–S cluster assembly [63]
Class II	AtGrxS14	At3g54900	PttGrxS14	CrGrx3	CGFS	FTR, not GSH [64]	No [64]	Yes [64]	Dithiol [64]	CrGrx3: deglutathionylation [64]; GrxS14 (At or Ptt): oxidant resistance [91]; arsenate tolerance [92]; Fe–S cluster assembly/biogenesis [61]
	AtGrxS16	At2g38270	PttGrxS16	CrGrx6	CGFS	Not GSH [76], (FTR?)	No [76]	No [76]	–	Fe–S cluster assembly/biogenesis [61]

[a] Name database.
[b] Disulfide reduction activity for chloroplast glutaredoxins have been determined following insulin-based assay.

glutaredoxins [78]. The standard redox potential of Trx-f and -m is linear with pH in the 6–8.5 range. Within this range, the standard redox potential decreased by 60 mV for each pH unit increase. This result strongly indicates that two protons together with two electrons were involved in the reaction ($S–S + 2e^- + 2H^+ = 2SH$). Trx-f and -m are thus predominantly in the protonated form at pH 8, implying a pK_a above 8.

This is in contrast with the general assumption that thioredoxins contain highly reactive (*i.e.* acidic) cysteines in their active site. However, although thioredoxins are potentially less reactive than glutaredoxins, they appear much more efficient than glutaredoxins in the insulin test, an assay to determine the ability to catalyze disulfide bonds reduction [64]. Indeed, thioredoxins have a higher specificity for protein homo-disulfide bonds while glutaredoxins are more specific to mixed disulfide bonds. Clearly, the ability to reduce disulfide bonds does not exclusively depend on the reactivity of the thiols involved in the nucleophilic attack. Other biochemical/structural features, possibly related to shape and charge distribution on the surface involved in protein–protein interactions, should be important for thioredoxin nucleophilic attack of target disulfides.

As glutaredoxins active site cysteines have lower pK_a values (*i.e.* they are more reactive) than thioredoxins, glutaredoxins are predicted to be more sensitive to redox modifications by small oxidants, such as sulfenation by H_2O_2 or $ONOO^-$, glutathionylation by H_2O_2 plus GSH, and nitrosylation by GSNO or other NO donors.

4. Protein deglutathionylation mechanisms catalyzed by Grxs

Chloroplast glutaredoxins catalyze protein deglutathionylation either through a dithiol or a monothiol mechanism (Fig. 2).

The reduction of *Chlamydomonas* Grx3 (CrGrx3, class II) was shown to follow a dithiol mechanism (Fig. 2A, [64]). The first step of this mechanism consists in the nucleophilic attack performed by the active site, N-terminal cysteine of the glutaredoxin on the GSH-mixed disulfide of the target (Prot-SSG). This reaction releases a reduced (*i.e.* deglutathionylated) protein substrate (Prot-SH) and a glutathionylated glutaredoxin intermediate (Grx-SSG). The intermediate glutaredoxin-SSG is internally attacked by a second cysteine (in a partially conserved position outside the active site sequence), leading to the formation of an intramolecular disulfide. This intramolecular disulfide cannot be reduced by GSH, but requires a reductase. Indeed the reduction of the disulfide of CrGrx3 occurs *via* a light-dependent reaction involving ferredoxin and ferredoxin:thioredoxin reductase [35]. The midpoint redox potential of CrGrx3 disulfide is close to the values determined for chloroplast thioredoxins and thus suitable for ferredoxin:thioredoxin reductase-dependent reduction [35], supporting this contention.

Based on homology with *Chlamydomonas* Grx3, class II GrxS14 from higher plants might be reduced by ferredoxin:thioredoxin reductase and be also involved in deglutathionylation processes (Fig. 3). However, preliminary results revealed that GrxS14 appears much less efficient in the deglutathionylation of A_4-GAPDH compared to CrGrx3 [authors unpublished observations]. Future studies are indeed required to investigate its function in the deglutathionylation reactions by testing other chloroplast glutathionylated target proteins. Another chloroplastic class II Grx from *Chlamydomonas*, CrGrx6, lacks the ability to form an internal disulfide and also to catalyze deglutathionylation [76]. Therefore, the possible role of class II glutaredoxins as deglutathionylating enzymes does not seem to be a general feature of this class. As reported above (Section 3.1), several members of class II may instead be involved in Fe–S cluster assembly/biogenesis [61] (Fig. 3).

