FEBS Letters

Biochemical characterization of glutaredoxins from *Chlamydomonas reinhardtii*: Kinetics and specificity in deglutathionylation reactions

Xing-Huang Gao [a], Mirko Zaffagnini [a,1], Mariette Bedhomme [a], Laure Michelet [a], Corinne Cassier-Chauvat [b], Paulette Decottignies [c], Stéphane D. Lemaire [a,*]

[a] *Institut de Biologie des Plantes, UMR 8618, CNRS/Univ Paris-Sud, Bâtiment 630, F-91405 Orsay Cedex, France*
[b] *CEA, iBiTec-S, SBIGeM, LBI, CNRS URA 2096, Bat 142 CEA-Saclay, F-91191 Gif sur Yvette Cedex, France*
[c] *Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619, CNRS/Univ Paris-Sud, Bâtiment 430, 91405 Orsay Cedex, France*

ARTICLE INFO

ABSTRACT

Protein deglutathionylation is mainly catalyzed by glutaredoxins (GRXs). We have analyzed the biochemical properties of four of the six different GRXs of *Chlamydomonas reinhardtii*. Kinetic parameters were determined for disulfide and dehydroascorbate reduction but also for deglutathionylation of artificial and protein substrates. The results indicate that GRXs exhibit striking differences in their catalytic properties, mainly linked to the class of GRX considered but also to the pK_a of the N-terminal catalytic cysteine. Furthermore, glutathionylated proteins were found to exhibit distinct reactivities with GRXs. These results suggest that glutathionylation may allow a fine tuning of cell metabolism under stress conditions.

Structured summary: MINT-7761120: *GRX6* (uniprotkb:A8HN52) and *GRX6* (uniprotkb:A8HN52) *bind* (MI:0408) by *comigration in non denaturing gel electrophoresis* (MI:0404)
MINT-7761098:*GRX5* (uniprotkb:A8I7Q4) and *GRX5* (uniprotkb:A8I7Q4) *bind* (MI:0408) by *comigration in non denaturing gel electrophoresis* (MI:0404)

1. Introduction

Glutathionylation is a reversible post-translational modification consisting of the formation of a mixed disulfide between glutathione and a protein cysteine residue. It is promoted by oxidative and nitrosative stresses but also occurs in unstressed cells. It can protect specific cysteine residues from irreversible oxidation to sulfinic or sulfonic acid forms but can also modulate protein activities, and thereby play a role in many cellular processes [1]. Glutathionylation has been mainly studied in mammals but emerging evidence suggests that it could also constitute an important mechanism of regulation and signaling in plants [2,3]. Glutathionylation can occur through several mechanisms but the one prevailing in vivo remains unclear. By contrast, the reverse reaction, called deglutathionylation, is mainly catalyzed by small disulfide oxidoreductases of the thioredoxin family named glutaredoxins (GRXs) [4,5].

Two types of GRXs, named CPYC and CGFS, are present in almost all organisms [6]. A third type, named CC, is specific to higher plant species [7]. CPYC-type GRXs are reduced by GSH and catalyze the deglutathionylation of artificial substrates (HED assay) or protein substrates [1,4]. They also possess dehydroascorbate reductase (DHAR) and disulfide reductase activities. On the other hand, CGFS-type GRXs are not efficiently reduced by GSH and present no activity in the HED (hydroxyethyl disulfide), DHA (dehydroascorbate) or disulfide reduction assays. However, three CGFS GRXs, namely *Escherichia coli* GRX4, *Saccharomyces cerevisiae* GRX5 and *Chlamydomonas reinhardtii* GRX3, were shown to catalyze protein deglutathionylation through a disulfide bridge which can be reduced by thioredoxin reductases [5,8,9]. In addition, different types of GRXs, including several plant CGFS GRXs are also able to coordinate an iron sulfur cluster and could play a role in iron–sulfur cluster assembly/biogenesis [10–13].

The unicellular green alga *C. reinhardtii* contains six GRXS: two CPYC-type GRXs (GRX1–2) and four CGFS-type GRXs (GRX3–6). We have previously characterized the biochemical properties of

cytosolic GRX1 and chloroplastic GRX3 [5]. GRX1 is a typical CPYC-type GRX which is reduced by GSH and can catalyze reduction of insulin, DHA and glutathionylated substrates. By contrast, GRX3 was shown to catalyze protein deglutathionylation using the photosynthetically reduced ferredoxin-thioredoxin reductase as electron donor.

In this work, we have determined and compared the biochemical properties of all Chlamydomonas GRXs that could be produced recombinantly and purified. We investigated their ability to form intra- or intermolecular disulfide bonds, their reduction by GSH and thioredoxin reductases and we kinetically studied the DHAR and disulfide reductase activities. Moreover, deglutathionylation activities were measured with the standard HED assay but also with two distinct glutathionylated proteins: isocitrate lyase (ICL) and A_4-glyceraldehyde-3-phosphate dehydrogenase (A_4-GAPDH). We show that GRX5 and GRX6 are able to form covalent homodimers but are not significantly active in any of the assays. We also show that GRX1 catalyzes deglutathionylation more efficiently than GRX2, likely because of the lower pK_a of its active site cysteine. Furthermore, A_4-GAPDH was found to be 10 times more efficiently deglutathionylated by GRXs compared to ICL, suggesting that GRXs exhibit substrate selectivity, a property which could be important for fine tuning of cell metabolism in vivo.

2. Materials and methods

2.1. Materials/chemicals

NAP-5 columns were from GE Healthcare. GSSG was from Roche Diagnostics Corporation. All the other reagents were from Sigma–Aldrich.

