Kinetic studies reveal a key role of a redox-active glutaredoxin in the evolution of the thiol-redox metabolism of trypanosomatid parasites

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Trypanosomes are flagellated protozoan parasites (kinetoplastids) that have a unique redox metabolism based on the small dithiol trypanothione (T(SH)2). Although GSH may still play a biological role in trypanosomatid parasites beyond being a building block of T(SH)2, most of its functions are replaced by T(SH)2 in these organisms. Consequently, trypanosomes have several enzymes adapted to using T(SH)2 instead of GSH, including the glutaredoxins (Grxs). However, the mechanistic basis of Grx specificity for T(SH)2 is unknown. Here, we combined fast-kinetic and biophysical approaches, including NMR, MS, and fluorescent tagging, to study the redox function of Grx1, the only cytosolic redox-active Grx in trypanosomes. We observed that Grx1 reduces GSH-containing disulfides (including oxidized trypanothione) in very fast reactions (k > 5 × 10^5 M^-1 s^-1). We also noted that disulfides without a GSH are much slower oxidants, suggesting a strongly selective binding of the GSH molecule. Not surprisingly, oxidized Grx1 was also reduced very fast by T(SH)2 (4.8 × 10^6 M^-1 s^-1); however, GSH-mediated reduction was extremely slow (39 M^-1 s^-1). This kinetic selectivity in the reduction step of the catalytic cycle suggests that Grx1 uses preferentially a dithiol mechanism, forming a disulfide on the active site during the oxidative half of the catalytic cycle and then being rapidly reduced by T(SH)2 in the reductive half. Thus, the reduction of glutathionylated substrates avoids GSSG accumulation in an organism lacking GSH reductase. These findings suggest that Grx1 has played an important adaptive role during the rewiring of the thiol-redox metabolism of kinetoplastids.

GSH metabolism requires enzymes, because the spontaneous reaction of GSH with different targets is too slow to be compatible with cell physiology (1, 2). Thiol-disulfide exchange reactions have been studied for decades and the chemistry involved in these reactions is common to both small molecules and proteins containing thiols (3–5). In the simplest form, a thiol-disulfide exchange reaction is a bimolecular nucleophilic substitution (S^2) in which a nucleophilic thiolate attacks an electrophilic disulfide, producing a linear transition state with a negative charge delocalized over the three sulfur atoms involved, leading to the formation of a new disulfide and a thiolate as leaving group (Scheme 1A). For small molecules, the rate of reaction and the equilibrium position depend on the nucleophilicity of the three possible (in unsymmetrical disulfides) thiolates and the pH of the reaction (5, 6). Several redox processes occurring in vivo involve thiol-disulfide exchange reactions that are catalyzed by enzymes called redoxins (e.g. thioredoxins (Trx) and glutaredoxins (Grxs)).

Grx are a diverse group of proteins characterized by a thioredoxin-fold and a number of functions including the enzymatic reduction of protein disulfides (7–9), catalysis of thiol-disulfide exchange, preferably with GSH as co-substrate (10, 11), and iron-sulfur cluster binding (12). Grx are classified in three classes based on sequence features: class I Grx are small, single-domain and redox-active proteins carrying a conserved CXXC/S active site sequence; class II are more diverse both in sequence and domain organization, most of them contain a CGFS active site and are redox inactive; class III Grx have an unique CCXC active site (13).

The redox reactions catalyzed by class I Grx are categorized by the number of cysteine residues involved in the reaction cycle. Some substrates, such as ribonucleotide reductase (7), PAPS^3 reductase (8), and OxyR (9) are reduced by Grx via a dithiol mechanism that involves both active-site cysteines cycling between a dithiol and disulfide state. However, the reduction of GSH-containing disulfides (deglutathionylation)