The two chloroplast glutaredoxins of class I, GrxS12 and GrxC5, catalyze deglutathionylation by a monothiol mechanism involving only the N-terminal active site cysteine [62,63] (Fig. 2B). The first step of the reaction is common to the dithiol mechanism but then the intermediate glutaredoxin-SSG is attacked by a second

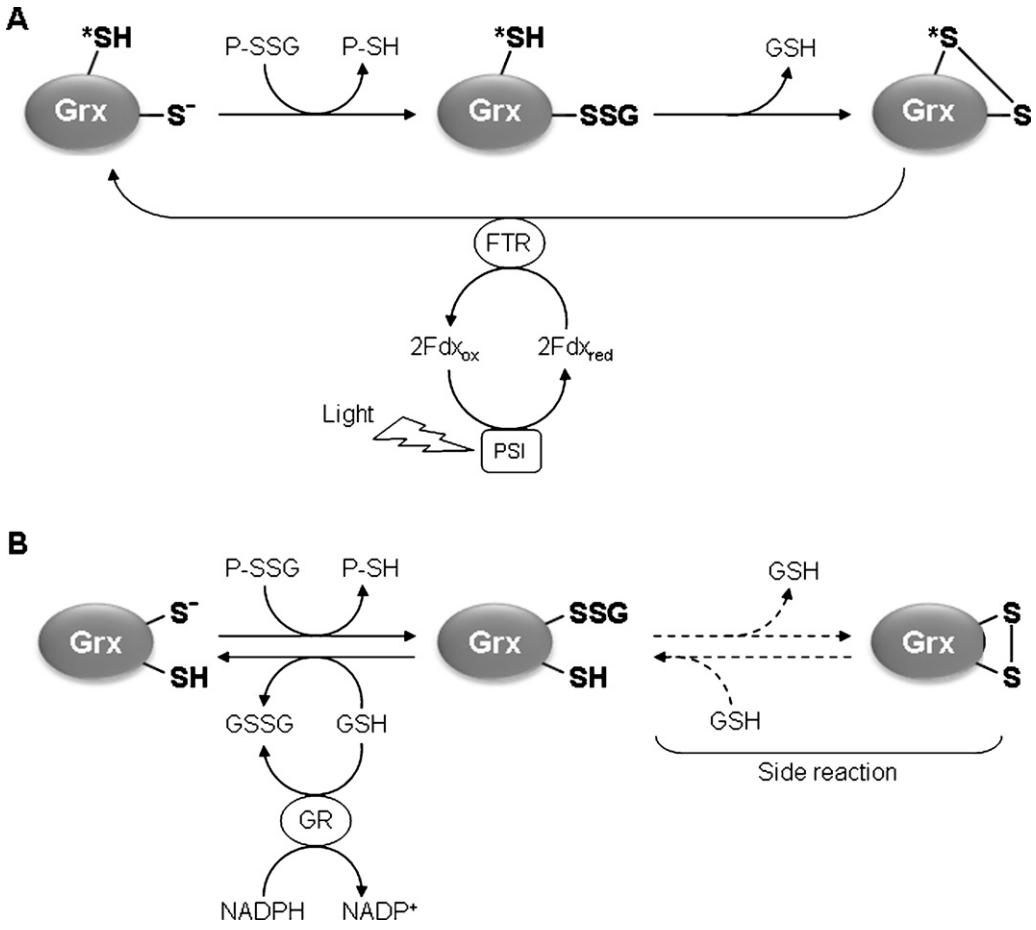

Fig. 2. Mechanisms of Grx-catalyzed deglutathionylation. The first step of protein deglutathionylation is common to both dithiol and monothiol mechanisms. The most reactive cysteine of Grx (the N-terminal active-site cysteine) performs a nucleophilic attack on the glutathione-mixed disulfide on a target protein (Prot-SSG), resulting in the release of a deglutathionylated substrate (Prot-SH) and the formation of a glutathionylated Grx intermediate (Grx-SSG). In the dithiol mechanism (A), the mixed disulfide (Grx-SSG) is then attacked by a second cysteine of the Grx, not belonging to the active site sequence motif (indicated by *). This mechanism has been described for chloroplast Grx3 from *Chlamydomonas* (ortholog of *Arabidopsis* GrxS14) and involves the ferredoxin:thioredoxin reductase (FTR) for reduction of the intramolecular disulfide [64]. In the monothiol mechanism (B), the mixed disulfide (Grx-SSG) is preferably reduced by a second molecule of GSH to form GSSG and reduced Grx. Class I Grxs catalyze deglutathionylation by this monothiol mechanism. In some Class I Grxs, the attack of GSH on the mixed disulfide (Grx-SSG) competes with the attack performed by the C-terminal cysteine of the active site. The side reaction of the monothiol mechanism is analogous to the second step of the dithiol mechanism, although the resulting intramolecular disulfide is sensitive to GSH and the glutathionylated form (Grx-SSG) can thus be regenerated. Note that both GrxS12 and GrxC5 are unable to form intramolecular disulfides [62,63], therefore their deglutathionylation mechanism is monothiolic with no side reaction.

molecule of GSH (rather than a second cysteine of Grx) thereby forming GSSG and reduced glutaredoxin. The latter reaction is usually the rate-limiting step of the overall process.