2.2. Expression and purification of recombinant proteins

The sequences coding the mature forms of GRX2, GRX5 and GRX6 were amplified by standard RT-PCR using total Chlamydomonas RNA extracts (primers listed in Supplementary Table 1) and cloned into the NcoI/NdeI and BamHI restriction sites of modified pET3c or pET3d vectors (Novagen) allowing expression of the protein with a polyhistidine tag at the N-terminus. The sequences were verified by sequencing. Expression and purification of recombinant GRXs were performed as described previously for GRX1 and GRX3 [5]. Recombinant ICL and A_4-GAPDH were produced and purified as previously described [14,15]. All purified proteins were checked by SDS–PAGE and MALDI-TOF mass spectrometry.

2.3. Activity assays

Insulin reduction, DHA reduction, HED assays and A_4-GAPDH glutathionylation and reactivation assays were performed as previously described [5,15]. The determination of kinetic parameters in the HED and DHA reduction assays was performed with non saturating concentrations of GSH in order to keep the rates of spontaneous reaction between GSH and the oxidized substrates low relative to those of the GRX-catalyzed reactions [16]. Glutathionylated ICL was prepared and assayed as described [14].

2.4. 3D structure modeling

CrGRX1 and CrGRX2 models were generated using the SWISS-MODEL modeling server (http://swissmodel.expasy.org). Yeast GRX1 (PDB code 3c1r) was selected as an appropriate template structure. Surface electrostatic potentials were generated and analyzed with Pymol (DeLano Scientific, Palo Alto, CA).

2.5. Determination of pK_a

The pK_a of the reactive cysteine of GRX1 and GRX2 was determined as described for human GRXs [17,18]. Briefly, GRXs were reduced by incubation with 20 mM reduced DTT for 1 h at room temperature and subsequently desalted in 30 mM Tris–HCl, pH 7.9 using NAP-5 columns. Reduced GRXs were then incubated for 3 min in different buffers with a pH range from 2.6 to 8.2 in the presence or absence of 0.3 M iodoacetamide (IAM), an alkylating agent reacting with thiolates but not thiols. Under each condition, the GRX activity in the HED assay was measured. The percentage of remaining activity at each pH was determined by comparing the activity of the enzyme incubated with and without IAM at the same pH. The pK_a was then calculated by fitting the experimental data to a derivation of the Henderson–Hasselbalch equation as described [17].
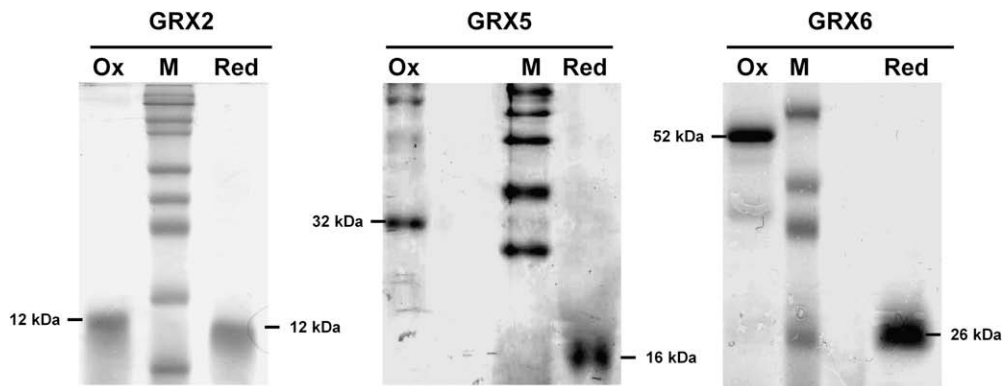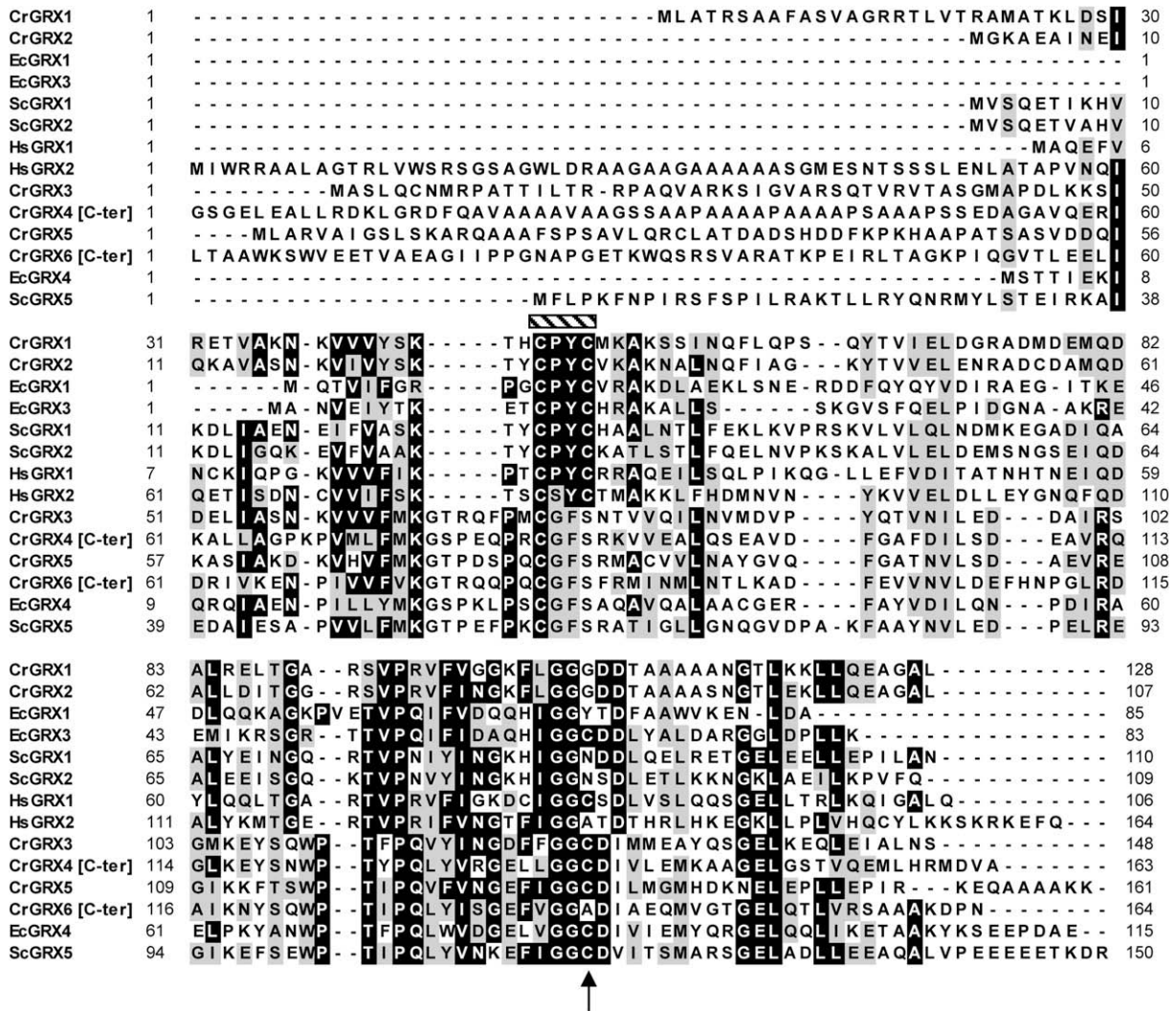
2.6. Replicates