3 The abbreviations used are: PAPS, adenosine 3'-phosphate 5'-phosphosulfate; GR, GSH reductase; HED, hydroxyethyl disulfide; HSA, human serum albumin; NHE, normal hydrogen electrode; TXN, T. brucei tryparedoxin; HMQC, heteronuclear multiple quantum coherence; GSP, glutathionyl-spermidine; mBBr, monobromobimane.
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Scheme 1. A, thiol-disulfide exchange mechanism. A nucleophilic thiolate \((R^1\text{-S}^-)\) attacks a symmetrical disulfide bond forming a linear transition state that breaks apart to produce a new disulfide and a leaving thiolate \((R^2\text{-S}^-)\). The whole reaction is reversible. B, reaction sequence of a GSH mixed disulfide (RSSG) being reduced by a glutaredoxin (Grx(SH)_2) using GSH as reductant via a monothiol mechanism (pathway involving reactions 1 and 2), by a dithiol mechanism (pathway involving reactions 1, 3, −3, and 2); or using an alternative two-electron reductant (AH₂ pathway involving reactions 1, 3, and 4). GSH GSH, GSSG GSH disulfide; Grx(SH)_2 reduced glutaredoxin; GrxS₂⁺, glutaredoxin internal disulfide; HSGxSSG, glutathionylated glutaredoxin.

proceeds mostly by a monothiol mechanism (Scheme 1, reactions 1 and 2) that involves only the N-terminal Cys of the Grx active site. The formation of the internal disulfide of Grx (Scheme 1, reaction 3) is considered a side reaction that can even slow down the deglutathionylation catalytic cycle (14). In any case, when formed, the internal disulfide of the Grx can be reduced back to dithiol by two consecutive reactions employing GSH as reductant (Scheme 1, reactions −3 and 2). Finally, GSSG is reduced back to GSH by GSH reductase (GR).

If GSH is the sole source of reducing equivalents, the dithiol mechanism consists of four steps involving thiol-disulfide exchange (Scheme 1, reactions 1, 3, −3, and 2), three of them being bimolecular; whereas the monothiol mechanism involves only two thiol-disulfide exchange reactions, both bimolecular (Scheme 1, reactions 1 and 2). In this context, the monothiol mechanism is kinetically faster than the dithiol mechanism. In fact, the internal disulfide formed in Grx during the dithiol mechanism can be considered a nonproductive intermediate that needs to be reintroduced in the catalytic cycle via the reaction with an additional GSH molecule. This potentially slow step may eventually be bypassed by a two-electron reductant of the Grx internal disulfide (Scheme 1, reaction 4). Worth noting, unlike Trx, which are efficiently reduced by a two-electron donor (thioredoxin reductase), Grx from most organisms lack a physiological and specific two-electron reductant, and rely on GSH as reducing agent.

The mechanism of deglutathionylation by class I Grx has been studied in detail for mammalian (15–17), yeast (18), bacterial (19, 20), and plant Grx (21). However, these studies used the hydroxyethyl disulfide (HED) assay, which couples the consumption of NADPH by GR upon reduction of GSSG generated by Grx during reduction of the mixed disulfide of GSH and 2-mercaptoethanol (GSSEtOH) (22). Hence, the HED assay yields limited information about the individual steps in the catalytic mechanism of Grx (18).

Grx are ubiquitous even in organisms equipped with redox systems that depend on low molecular weight thiols other than GSH, like actinomycetes (23), firmicutes (24), and trypanosomatids (25). African trypanosomatids, which represent an important public health problem, synthesize and use bis-glutathionyl spermidinium (trypanothione, T(SH)₂) as major low molecular mass redox substrate and are devoid of genes encoding for thioredoxin reductase and GR (25). They also harbor genes for two class I (named 2CGrx1 and 2CGrx2) and three class II Grx (25, 26). 2CGrx1 (hereinafter Grx1) is a cytosolic and relatively abundant protein with a canonical CPYC active site motif and a third cysteine (Cys-78) that, in vitro, is target of glutathionylation (27). Similar to human Grx2 (12), the trypanosomal Grx1 has been shown to use its active site cysteine residues either to reduce GSH-containing disulfides or to bind an iron-sulfur cluster, employing indistinctly GSH and T(SH)₂ as reductors or iron ligands, respectively (28, 29). Grx1, and to a minor extent 2CGrx2, display a significant T(SH)₂-GSSG oxidoreductase activity (28). This, together with the fact that trypanosomatids lack GR and convert a large amount of GSH into T(SH)₂ (30), has led us to propose that Grx1 surrogates for GR function (26). The biochemical and kinetic basis for trypanosomal Grx1 specificity has so far not been addressed, and this deems relevant to understand the physiological role the protein may have fulfilled during the evolution of this lineage.