Interestingly, in some glutaredoxins that follow a monothiol mechanism but contain a second active site cysteine (C-terminal), the attack of GSH on Grx-SSG competes with a side reaction in which the second active site cysteine performs the nucleophilic attack on the mixed disulfide (Grx-SSG), thus generating an intramolecular disulfide on the glutaredoxin [39]. The side reaction generally affects the turnover number of the protein as two molecule of GSH are required to regenerate reduced Grx form its disulfide form. The occurrence of the side reaction was demonstrated in yeast and human Grxs of class I (active site: CPYC, [39,75,76 refs]), but chloroplast glutaredoxins involved in deglutathionylation seem not to be affected by the side reaction. GrxS12 contains only one Cys in the active site motif (CSYS) and has no other cysteines that can form an intramolecular disulfide [50]. The active site sequence of GrxC5 contains the second cysteine (CSYC) but the two cysteine residues do not form a disulfide bond, at least in the conditions tested [63].

The absence of disulfide formation in the active site of these glutaredoxins has important consequences in their mechanism of action. These two glutaredoxins (GrxS12 and GrxC5) can only exist in two forms, either the reduced form (Grx-SH) or the glutathionylated one (Grx-SSG). In terms of activity, the glutathionylated form is temporarily inactive toward the glutathionylated substrates until Grx-SSG is reduced by GSH. Both proteins are stable *in vitro* in their glutathionylated form [50,63] and GrxS12 is steadily glutathionylated *in vivo* under stress [8]. Moreover, GrxS12 is hypersensitive to GSH/GSSG ratios [50] such that even a marginal oxidation of the glutathione pool would cause GrxS12 to become glutathionylated and therefore unable to deglutathionylate its targets that would remain in the glutathionylated form that, in most cases, corresponds to the inactive form (Fig. 3).

A similar scenario could also be proposed for GrxC5 but additional experiments are required to verify the sensitivity of the redox state of the protein (reduced *versus* glutathionylated) to variation of GSH/GSSG ratios. In addition, GrxC5 can assemble Fe–S clusters and the assembly/disassembly of the cluster, likely dependent on GSH and GSSG, respectively, might constitute an additional