All the results reported are representative of at least three independent experiments and expressed as mean ± standard deviation.

3. Results

3.1. Chlamydomonas glutaredoxins

The genome of C. reinhardtii contains six genes encoding GRXs. GRX1 and GRX2 are typical CPYC-type GRXs predicted to localize to the cytosol while the other four GRXs belong to the CGFS type (Fig. 1). Sequence analyses suggested that GRX3 and GRX6 are plastidial, GRX5 is mitochondrial and GRX4 is cytosolic/nuclear [7]. These predictions of subcellular localizations were confirmed for homologous GRXs (GrxS14–S17) from Arabidopsis or poplar [10], suggesting that the functions of these GRXs might be conserved in photosynthetic eukaryotes. GRX1–3 and GRX5 are single module GRXs while GRX4 and GRX6 contain an N-terminal domain upstream of the C-terminal CGFS GRX module. GRX4 is a bipartite protein containing two CGFS GRX modules, while the N-terminal domain of GRX6 shares strong homology with the Synechocystis sp. PCC6803 slr1035 sequence of unknown function. GRX1 and GRX3 have been previously purified as recombinant proteins [5]. The sequences encoding the mature form of the other four GRXs were amplified by RT-PCR and cloned into pET3c or pEt3d vectors fused to an N-terminal histidine tag. The proteins were produced in E. coli and purified by nickel affinity chromatography with the exception of GRX4 which accumulated in inclusion bodies and was not further characterized.

All typical CPYC GRXs characterized to date contain an intramolecular disulfide bond between the two vicinal cysteines of their active site. Moreover, CGFS type GRXs, despite the absence of a vicinal cysteine within their active site motif, contain a partially conserved cysteine in their C-terminal part (Fig. 1) which was shown to form a disulfide bond with the active site cysteine in E. coli GRX4, S. cerevisiae GRX5 and Chlamydomonas GRX3 [5,8,9]. This C-terminal cysteine is present in all Chlamydomonas CGFS GRXs except GRX6 (Fig. 1). In order to investigate the redox state and oligomerization of Chlamydomonas GRXs, purified recombinant GRX2, GRX5 and GRX6 were analyzed by non-reducing SDS–PAGE and thiol titrations (DTNB assay) after incubation with reduced DTT or oxidized DTT (Fig. 2). GRX2 was found to migrate, either in the oxidized or reduced form, as a single band of ca. 12 kDa, the size expected for monomeric GRX2. By contrast, after treatment of GRX5 or GRX6 with oxidized DTT, we observed a major band of 32 kDa and 52 kDa, respectively, corresponding to the size of the dimeric forms. After incubation with reduced DTT, GRX5 and

Fig. 1. Multiple sequence alignment of GRXs from diverse organisms. The proteins were aligned with the ClustalW program and corrected manually for N-terminal extensions. Residues boxed in black correspond to 50% identity. Residues boxed in gray correspond to 50% conservation with the PAM120 matrix. Accession numbers and abbreviations: *Chlamydomonas reinhardtii* (Cr) GRX1, XP_001702999; GRX2, XP_001694001; GRX3, XP_001702880; GRX4 [C-Ter], XP_001703697 with deletion of N terminal extension up to G72; GRX5, XP_001700896 with correction of exon 2 based on cDNA sequencing (insertion of KVHVFM between residues D63 and K64); GRX6 [C-ter]; XP_001689744 with deletion of N-terminal extension up to L118; *Escherichia coli* (Ec) GRX1, P00277; GRX3, P37687; *Saccharomyces cerevisiae* (Sc) GRX1, S19363; GRX2, P17695; GRX5, NP_015266; and *Homo sapiens* (Hs) GRX1, NP_002055; GRX2, NP_932066. The dashed line corresponds to the active site. The arrow indicates the position of the C-terminal cysteine conserved in several members of the CGFS family.