With this aim, we investigated the individual steps in the catalysis of Grx1 with monothiol and dithiol substrates applying kinetic and biophysical approaches. The kinetic determinations relied on changes in fluorescence emission of a tryptophan residue (Trp₁₈) located less than 5 Å away from the N-terminal cysteine of the Grx1 active site (PDB 2MYG (31)), and thus provided a reliable measurement of enzyme kinetics. Our data show that Grx1 evolved a remarkable capacity to sustain GSSG reduction at the expense of T(SH)₂ and to catalyze thiol/disulfide-exchange reactions by a dithiol mechanism. These findings reinforce the idea that Grx1 has played an important adaptive role during the rewiring of the thiol-redox metabolism of kinetoplastids, and add a new example of functional versatility offered by the Grx domain.

Results

Fluorescence emission spectra of Grx1 and pKₐ of its catalytic cysteine

Oxidation, alkylation, and protonation of the catalytic Cys²¹ have a dramatic effect on Trp₁₈ fluorescence, quenching over two-thirds of its intensity at 345 nm (Fig. I). Such changes in the intrinsic fluorescence of the protein are likely associated to conformational transitions occurring at its active site upon modification of the Cys residues. This feature of Grx1 provides a sensitive and specific tool to monitor reactions of the active site, and was used herein to follow the redox (Fig. IÀ) and acid-base behavior of the enzyme (Fig. I, B and C).

The pH titration of reduced Grx1 observed through tryptophan fluorescence (Fig. 1C), reaction with mBBr (Fig. S1) or
oxidation with GSSG (Fig. 2) all yield $pK_a$ values between 5.1 and 5.5, higher than those reported for most other class I Grx that were measured by different techniques (Table 1). For instance, differences of 1 to 3 $pK_a$ units are observed when comparing the trypanosomal protein with different Grx from yeast, human, and pig. The comparatively less acidic nature of the catalytic Cys from Grx1 is to some extent shared also by some Grx from bacteria (Escherichia coli Grx1 and Grx2 $pK_a$ ~5, and Chlamydomonas reinhardtii Grx2 $pK_a$ 4.8) and plant (Populus tremula GrxC1 and GrxC2 $pK_a$ ~5). For most class I Grx, a basic residue (Lys/H11022/Arg) located three residues upstream the active site Cys appears to contribute to the stabilization of the thiolate form (32–34). In Grx1, this position is occupied by a bulky and hydrophobic Trp residue (Fig. S2), which may explain the higher $pK_a$ of the trypanosomal protein.

**Kinetics of Grx1 oxidation and reduction**

Grx1 is rapidly oxidized by GSH containing disulfides—Oxidized GSH and a number of GSH-containing disulfides react with Grx1 with rate constants in the order of $10^6\ M^{-1}\ s^{-1}$ (Table 2, Fig. 2), which is 1 order of magnitude higher than that determined previously (28). The rate of Grx1 in these reactions is comparable with that of EcGrx1 with glutathionylated RNase ($k_{cat}/K_m = 3 \times 10^6\ M^{-1}\ s^{-1}$ (35)) and somewhat higher than the previously reported for Grx from yeast ($3 \times 10^5\ M^{-1}\ s^{-1}$ (36)), pig ($7.1 \times 10^5\ M^{-1}\ s^{-1}$ (37)), or human Grx1 ($1.24 \times 10^6\ M^{-1}\ s^{-1}$ (38)) with similar substrates. For comparison, the reaction of GSSG with other protein thiols is much slower, with rate constants of 600 $M^{-1}\ s^{-1}$ (protein-disulfide isomerase (39)), 570 $M^{-1}\ s^{-1}$ (Trx (40)), 1.2 $M^{-1}\ s^{-1}$ (redox-sensitive yellow fluorescent protein (36)), and 0.1 $M^{-1}\ s^{-1}$ (human serum albumin, HSA (41)). The time course of the reaction of Grx1 with excess GSSG shows a very fast phase, lasting less than 4 ms, followed by an exponential decay in emission (Fig. 2A). The slower phase fits very well to a first-order function with a $k_{obs}$ that depends linearly with [GSSG]$_o$ (Fig. 2B).