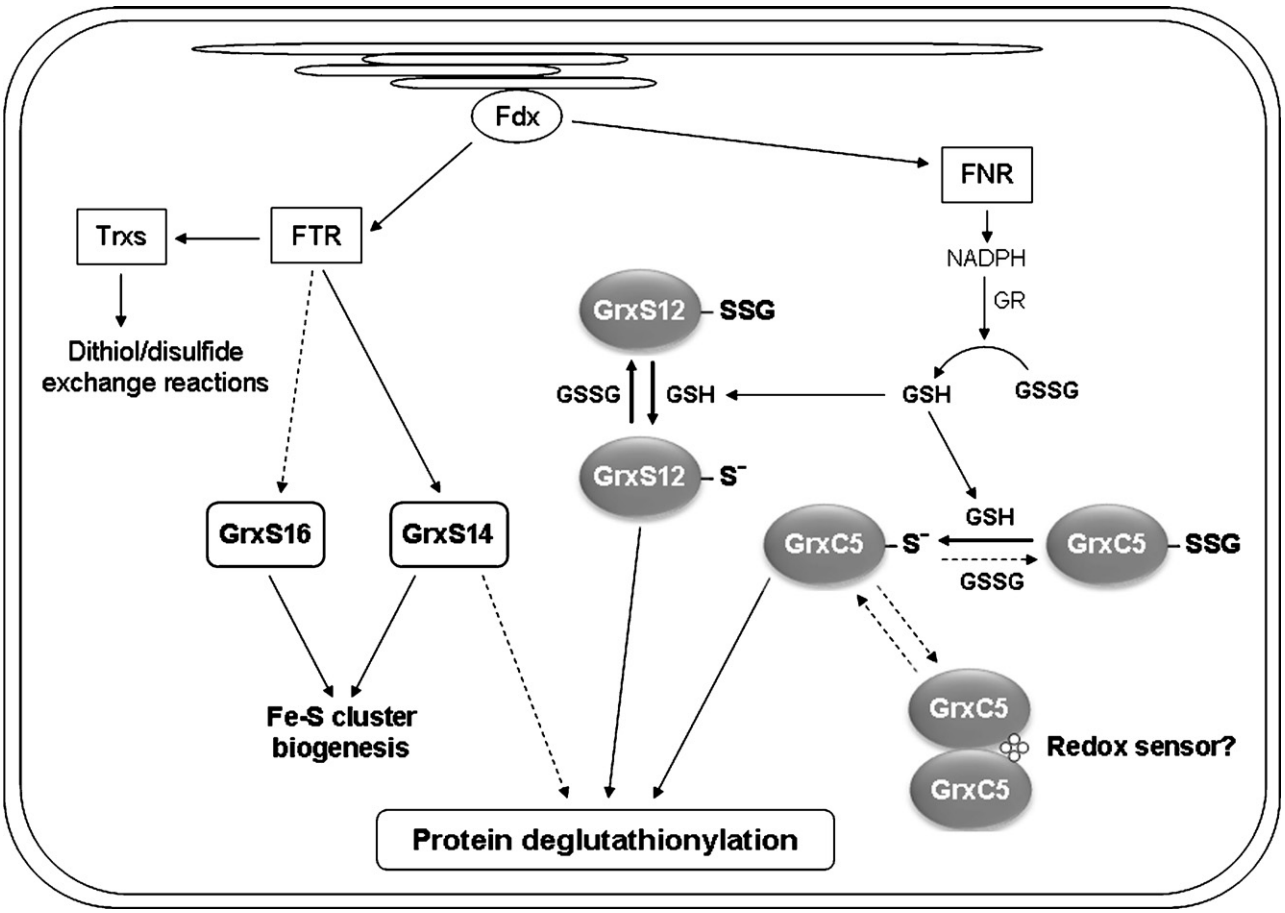

Fig. 3. Reduction pathways and functions of chloroplast glutaredoxins. Only glutaredoxins of Class I (GrxS12, GrxC5, depicted in gray) and of Class II (GrxS14, GrxS14, white symbols) are represented, since Class III Grxs have not been characterized. Broken arrows indicate uncertain reactions or speculative functions. Photosynthetically reduced ferredoxin (Fdx) provide electrons in chloroplasts to both ferredoxin:thioredoxin reductase (FTR) and ferredoxin:NADP reductase (FNR). FTR-reduced thioredoxins (Trxs) reduce of intra- or intermolecular protein disulfides. GrxS14 and GrxS16 (Class II) are also potentially reduced by FTR and may be involved in Fe–S cluster biogenesis/assembly. Class I GrxS12 and GrxC5 are reduced by GSH and their redox state is dependent on the GSH/GSSG ratio which is ultimately controlled by glutathione reductase (GR). Whereas GSH stabilizes the reduced/active form of the protein, GSSG would induce the glutathionylated/inactive state (to date, demonstrated only for GrxS12). GrxS12 and GrxC5 are both active in protein deglutathionylation. In addition, GrxC5 can function as redox sensor oscillating between an apomonomer and a holodimer state binding a Fe–S cluster.

regulatory mechanism. Based on these properties, GrxC5 might play a role as a redox sensor, similar to human Grx2 [79,80] (Fig. 3).

5. Possible functions of protein glutathionylation in chloroplasts

Some aspects of protein glutathionylation in chloroplasts are well established; others are still hypothetical and matter of debate. It is well established that chloroplasts contain a number of proteins that are amenable to glutathionylation [7,9,13] and for some of them, the process has been thoroughly characterized [7,9,13]. It is also well known that chloroplasts contain a number of glutaredoxins that can catalyze the deglutathionylation of specific targets [37,38,50,62,63]. Two higher