Fig. 2. Oligomerization of Chlamydomonas GRXs under oxidizing and reducing conditions. Ox: oxidized GRX2, GRX5 and GRX6 were prepared by incubation with 20 mM oxidized DTT for 4 h at room temperature in 30 mM Tris–HCl, pH 7.9, then desalted through NAP-5 columns using the same buffer. Red: reduced GRXs were prepared by incubation of oxidized GRXs with 20 mM reduced DTT for 1 hour. M: molecular weight markers. The faint degradation of GRX2 was only observed after long incubations at room temperature. Five micrograms oxidized and reduced GRXs were analyzed by non-reducing SDS–PAGE and stained with Coomassie brilliant blue.

GRX6 migrated as a single monomeric band of 16 kDa and 26 kDa, respectively. Thiol titrations revealed that reduction of the oxidized forms led to the appearance of approximately two thiols per monomer in the case of GRX2 but only one for GRX5 and GRX6 (Table 1). All these results are consistent with the formation of an intramolecular disulfide between Cys27 and Cys30 within the CPYC active site of GRX2 and indicate that the third cysteine, Cys56, is presumably buried and therefore unable to mediate the formation of an intermolecular disulfide bond. In contrast, GRX5 and GRX6 can form covalent homodimers through intermolecular disulfide bonds that were only poorly reduced by GSH (data not shown). As all these GRXs can form disulfide bonds, they might be able to catalyze disulfide reduction through dithiol/disulfide exchange. This prompted us to analyze the activity of these GRXs in different classical activity assays.

3.2. Insulin and DHA reduction

Insulin disulfide bonds are very efficiently reduced by TRXs [19]. Although much less efficient, CPYC-type GRXs are known to catalyze insulin reduction using DTT or GSH as electron donor [20]. Consistently, GRX2 was found to catalyze insulin reduction in the presence of these reductants though the enzyme appeared slightly less efficient than GRX1 (Fig. 3; [5]). However, we cannot

Table 1
Free thiol content in oxidized and reduced GRXs. The content of free thiols in reduced and oxidized Chlamydomonas GRXs was titrated using the standard DTNB assay as described in [8]. Data presented are the means ± S.D. (n = 3).

	Number of free thiols measured (mol SH/mol monomer)		
	Reduced	Oxidized	Decrease in free thiols (reduced−oxidized)
GRX2	1.9 ± 0.06	0.2 ± 0.06	1.7 ± 0.06
GRX5	1.4 ± 0.06	0.2 ± 0.02	1.2 ± 0.06
GRX6	1.1 ± 0.10	0.2 ± 0.04	0.9 ± 0.10

exclude that relatively small differences between GRX1 and GRX2 could be accounted for by variation of enzyme batches. On the other hand, GRX5 or GRX6 did not exhibit a significant activity in the insulin reduction assay, either in the presence of DTT or GSH (Fig. 3). This result is consistent with the lack of insulin disulfide reductase activity observed for other CGFS GRXs including Chlamydomonas GRX3 [5,8].

GRXs also have the ability to catalyze the reduction of DHA using glutathione as reductant [5,21,22]. As above, GRX1 and GRX2, but not GRX5 and GRX6, were found to catalyze DHA reduction. The catalytic parameters of DHA reduction by GRX1 and GRX2 were determined (Table 2). Compared to GRX1, GRX2 exhibited, for both DHA and GSH, a lower apparent K_m (nearly 2-fold), but also a lower apparent k_{cat} (2-fold). As a consequence, the catalytic efficiencies (k_{cat}/K_m) of GRX1 and GRX2 were almost comparable. Moreover, these kinetic parameters are comparable to those previously reported for several mammalian or higher plant GRXs [21–23].

3.3. Deglutathionylation of an artificial substrate: HED assay
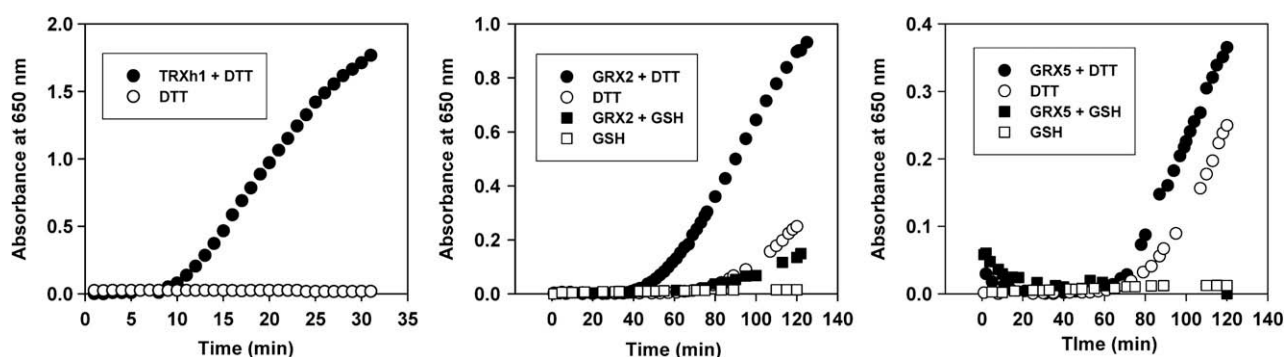
The deglutathionylation activity of GRXs is generally measured with an artificial substrate in the HED assay [24]. In this assay, GRX activity is followed as the oxidation of NADPH in a coupled system with GSH and glutathione reductase (GR). The substrate of GRX, the glutathionylated β-mercaptoethanol (β-ME-SG), is formed during an initial preincubation where HED reacts with GSH. The reaction proceeds in two steps. First, GRX performs an initial nucleophilic attack on the β-ME-SG mixed disulfide giving rise to GRX-SG and releasing the deglutathionylated substrate. In the second step, reaction of GSH with GRX-SG allows reduction of GRX and releases GSSG. This second step is the rate-limiting step of the reaction [4].