Other GSH containing disulfides, including GSSEtOH (Fig. 3A), HSA-SSG (Fig. 3B), Tb1CGGrx1 Cys$^{381}$–SSG, FGSSGF, and GSP disulfide all react with rate constants in the range $0.5–6 \times$...
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Table 1

| Organism       | Protein | Active site | Cys | pK_a | Method                        | Reference         |
|----------------|---------|-------------|-----|------|-------------------------------|-------------------|
| *T. brucei*    | Grx1    | CPYC        | 21  | 5.45 ± 0.02 | Intrinsic fluorescence | This work         |
|                |         |             |     | 5.4 ± 0.1  | mBBr alkylation              | This work         |
|                |         |             |     | 5.15 ± 0.05 | Oxidation by GSSG            | This work         |
| *E. coli*      | Grx1    | CPYC        | 11  | 4.9   | Ellman reaction               | 76                |
| *E. coli*      | Grx3    | CPYC        | 11  | <5.5  | NMR chemical shift            | 77                |
| *S. cerevisiae*| Grx1    | CPYC        | 27  | 3.2   | mBBr alkylation               | 18                |
|                | Grx2    | CPYC        | 61  | 4.0   | Inactivation by iodoacetamide | 78                |
| *Homo sapiens* | Grx1    | CPYC        | 22  | 3.5   | Inactivation by iodoacetamide | 16                |
|                | Grx1    | CPYC        | 22  | 3.5   | Inactivation by iodoacetamide | 79                |
| *Sus scrofa*   | Grx1    | CPYC        | 47  | 4.8   | Inactivation by iodoacetamide | 80                |
| *C. reinhardtii*| Grx1  | CPYC        | 47  | 4.8   | Inactivation by iodoacetamide | 80                |
| *P. tremula*   | GrxS12  | WCSSYC      | 29  | 5.5   | NMR chemical shift            | 77                |
| *GrxC1*        |         | CGYC        | 31  | 4.7   | Inactivation by iodoacetamide | 83                |
| *GrxC2*        |         | CPFC        | 23  | 5.0   | Inactivation by iodoacetamide | 21                |
| *GrxC3*        |         | CPYC        | 37  | 4.6   | Inactivation by iodoacetamide | 21                |
| *GrxC4*        |         | CPYC        | 27  | 4.6   | Inactivation by iodoacetamide | 21                |

Table 2

| Reactant                                 | k (s⁻¹) | pH     |
|------------------------------------------|---------|--------|
| Glutathione containing disulfides         |         |        |
| GSSG                                     | 7.0 ± 0.1 x 10⁶ | 7.07   |
|                                          | 6.7 ± 0.1 x 10⁶ | 5.98   |
|                                          | 2.0 ± 0.1 x 10⁶ | 4.83   |
|                                          | 7.5 ± 0.2 x 10⁶ | pH-independent |
|                                          | 5.7 ± 0.2 x 10⁶ | 7.05   |
| TS₂                                      | 5.2 ± 0.08 x 10⁷ | 7.05   |
| Glutathionylspermidine disulfide          | 4.2 ± 0.09 x 10⁶ | 7.05   |
| FGSSGF                                   | 5.7 ± 0.2 x 10⁷ | 7.11   |
|                                          | 6.2 ± 0.05 x 10⁸ | 6.85   |
|                                          | 1.1 ± 0.04 x 10⁷ | 6.85   |
|                                          | 5.4 ± 0.1 x 10⁷ | 6.85   |
| Glutathionylhydroxyethyl disulfide       | 6.4 ± 0.04 x 10⁷ | 7.11   |
| Glutathionylcysteinylmethyl ester disulfide | 5.3 ± 1.1 x 10⁸ | 7.03   |
| GlutathionylHSA                          | 2.6 ± 0.3 x 10⁸ | 7.05   |
| Other disulfides                         |         |        |
| Cystine dimethyl ester                   | 3.7 ± 0.03 x 10⁴ | 7.04   |
| Bishydroxyethyl disulfide                | 30 ± 0.4 | 7.07   |
| Cystamine                                | 20 ± 0.2 | 7.10   |
| Cystine                                  | 99 ± 5  | 7.10   |
| Thiods                                   |         |        |
| T(SH)₂                                   | 4.8 ± 0.2 x 10⁷ | 7.05   |
| GSH                                      | 39 ± 3  | 7.10   |
| Unspecific oxidation                     |         |        |
| H₂O₂                                     | 3.77 ± 0.06 | 7.13   |