plants glutaredoxins of class I (GrxS12 and GrxC5) seem particularly active as deglutathionylating agents using GSH as a reductant [50,62,63]. In *Chlamydomonas*, these Grxs are apparently substituted by class II Grx3 (Table 1), which uses electrons provided by photosynthesis through ferredoxin:thioredoxin reductase [64]. Glutathionylation is probably a reversible process *in vivo* and we speculate that it might fulfill several important functions in plant cells, particularly in chloroplasts.

5.1. Glutathionylation as a protecting mechanism of hypersensitive thiols

The first possible function of glutathionylation is protection against oxidative stress. The photosynthetic machinery of chloroplasts can be a strong source of ROS under stress conditions, and several members of the chloroplast proteome contain cysteines that are both reactive and accessible. Other than their spatial accessibility, a key point determining the reactivity of cysteines is their acidity or (de)protonation state, *i.e.* reactive cysteines have low pK_a values because they are found in molecular environments that favor deprotonation. In some cases this reactivity is necessary for the catalytic function of the protein. Reactive cysteines can also be very sensitive to ROS and undergo reversible and irreversible oxidation to sulfenic and sulfinic/sulfonic acid forms, respectively. Glutathionylation, occurring *via* the reaction of GSH with protein sulfenates, may be a general mechanism that protects hypersensitive cysteines under stress condition. Consistently, proteomic studies aimed at the identification of the largest possible number of glutathionylated proteins often use oxidant molecules such as H_2O_2 to stimulate glutathionylation [8–11].

Cysteine-149 of chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a typical example of a catalytic cysteine that is highly sensitive to oxidation [12]. This active site residue of the