As previously observed for GRX3 [5], GRX5 and GRX6 exhibited no significant activity in the HED assay. On the other hand, GRX1 and GRX2 were found to be active although GRX1 appeared more

Fig. 3. Insulin disulfide reductase activity of Chlamydomonas GRXs. The rate of insulin reduction was assessed by measuring the turbidity at 650 nm in reaction assays containing 0.33 mM DTT or 1 mM GSH in the presence of 5 μM Chlamydomonas TRXh1 (left panel) or 5 μM GRX2 (central panel). The two CGFS-type GRXs, GRX5 and GRX6, were both inefficient and yielded similar results. Typical results obtained with 5 μM GRX5 are presented (right panel).

Table 2
Kinetic parameters of Chlamydomonas GRX1 and GRX2 in the dehydroascorbate reductase assay. The apparent K_m value for DHA was determined using a DHA concentration range of 0.125–1 mM for GRX1 and 0.25–3 mM for GRX2, in the presence of 2 mM GSH. The apparent K_m value for GSH was determined using a GSH concentration range of 0.2–4 mM for GRX1 and 0.5–3.5 mM for GRX2, in the presence of 1 mM DHA. The concentration of GRX1 and GRX2 was set to 0.5 μM. The apparent K_m and apparent k_{cat} were calculated by non-linear regression using the Michaelis–Menten equation. Data are represented as mean ± S.D.

	GSH			DHA		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
GRX1	1.63 ± 0.29	1.82 ± 0.15	1.11 × 10^3	0.39 ± 0.03[a]	1.67 ± 0.09[a]	4.3 × 10^{3a}
GRX2	0.96 ± 0.11	0.94 ± 0.03	0.98 × 10^3	0.17 ± 0.05	0.84 ± 0.06	4.92 × 10^3

[a] Data are from Ref. [8].

efficient than GRX2. Kinetic parameters were measured for both substrates, GSH and HED, the concentration of the latter representing an approximation of the levels of β-ME-SG formed during the initial preincubation. GRX1 exhibited a 2.5- to 8.6-fold higher catalytic efficiency compared to GRX2, depending on the substrate considered (Table 3). The higher efficiency of GRX1 is essentially due to higher k_{cat} values. Similar differences were previously observed between human GRX1 and GRX2 [17,25].

3.4. Deglutathionylation of protein substrates

We have recently shown that Arabidopsis A_4-GAPDH and Chlamydomonas ICL were inactivated by glutathionylation and could be reactivated by GRXs [14,15]. Both glutathionylated proteins were therefore used in the present study as protein substrates to measure Chlamydomonas GRX deglutathionylation activities and to examine their possible substrate specificities.

For glutathionylated A_4-GAPDH, chloroplastic CGFS GRX3 was previously shown to catalyze deglutathionylation using ferredoxin–thioredoxin reductase as electron donor [5]. However, the two CGFS GRXs tested here, GRX5 and GRX6, did not exhibit any ability to reactivate A_4-GAPDH using ferredoxin- or NADPH-dependent thioredoxin reductases (data not shown). Moreover, GRX5 and GRX6 were also not active in the ICL reactivation assay where DTT serves as a reductant (Fig. 4A). This suggests that contrary to GRX3, GRX5 and GRX6 are not efficient catalysts of protein deglutathionylation or that they exhibit distinct substrate specificities.

By contrast, both CPYC type GRXs, GRX1 and GRX2, proved to be efficient catalysts of protein deglutathionylation as illustrated by their efficiency to catalyze reactivation of glutathionylated ICL using either DTT or GSH as electron donor (Fig. 4A and B). However, GRX1 appeared more efficient than GRX2 in these assays. Indeed, determination of half-saturation values revealed that, compared to GRX2, GRX1 has a 2.2- and 2.6-fold higher catalytic efficiency for reactivation of ICL and A_4-GAPDH, respectively (Table 4). These differences are consistent with those observed in the HED assay (Table 3).

Moreover, comparison of ICL and A_4-GAPDH reactivation by GRX1 and GRX2 revealed that A_4-GAPDH is ca. 12 times more efficiently deglutathionylated than ICL. This suggests that glutathionylated proteins can exhibit distinct reactivities with GRXs and may therefore be deglutathionylated at different rates under similar conditions.
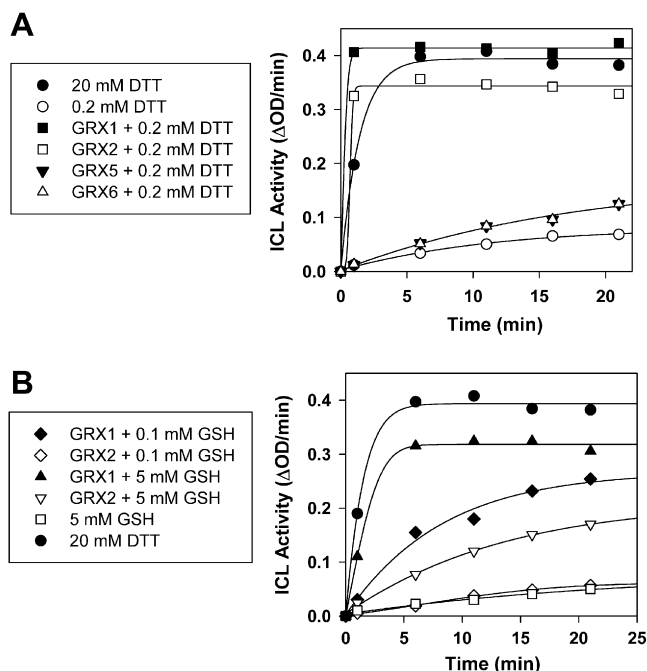
3.5. Origin of the higher catalytic efficiency of GRX1 compared to GRX2