* Reaction of *E. coli* Grx1 followed by changes in Trp78 intrinsic fluorescence, as described under “Experimental procedures.”
* *TbGrx1C24S.*
* *TbGrx1C178S.*

10⁶ M⁻¹ s⁻¹ at neutral pH (Table 2). *EcGrx1* was also studied by the same methodology, taking advantage of the fluorescence emission of Trp⁷⁸, which changes slightly upon oxidation of the active site. The reaction of *EcGrx1* with GSSG has a rate constant of 5.7 x 10⁶ M⁻¹ s⁻¹.

In contrast, disulfides lacking a GSH moiety, such as HED, cystamine (Fig. 3C), or cystine, are slow oxidants of Grx1, highlighting the selectivity for the substrate during the oxidative step of the catalysis. An important molecular factor that seems to determine the specificity exhibited by Grx1 is the recognition and interaction with the α-carboxylate of the γ-glutamyl of GSH (42, 43). This is supported by the fact that GSP disulfide and TS₂, both having a free γ-glutamyl carboxylate but lacking the Gly carboxylate, react with similar or higher rate constant with Grx1(SH)₂ than GSSG. Additionally, FGSSGF, which has the amino group of the γ-glutamyl blocked also reacts remarkably fast with Grx1(SH)₂ (Table 2).

![Figure 3. Oxidation of *TbGrx1* by different oxidants.](image-url)

A. rate constants for 0.25 μM *TbGrx1(SH)₂* oxidation with different concentrations of GSH previously equilibrated with 10 mM HED for 2 h. *Inset,* time course of the reaction mixture containing 25 μM GSSEtOH. B. rate constants of different concentrations of *TbGrx1(SH)₂* with 0.4 μM HSA-SGG. *Inset,* time course of the reaction mixture containing 7.5 μM *TbGrx1(SH)₂*. C. rate constants of *TbGrx1(SH)₂* (0.4 μM) oxidation with different concentrations of cystamine. *Inset,* time course of the reaction mixture containing 13.4 mM H₂O₂. All time course plots show the fluorescence emission values in arbitrary units. Points are plotted as mean ± S.D. of n = 10 (GSSEtOH, HSA-SGG and H₂O₂) or n = 4 (cystamine). The numbers below the straight lines are the slopes (second-order rate constants) in units of M⁻¹ s⁻¹.
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oxidation by H$_2$O$_2$ is extremely slow (3.77 m$^{-1}$ s$^{-1}$ at pH 7.4; Fig. 3D) and comparable with that of low molecular weight thioclates (44).

Reduction of Grx1S$_2$ by GSH is extremely slow—The disulfide formed between the active site cysteines of Grx needs to be reduced to maintain the enzyme in the monothiol catalytic cycle. Synthetic dithiols (e.g. DTT) can reduce the oxidized forms of Grx. However, the reaction of Grx1S$_2$ with DTT is not specific and occurs with a moderate rate constant (690 ± 6 m$^{-1}$ s$^{-1}$, Fig. S3) in the expected range for the reduction of any disulfide by this dithiol (6, 45) and is mainly driven by the entropic effect of the formation of a six-membered cyclic disulfide of DTT (trans-4,5-dihydroxy-1,2-dithiane).