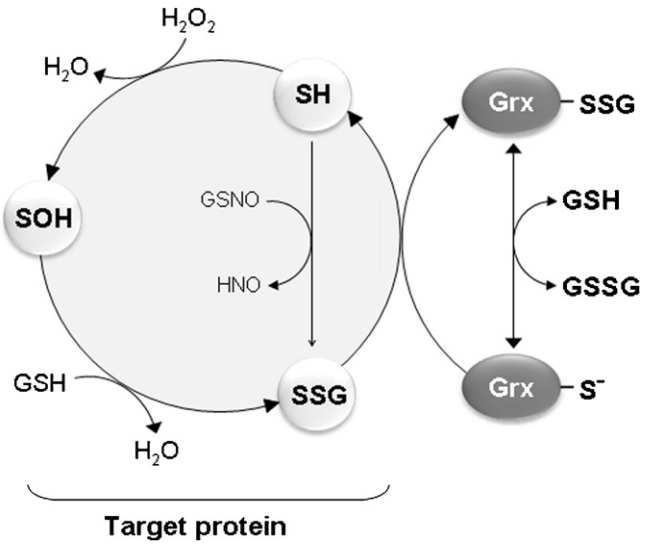

Fig. 4. The glutathionylation–deglutathionylation cycle (GD-cycle). In this view target proteins are first primarily oxidized by H_2O_2 to sulfenic acid (SOH) and then glutathionylated by GSH. Alternatively, nitrosoglutathione (GSNO) might directly glutathionylate some targets. Based on the K_{ox}, GSSG-mediated glutathionylation of target proteins is excluded (see text for details). The glutathionylated target protein (SSG) is deglutathionylated by reduced glutaredoxin (Grx-S^-) that becomes itself glutathionylated (Grx-SSG) and then reduced back by GSH. Direct deglutathionylation of target proteins by GSH (not shown) is a thermodynamically favored process that is probably too slow to have any physiological meaning. In some cases, *e.g.* GrxS12, the deglutathionylation activity of Grx is strongly dependent on the GSH/GSSG ratio that establishes which percentage of the GrxS12 pool is in the reduced/active form. Overall, the GD-cycle may be envisioned as either a mechanism of (i) protection against over-oxidation, (ii) ROS scavenging and (iii) redox signaling (see text for further details).

enzyme is made reactive by the vicinity of the side chain of a histidine (His176) that attracts the proton of the cysteine thiol [81]. As a result, the pK_a of Cys149 of *Arabidopsis* GAPDH is about 6 [authors unpublished observation]. The thiolate of Cys149 is essential for catalysis, as it covalently binds the substrate bisphosphoglycerate during the catalytic cycle of the enzyme. However, because of its prominent reactivity, Cys149 is also very sensitive to oxidation by H_2O_2 [12]. A sulfenic acid is formed (—SOH) in the first step of H_2O_2-dependent oxidation, but further steps may lead to sulfinic ($—SO_2H$) and sulfonic ($—SO_3H$) acid formation [12]. The rapid reaction of sulfenic Cys149 with GSH prevents irreversible oxidation and allows recovery of the active protein in a further step. Glutathionylated GAPDH is completely inactive, but can be fully and rapidly reactivated by GSH in the presence of glutaredoxins like GrxS12 [62]. Similar results were obtained with *Chlamydomonas* isocitrate lyase [82], suggesting that glutathionylation can be an effective means of protection against over-oxidation of specific protein cysteine thiols.

5.2. The GD-cycle as a potential mechanism of ROS scavenging

Protein glutathionylation is a reversible process and glutathionylated proteins are reduced back to their thiol form by glutaredoxins with concomitant recover, in most cases, of enzyme activity [7]. As a whole, protein glutathionylation and Grx-dependent deglutathionylation reactions constitute an integrated system, here named glutathionylation–deglutathionylation cycle (GD-cycle) (Fig. 4), which allows protection and regeneration of protein targets that are particularly prone to oxidative modifications.

Intriguingly, the GD-cycle may also be looked at a different perspective as a mechanism of ROS scavenging. MSRB1 and Prx IIE are two enzymes involved in the antioxidant stress response in which glutathionylation is part of the catalytic cycle [37,38]. However, aside from these special cases, we propose that glutathionylation of a subset of chloroplast proteins may have a general ROS scavenging effect. Though speculative, this hypothesis is supported by peculiar features of the chloroplast proteome and glutaredoxins active in deglutathionylation.

First, the actual concentration of reactive cysteines that are accessible to H_2O_2 in the chloroplast proteome may be very high. Rubisco accounts for about 50% of total soluble proteins in chloroplasts and its concentration in the stroma is around 0.5 mM [83]. Interestingly, Rubisco was identified as a target of protein glutathionylation [8,13] with three putative glutathionylation sites in the large (L) subunit and an additional one in the small (S) subunit [13]. Since Rubisco is a hexadecamer (L_8S_8), the *in vivo* concentration of each of these reactive cysteines would be around 4 mM, and the rest of the chloroplast proteome would further contribute to the total pool of reactive/glutathionylable cysteines. The cumulative concentration of cysteines of the chloroplast proteome that can potentially react with H_2O_2 and GSH is thus even higher than the concentration of the free glutathione pool that is estimated to be 1–3 mM in chloroplasts [15,16]. Overall, the chloroplast proteome seems to have the potentiality to reduce substantial amounts of H_2O_2 *via* primary oxidation of reactive cysteines.