In the case of TRXs, the differences of substrate specificity and reactivity with different isoforms of TRXs have been suggested to be linked to differences in charge distribution around the active site [26]. The local environment of GRX active site has also been suggested to contribute to the reactivity of GRXs [4]. Therefore, we modeled the 3D structure of Chlamydomonas GRX1 and GRX2 and analyzed surface charge distribution (Fig. 5). Positively charged residues surrounding the GSH binding site were found to

Fig. 4. Reactivation of glutathionylated ICL by Chlamydomonas GRXs. Glutathionylated ICL (10 μM) was reactivated by treatment with 5 μM Chlamydomonas GRXs in the presence of (A) 0.2 mM DTT or (B) 5 mM or 0.1 mM GSH. Positive control (20 mM DTT) and negative controls (0.2 mM DTT or 5 mM GSH) are also represented.
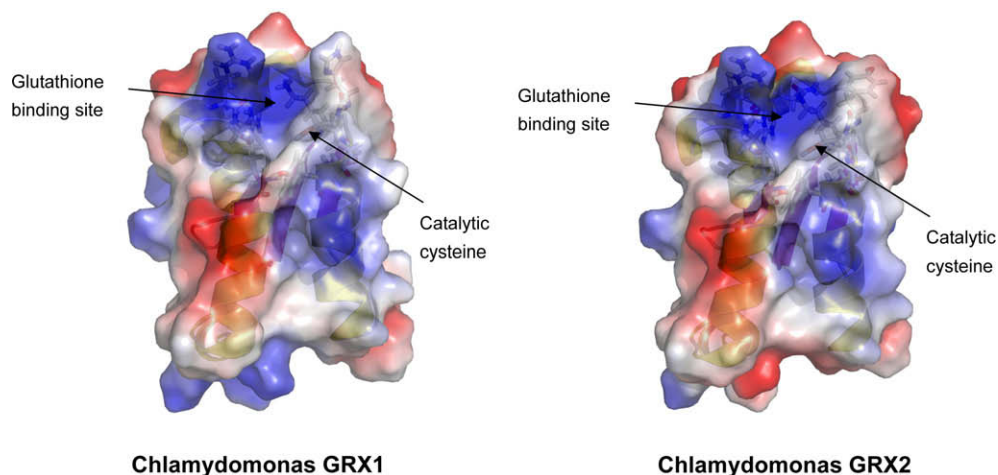
Table 4
Half-saturation values for reactivation of glutathionylated ICL and glutathionylated A_4-GAPDH by GRX1 and GRX2. Deglutathionylation of ICL-SG and A_4-GAPDH-SG was measured by following reactivation of the enzymes using corresponding activity assays. Reactivation was measured in the presence of varying concentrations of GRX1 and GRX2 (0.1–100 μM) and 5 mM GSH or 0.2 mM DTT.

$S_{0.5}$	ICL-SG		A_4-GAPDH-SG
	5 mM GSH	0.2 mM DTT	5 mM GSH
GRX1	12 μM ± 0.13	8.2 μM ± 0.76	0.87 μM ± 0.13[a]
GRX2	26.5 μM ± 2.64	16.4 μM ± 0.63	2.27 μM ± 0.35

[a] Data are from Ref. [8].

be conserved and no significant difference in charge distribution around the active sites of GRX1 and GRX2 could be observed. This suggested that the higher catalytic efficiency of GRX1 is likely not linked to differences of charge distribution. Another important factor that has been previously shown to contribute to the reactivity of GRXs is the pK_a of the active site catalytic cysteine [17]. This pK_a was therefore determined for both GRXs. The catalytic cysteines of GRX1 and GRX2 were found to have pK_a of 3.9 ± 0.1 and 4.8 ± 0.1, respectively. This one unit pH difference is likely a major factor contributing to the higher reactivity of GRX1. Indeed, such a differ-

Table 3
Kinetic parameters of Chlamydomonas GRX1 and GRX2 in the HED assay. The apparent K_m value for GSH was determined using a GSH concentration range of 0.5–3.5 mM in the presence of 0.7 mM HED for GRX1 (10 nM) and GRX2 (100 nM). The apparent K_m value for HED was determined using an HED concentration range of 0.1–2 mM for GRX1 (30 nM) and 0.05–0.8 mM for GRX2 (200 nM), in the presence of 1 mM GSH. The apparent K_m and k_{cat} were calculated by non-linear regression using the Michaelis–Menten equation. Data are represented as mean ± S.D.

	GSH			HED		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
GRX1	2.65 ± 0.51[a]	161.6 ± 15[a]	6.1 × 10^{4a}	0.34 ± 0.04	30.4 ± 4.31	8.9 × 10^4
GRX2	3.75 ± 0.66	26.5 ± 4.1	7.1 × 10^3	0.20 ± 0.04	7.1 ± 0.27	3.5 × 10^4

[a] Data are from Ref. [8].