Contrary to dithiol reagents, the reduction of Grx1 by GSH is very slow. Our first attempts to reduce Grx1S$_2$ with an excess GSH were uncondusive, apparently due to the rapid reoxidation of the enzyme by the contaminating GSSG present in our commercial GSH (about 0.5%). Assays conducted with a GSSG-free GSH sample yielded a rate constant for the reduction of Grx1 of 39 ± 3 m$^{-1}$ s$^{-1}$, identical to the previously reported value of 37 m$^{-1}$ s$^{-1}$ (28) (Fig. 4). Thus, under physiological conditions, the reduction of Grx1 by GSH will not be kinetically favored.

The glutathionylated form of Grx1 is not particularly stable—The reactions of Grx1S$_2$ with GSH (i.e. reaction – 3) or Grx1(SH)$_2$ with GSSG (i.e. reaction 1), produce a transient covalent intermediate, namely Grx1 glutathionylated in Cys$_{21}$ (Grx1SSG). We conducted several experiments to identify the occurrence of this mixed disulfide.

First, the SOFAST-HMQC spectra of $^{15}$N-labeled Grx1S$_2$ was monitored during titration with GSH. Upon addition of GSH, for some of the amino acids new peaks appeared at positions different from Grx1(SH)$_2$ and at the same time the corresponding peaks of Grx1S$_2$ disappeared. The spectra showed that even with excess GSH, up to 5 eq, Grx1(SH)$_2$ is not formed. Grx1SSG cannot be unambiguously identified by this experiment but, compared with the spectrum of the fully reduced and fully oxidized protein, the chemical shift changes observed for several amino acids of Grx1 upon addition of GSH, indicate the formation of a distinct intermediate (Fig. 5). Interestingly, although the assignment of the oxidized protein is not available, from the assignment of Grx1(SH)$_2$, (31) it is evident that the most prominent shifts occurred at residues that partake in the GSH-binding cleft (Fig. 5).

The formation of Grx1SSG was confirmed by MS analysis (Table 3; Fig. S4). Treatment of Grx1(SH)$_2$ with GSSG in a 1:1 ratio resulted in the formation of the internal disulfide on Grx1 active site and a minor peak of $m/z$ coincident with the mass estimated for the Cys$_{21}$-glutathionylated form. Incubation of Grx1(SH)$_2$ with an excess of GSSG produced Grx1S$_2$ and a minor peak of $m/z$ coincident with Cys$_{78}$-glutathionylated Grx1S$_2$. Reaction of Grx1S$_2$ with 1 eq of GSH did not produce any new peaks and the Cys$_{21}$-glutathionylated form was observed only when a 10-fold excess GSH was used (Table 3).

To provide further evidence on the occurrence of glutathionylated Grx1 during the reaction with GSH-mixed disulfides, Grx1(SH)$_2$ was reacted with GSSG labeled at the amino group with fluorescein (FGSSGF) and the products were separated by gel filtration. As shown in Fig. 6A, the protein-containing fraction (Grx1 10.8 kDa) is excluded from the column and separated from the low-molecular weight components present in the reaction sample: nonreacted FGSSGF (1.34 kDa) and FGH (0.69 kDa) released upon reduction of the corresponding disulfide by Grx1(SH)$_2$. The peak eluting at 4.66 min did not contain protein but only fluorescein conjugated to GSH.

Spectra of the first peak confirmed the presence of protein (absorbance at 280 nm) and a small amount of fluorescein (absorbance at 495 nm; Fig. 6B). Using the molar extinction coefficient of Grx1-SSGF (about 15,000 m$^{-1}$ cm$^{-1}$ at 280 nm) and fluorescein (70,000 m$^{-1}$ cm$^{-1}$ at 495 nm), we estimated that 0.7% of the Grx1 appeared glutathionylated after separation.
To confirm the finding, we also subjected two mutants of Grx1 to the same treatment. First, Grx1 C78S, lacking the cysteine outside the active site, behaved almost exactly as Grx1 WT (Fig. 6, C and D), with marginally lower glutathionylation. On the other hand, the C24S mutant, lacking the C-terminal cysteine of the active site (therefore unable of forming the internal disulfide) yielded the mixed disulfide quantitatively (Fig. 6, E and F).