Second, glutaredoxins have a highly reactive catalytic cysteine (pK_a around 4 for GrxS12; [50]) that can perform nucleophilic attack on protein-glutathione mixed disulfide with high catalytic efficiency (k_{cat}/K_m 10^5–10^6 M^{-1} s^{-1} for GrxS12, [50]) at any physiological pH. Provided that primary oxidation of reactive cysteines (*i.e.* H_2O_2 reduction) might be followed by spontaneous glutathionylation of sulfenic cysteines, glutaredoxins would allow a rapid cycling of glutathione-bound proteins to their reduced forms by means of the GD-cycle (Fig. 4). Interestingly, a consequence of the GD-cycle consists in the oxidation of two GSH molecules per each H_2O_2 reduced, with concomitant formation of one GSSG. Different types of stress conditions have been shown to perturb the glutathione redox state *in vivo* [17], and it is likely that catalase deficient mutants *cat2* accumulate GSSG in chloroplasts under photorespiratory conditions [16,47]. It is possible that this H_2O_2-triggered GSSG accumulation may be a consequence of the ongoing GD-cycle.

By this cycle, Grxs would finally function as a "proteome-dependent" glutathione peroxidase ($H_2O_2 + 2GSH = GSSG + 2H_2O$), an interesting possibility if we consider that the activity of true glutathione peroxidases in chloroplasts is very low [84]. However, the efficiency of the GD-cycle *in vivo* as compared with other processes of ROS scavenging cannot be easily estimated in the light of the current knowledge. Nonetheless, this potential function of glutathionylation has intriguing aspects that need to be explored in the coming future.

5.3. Glutathionylation as a potential signaling mechanism in chloroplasts

Glutathionylation plays an important role in the regulation of many mammalian cell signaling pathways and transcription factors regulating cell growth, differentiation and apoptosis [27,28]. A similar signaling role of glutathionylation in chloroplasts is also possible. Glutaredoxins, particularly GrxS12, seem to have the properties needed for regulating this type of signaling. In a signaling context, some proteins of the chloroplasts would be oxidized to sulfenic acid by controlled amounts of H_2O_2. Stress is not a prerequisite, as redox signaling is expected to take place also under physiological growth conditions.

The antioxidant activity of the chloroplast (ascorbate peroxidases and peroxiredoxins) is powerful [14,25]. Thus, it is possible that high local concentrations of H_2O_2 can occur where

scavenging activities are temporarily inactivated. According to the floodgate hypothesis [85], redox signaling in human cells is triggered after the specific inactivation (*e.g.* by over-oxidation) of a small fraction of 2-cys-peroxiredoxin that interacts with lipid rafts. In the absence of scavenging activities, the concentration of H_2O_2 can locally build up and promote signaling by interacting with proteins other than peroxiredoxins. Over-oxidized chloroplast 2-Cys-peroxiredoxin interacts with photosystem II [86]. It is thus tempting to speculate that redox signaling in this organelle is also made possible by a local inactivation of scavenging enzymes.