Fig. 5. Surface electrostatic potentials around the active site of Chlamydomonas GRX1 and GRX2. Positively charged regions are colored in blue and negatively charged regions in red. The catalytic cysteine and glutathione binding domain are indicated.

ence between the pK_a of the leaving group is predicted, according to the Brønsted theory, to result in a 4-fold higher rate enhancement for GRX1 compared to GRX2 [17,27]. In yeast the higher catalytic activity of yGRX2 compared to yGRX1 was suggested not to be linked to differences in the pK_a of these GRXs but to the orientation of the side chain of the second active site cysteines due to surrounding amino acids [27]. The high reactivity of yGRX2 was shown to depend on the presence of Ser23 and Glu22, which are replaced by Ala23 and Gln52 in yGRX1. However, Chlamydomonas GRX1 and GRX2 both possess a Ser/Glu in the corresponding positions (Fig. 1) as in the highly reactive yGRX2, indicating that the higher deglutathionylation activity of GRX1 cannot be attributed to these residues. Other factors may also contribute to the differences of reactivity between the two Chlamydomonas GRXs, including enhancement of the nucleophilicity of GSH, the GRX helix two dipole moment and the reactivity of the second active site cysteine which can participate in a non-productive side reaction by formation of an intramolecular disulfide from the GRX-SG intermediate [4,17]. However, the higher reactivity of GRX1 was maintained when DTT was used as a reductant instead of GSH in the ICL reactivation assay (Table 4). This indicates that the differences between Chlamydomonas GRXs are more likely linked to the differences between the pK_a of their active site catalytic cysteines than to differences in their ability to enhance the nucleophilicity of GSH. Furthermore, these results confirm that reduction of the GRX-SG intermediate by GSH is the rate determining step of the overall deglutathionylation reaction as previously described [4,28].

4. Discussion

The biochemical characterization of Chlamydomonas GRXs clearly indicates that the CPYC and CGFS-type GRXs, initially distinguished based on sequence and phylogenetic analyses [7], exhibit distinct biochemical properties. Indeed, Chlamydomonas CGFS type GRXs, GRX5 and GRX6, do not possess classical GRX activities such as disulfide reductase and DHA reductase activities and they also poorly catalyze deglutathionylation of artificial and protein substrates. These results are consistent with previous studies on CGFS-type GRXs from diverse sources [5,8,9,29]. Moreover, GRX5 and GRX6 were both found to form covalent homodimers through intermolecular disulfide bonds while the CPYC-type GRXs, GRX1 and GRX2, only form an intramolecular disulfide bond between their vicinal cysteines. Although it cannot be excluded that GRX5 and GRX6 could catalyze deglutathionylation of more specific sub-

strates, all these results suggest that GRX5 and GRX6 may have functions distinct from those of classical CPYC GRXs. One of these potential functions might be related to iron–sulfur cluster assembly/biogenesis. Indeed, yeast GRX5, the homologue of Chlamydomonas GRX5, is required for the biogenesis of iron–sulfur containing proteins [30]. Furthermore, the absence of yeast GRX5 can be complemented by GrxS16, the Arabidopsis homologue of GRX6 [10]. Further studies will be required to unravel the exact functions of GRX5 and GRX6 and their higher plant homologues and to determine whether formation of the intermolecular disulfide may play a role in the regulation of the activity of these GRXs.

GRX1 and GRX2, the two CPYC type GRXs of Chlamydomonas, were found to catalyze disulfide reduction, DHA reduction and deglutathionylation of β-ME-SG with kinetic parameters similar to those of in the same range as numerous other GRXs from bacteria to human. Both GRXs were also found to catalyze protein deglutathionylation using the glutathionylated forms of ICL and GAPDH as substrates. However, compared to GRX2, GRX1 was a more efficient catalyst of deglutathionylation, a property which appears to rely mainly on the lower pK_a of GRX1 active site catalytic cysteine. These properties are strikingly similar to those of human GRX1 and GRX2 that also have a one pH unit difference in the pK_a of their catalytic cysteines [17]. This similarity with human proteins and genes is reminiscent of Chlamydomonas and has been emphasized previously [31].

While the results confirm that different CPYC-type GRXs can catalyze deglutathionylation at different rates, the most striking differences were observed when the rates of deglutathionylation were compared between the two protein substrates, ICL and A_4-GAPDH. Indeed, A_4-GAPDH was reactivated 12 times more efficiently than ICL. The reactivation of these enzymes depends on the deglutathionylation of a single catalytic cysteine [14,15]. Moreover the difference was consistently observed with both GRX1 and GRX2, suggesting that it is most likely linked to intrinsic properties of the glutathionylated protein substrates rather than to the properties of GRXs. Further studies will be required to determine whether this is also true for other glutathionylated proteins and to delineate the structural and molecular determinants of the reactivity of glutathionylated proteins with GRXs. These differences in the reactivity of glutathionylated proteins may have a functional importance. Indeed, in vivo, different kinetics of deglutathionylation/glutathionylation, depending on the intracellular redox state, might exist for different metabolic pathways, thereby allowing a fine tuning of cell metabolism under stress conditions.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.034.

References

[1] Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D. and Milzani, A. (2009) Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends Biochem. Sci. 34, 85–96.

[2] Gao, X.H., Bedhomme, M., Michelet, L., Zaffagnini, M. and Lemaire, S.D. (2009) Glutathionylation in photosynthetic organisms in: Oxidative Stress and Redox Regulation in Plants (Jacquot, J.-P., Ed.), pp. 363–403, Academic Press, Burlington.

[3] Rouhier, N., Lemaire, S.D. and Jacquot, J.P. (2008) The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu. Rev. Plant Biol. 59, 143–166.

[4] Gallogly, M.M., Starke, D.W. and Mieyal, J.J. (2009) Mechanistic and kinetic details of catalysis of thiol-disulfide exchange by glutaredoxins and potential mechanisms of regulation. Antioxid. Redox Signal 11, 1059–1081.

[5] Zaffagnini, M., Michelet, L., Massot, V., Trost, P. and Lemaire, S.D. (2008) Biochemical characterization of glutaredoxins from Chlamydomonas reinhardtii reveals the unique properties of a chloroplastic CGFS-type glutaredoxin. J. Biol. Chem. 283, 8868–8876.