To exclude potential artifacts arising from differential kinetics among the mutants we studied the reaction of FGSSGF by Grx1(SH)2 (WT and the two mutants). The obtained rate constants were slightly smaller than with unlabeled GSH, which is consistent with a relative instability of Grx1SSG with respect to Grx1S2 + GSH.

Both reduction and oxidation of Grx1 by trypanothione are extremely fast

Contrary to what we observed with GSH, the thiol-disulfide exchange between trypanothione and Grx1 is very fast in both oxidation and reduction. The kinetics are somewhat complex, both the reduction of TS2 by Grx1(SH)2 and the oxidation of T(SH)2 by Grx1S2 are biphasic when monitored through the intrinsic fluorescence of the protein (Fig. 7). In both reactions, there is a faster phase with a rate constant that is first order on trypanothione and a slower phase that appears to be zero order on trypanothione. This kinetic behavior is consistent with the expected sequence shown in reactions C and D of Table 4, which includes a mixed trypanothionylated intermediate.

Both dissociation reactions of the intermediate are quite rapid with rate constants $k_f^C = 30 \text{s}^{-1}$ and $k_f^D = 43 \text{s}^{-1}$ and both dissociation equilibrium constants ($K_D$) indicate that the mixed disulfide is a rather unstable species. The equilibrium constant of the global reaction

$$\text{TS}_2 + \text{Grx} \text{(SH)}_2 \rightleftharpoons \text{T(SH)}_2 + \text{GrxS}_2$$

(Eq. 1)

can be calculated as the following.

$$K_D = \frac{K_D^C}{K_D^D} = 7.5$$

(Eq. 2)

Where $K_D^C$ and $K_D^D$ are the dissociation constants of reactions C and D in Table 4. Using $K_D$ and the known reduction potential of the TS2/T(SH)2 couple ($E' = -242 \text{mV/NHE}$, (46)) and combining the Nernst equation for both couples,
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Initial concentrations taken from Refs. 26 and 28; pH 7. Rate constants referenced to this work also appear in Table 2.

\[
\begin{align*}
[T(SH)_2] &= 300 \mu M \\
[ThGrx1(SH)_2] &= 2 \mu M \\
[TXN(SH)_2] &= 85 \mu M \\
[GSH] &= 300 \mu M \\
[TS_2] &= 0 \mu M \\
[ThGrx1S_2] &= 0 \\
[TXN_S_2] &= 0 \\
[GSSG] &= 10 \mu M
\end{align*}
\]

| Reaction | Rate constant | Reference |
|----------|---------------|-----------|
| (A) \(T(SH)_2 + GSSG \rightarrow TS_2 + 2 GSH\) | \(k_f = 0.31 \text{ M}^{-1} \text{s}^{-1}\) | (6) |
| (B) \(Grx(SH)_2 + GSSG \rightleftharpoons GrxS_2 + 2 GSH\) | \(k_f = 7.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) | This work |
| | \(k_r = 39 \text{ M}^{-1} \text{s}^{-1}\) | This work |
| (C) \(TS_2 + Grx(SH)_2 \rightleftharpoons TSSGrx\) | \(k_f = 2.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) | This work |
| | \(k_r = 30 \text{ s}^{-1}\) | This work |
| (D) \(TSSGrx \rightleftharpoons (T(SH)_2 + GrxS_2\) | \(k_f = 43 \text{ s}^{-1}\) | This work |
| | \(k_r = 4.8 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) | This work |
| (E) \(TXN(SH)_2 + GSSG \rightarrow TXN_S_2 + 2 GSH\) | \(k_f = 1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) | This work |
| (F) \(TXN_S_2 + T(SH)_2 \rightleftharpoons TXN(SH)_2 + TS_2\) | \(k_f = 3.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1}\) | (84) |
| | \(k_r = 5.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) | (52, 84) |

(6) \(k_r\) refers to the reaction going left to right as written, \(k_f\) refers to the reaction going right to left as written.