In any case, sulfenation of reactive/catalytic cysteines would be followed by spontaneous glutathionylation. This chain of reactions would also affect glutaredoxins that are sensitive to oxidation and, of course, also undergo glutathionylation. However, since glutathionylated GrxS12 reacts very rapidly with GSH, the stability of GrxS12-SSG *in vivo* is dictated by the GSH/GSSG ratio and by the K_{ox} of the reaction (Fig. 4). Indeed, under conditions of GSH/GSSG ratios highly above the K_{ox} of GrxS12 (309 at pH 7.0; 69 at pH 7.9; [50]), we can predict that no glutathionylated targets should accumulate, because fully active GrxS12 would efficiently mediate the removal of glutathione from glutathionylated targets. However if we assume that the GSH/GSSG ratio could locally change, even in the absence of stress, then the signal could be transmitted and modulated by glutaredoxins. If we speculate, for example, that a localized concentration of H_2O_2 caused the oxidation of 1% of the local pool of glutathione, the resulting GSH/GSSG ratio of 200 will strongly affect GrxS12. Indeed, the K_{ox} of 300 implies that 60% of GrxS12 is glutathionylated under these conditions, causing a 60% decrease of the deglutathionylating activity. From these calculations, we can predict that GrxS12 is more than 90% reduced (active) at GSH/GSSG ratios above 3000, and more than 90% glutathionylated (inactive) at ratios below 30. Any change of the glutathione redox state within these limits would have an effect on the deglutathionylating activity of GrxS12, and consequently on the glutathionylated state of the targets. In analogy with the floodgate hypothesis, the unique biochemical properties of GrxS12 would allow glutathione to have a signaling role through glutathionylation of GrxS12 targets. The rapid glutathionylation of GrxS12 under mild oxidative stress would inactivate the glutaredoxin itself and consequently stabilize a number of glutathionylated proteins that could amplify the oxidative stress signal and maybe activate specific adaptative responses.

A remarkable example of redox signaling involving glutathionylation concerns thioredoxin-f and the Calvin–Benson cycle. This type of chloroplast thioredoxin is characterized by the presence of an additional cysteine that has no catalytic role but is located in an exposed position close to the active site [36]. This residue is strictly conserved in all f-type thioredoxins but absent in other chloroplast thioredoxins. Treatment of Trx-f with H_2O_2 plus GSH leads to specific glutathionylation of this additional cysteine. Indeed Trx-f is the only chloroplast thioredoxin known to be sensitive to glutathionylation. Interestingly, glutathionylation of the additional cysteine does not alter the capability of the protein to perform thiol/disulfide exchange reactions, but strongly hinders the functional interaction between Trx-f and ferredoxin:thioredoxin reductase [36]. Glutathionylated Trx-f cannot be efficiently reduced by ferredoxin:thioredoxin reductase and lacks the capacity to link the light reactions of photosynthesis with the Calvin–Benson cycle. Trx-f specifically regulates GAPDH and fructose-1,6-bisphosphatase of the Calvin–Benson (a rare property among thioredoxin targets) [4,87]. Moreover, Trx-f is also the preferential electron donor for activation of other Calvin–Benson cycle enzymes. Although glutathionylation of Trx-f still lacks *in vivo* confirmation, we propose that this redox modification (*i.e.* glutathionylation of Trx-f) may contribute to the down-regulation of the Calvin–Benson cycle under oxidative stress conditions. Besides this indirect regulation of the Calvin–Benson cycle mediated by Trx-f, glutathionylation may also have a direct effect on this pathway as the 11 enzymes of the cycle all undergo glutathionylation [8,10,11,13]. However, the effect of glutathionylation on the activity of most of these enzymes remains to be established.

Interestingly, a glutathione-dependent, Trx-f-mediated regulation of the Calvin–Benson cycle under stress may allow redistribution of reducing equivalents within chloroplasts in the light. Notably, carbon fixation is among the first chloroplast processes that are affected by stress, and the inhibition of the Calvin–Benson cycle typically precedes the inhibition of light reactions of photosynthesis at the onset of stress [88,89]. Down-regulation of the Calvin–Benson cycle would lead to a decreased consumption of NADPH (by GAPDH) and increased production of ROS. The latter effect would be a consequence of the over-reduction of photosystem I caused by decreased availability of electron acceptors. On the one hand the increased ROS production would probably reinforce the initial oxidant signal, but on the other the decreased consumption of NADPH by the Calvin–Benson cycle would make more NADPH available for ROS detoxification systems [4,90]. Clearly, further studies are required to determine the timing and the relative importance of these alternative routes.

Overall, it seems that the light-dependent (thioredoxin-mediated) regulation of the Calvin–Benson cycle interacts with the ROS-dependent glutathionylation in an apparently complex network, but the understanding of this network is still in its infancy.

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