[6] Herrero, E. and de la Torre-Ruiz, M.A. (2007) Monothiol glutaredoxins: a common domain for multiple functions. Cell Mol. Life Sci. 64, 1518–1530.

[7] Lemaire, S.D. (2004) The glutaredoxin family in oxygenic photosynthetic organisms. Photosynth. Res. 79, 305–318.

[8] Fernandes, A.P. et al. (2005) A novel monothiol glutaredoxin (Grx4) from Escherichia coli can serve as a substrate for thioredoxin reductase. J. Biol. Chem. 280, 24544–24552.

[9] Tamarit, J., Belli, G., Cabiscol, E., Herrero, E. and Ros, J. (2003) Biochemical characterization of yeast mitochondrial Grx5 monothiol glutaredoxin. J. Biol. Chem. 278, 25745–25751.

[10] Bandyopadhyay, S. et al. (2008) Chloroplast monothiol glutaredoxins as scaffold proteins for the assembly and delivery of [2Fe–2S] clusters. EMBO J. 27, 1122–1133.

[11] Johansson, C., Kavanagh, K.L., Gileadi, O. and Oppermann, U. (2007) Reversible sequestration of active site cysteines in a 2Fe–2S-bridged dimer provides a mechanism for glutaredoxin 2 regulation in human mitochondria. J. Biol. Chem. 282, 3077–3082.

[12] Picciocchi, A., Saguez, C., Boussac, A., Cassier-Chauvat, C. and Chauvat, F. (2007) CGFS-type monothiol glutaredoxins from the cyanobacterium Synechocystis PCC6803 and other evolutionary distant model organisms possess a glutathione-ligated [2Fe–2S] cluster. Biochemistry 46, 15018–15026.

[13] Rouhier, N. et al. (2007) Functional, structural, and spectroscopic characterization of a glutathione-ligated [2Fe–2S] cluster in poplar glutaredoxin C1. Proc. Natl. Acad. Sci. USA 104, 7379–7384.

[14] Bedhomme, M., Zaffagnini, M., Marchand, C.H., Gao, X.H., Moslonka-Lefebvre, M., Michelet, L., Decottignies, P. and Lemaire, S.D. (2009) Regulation by glutathionylation of isocitrate lyase from Chlamydomonas reinhardtii. J. Biol. Chem. 284, 36282–36291.

[15] Zaffagnini, M. et al. (2007) The thioredoxin-independent isoform of chloroplastic glyceraldehyde-3-phosphate dehydrogenase is selectively regulated by glutathionylation. FEBS J. 274, 212–226.

[16] Mieyal, J.J., Starke, D.W., Gravina, S.A. and Hocevar, B.A. (1991) Thioltransferase in human red blood cells: kinetics and equilibrium. Biochemistry 30, 8883–8891.

[17] Gallogly, M.M., Starke, D.W., Leonberg, A.K., Ospina, S.M. and Mieyal, J.J. (2008) Kinetic and mechanistic characterization and versatile catalytic properties of mammalian glutaredoxin 2: implications for intracellular roles. Biochemistry 47, 11144–11157.

[18] Jao, S.C., English Ospina, S.M., Berdis, A.J., Starke, D.W., Post, C.B. and Mieyal, J.J. (2006) Computational and mutational analysis of human glutaredoxin (thioltransferase): probing the molecular basis of the low pK_a of cysteine 22 and its role in catalysis. Biochemistry 45, 4785–4796.

[19] Holmgren, A. (1979) Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. 254, 9627–9632.

[20] Mannervik, B., Axelsson, K., Sundewall, A.C. and Holmgren, A. (1983) Relative contributions of thioltransferase-and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides. Biochem. J. 213, 519–523.

[21] Washburn, M.P. and Wells, W.W. (1999) The catalytic mechanism of the glutathione-dependent dehydroascorbate reductase activity of thioltransferase (glutaredoxin). Biochemistry 38, 268–274.

[22] Wells, W.W., Xu, D.P., Yang, Y.F. and Rocque, P.A. (1990) Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. J. Biol. Chem. 265, 15361–15364.

[23] Couturier, J. et al. (2009) Structure–function relationship of the chloroplastic glutaredoxin S12 with an atypical WCSYS active site. J. Biol. Chem. 284, 9299–9310.

[24] Luthman, M. and Holmgren, A. (1982) Glutaredoxin from calf thymus. Purification to homogeneity. J. Biol. Chem. 257, 6686–6690.

[25] Johansson, C., Lillig, C.H. and Holmgren, A. (2004) Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. J. Biol. Chem. 279, 7537–7543.

[26] Collin, V., Issakidis-Bourguet, E., Marchand, C., Hirasawa, M., Lancelin, J.M., Knaff, D.B. and Miginiac-Maslow, M. (2003) The Arabidopsis plastidial thioredoxins: new functions and new insights into specificity. J. Biol. Chem. 278, 23747–23752.

[27] Discola, K.F. et al. (2009) Structural aspects of the distinct biochemical properties of glutaredoxin 1 and glutaredoxin 2 from Saccharomyces cerevisiae. J. Mol. Biol. 385, 889–901.

[28] Srinivasan, U., Mieyal, P.A. and Mieyal, J.J. (1997) PH profiles indicative of rate-limiting nucleophilic displacement in thioltransferase catalysis. Biochemistry 36, 3199–3206.

[29] Deponte, M., Becker, K. and Rahlfs, S. (2005) Plasmodium falciparum glutaredoxin-like proteins. Biol. Chem. 386, 33–40.

[30] Rodriguez-Manzaneque, M.T., Tamarit, J., Belli, G., Ros, J. and Herrero, E. (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. Mol. Biol. Cell 13, 1109–1121.

[31] Merchant, S.S. et al. (2007) The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science 318, 245–250.