(52) Value from Fig. 7B.

(51) Calculated from the reported \(k_r\) and the reported redox potentials of TXN and \(T(SH)_2\).

\[
E^\circ_{TS_2/T(SH)_2} - E^\circ_{GrxS_2/Grx(SH)_2} = \Delta E^\circ = 0.03 \log K_2 = 0.026 \text{ mV}
\]  
(Eq. 3)

yields a \(E^\circ\) value for the couple \(GrxS_2/Grx(SH)_2\) of about −268 mV versus the normal hydrogen electrode (NHE), remarkably low for a Grx (approximately −170 mV/NHE for \(EcGrx1\) and \(HsGrx1\) but close to the −270 mV/NHE reported for \(EcTrx\)).

Reduction of GSSG by trypanosomal redoxins—To provide a semiquantitative idea of the relative relevance of the various reduction routes of GSSG in trypanosomatids, which are devoid of GR activity, we performed kinetic simulations with the system described in Table 4. The system was perturbed by the introduction of 10 μM GSSG with a time constant of 0.02 s, it was then allowed to equilibrate monitoring the concentration of GSSG over time in the absence or presence of Grx1 and TXN at their reported intracellular concentrations in the infective form of Trypanosoma brucei (28, 50).

In our simulated system under conditions resembling an intracellular reducing milieu, the reduction by \(T(SH)_2\) alone (reaction A) can revert the pulse of GSSG very slowly, in not less than 15 h (Fig. 8A). If the system also contains 2 μM Grx1, GSSG reduction is completed in less than 3 s, whereas in the presence of 85 μM TXN, the \(T(SH)_2\)–dependent reduction of GSSG is completed in ~5 s. In a model were both Grx1 and TXN are present, GSSG reduction is evidently faster (>2 s) with Grx1 accounting for 76% of the reductase activity and TXN contributing with the remaining 24%. Worth noting, the model assumes that Grx1 is reduced by \(T(SH)_2\) (reverse reaction D), but according to our rate constants, this reduction system is very sensitive to the presence of \(TS_2\) (reaction C). Also TXN has been reported to be susceptible to inhibition by \(TS_2\) (51).

Therefore, we tested this experimental information by running a simulation of the reaction at different initial concentrations of \(TS_2\) from 0 to 50 μM, and verified that the accumulation of \(TS_2\) slows down the overall reduction of GSSG due to a drop in the steady-state concentrations of both \(Grx(SH)_2\) and \(TXN(SH)_2\) (Fig. S5). Above 15 μM \(TS_2\) ([\(T(SH)_2\])/[\(TS_2\]) < 20), the reduction of GSSG by TXN becomes predominant. In principle, TXN is comparatively less sensitive than Grx1 to inhibition by \(TS_2\) due to the smaller difference in redox potentials (\(E^\circ_{TXN} = −249 \text{ mV/NHE}\) (52), \(E^\circ_{TS_2} = −242 \text{ mV/NHE}\) (46), \(E^\circ_{Grx_1} = −268 \text{ mV/NHE}\)). However, previous reports indicate that the reductase activity of TXN over ribonucleotide reductase is inhibited 60% by a ([\(T(SH)_2\])/[\(TS_2\])] ratio of 10 (51), which would indicate a \(\Delta E^\circ\) of 25 mV between TXN and trypanothione. In any case, the difference is subtle and the trend in the reduction of GSSG holds. The simulation of GSSG reduction assuming a \(\Delta E^\circ\) of 25 mV between TXN and trypanothione is also first order in \(TS_2\).

Discussion

Oxidation of \(Grx1(SH)_2\) by GSSG proceeds mainly to the internal disulfide

The oxidation of \(Grx1(SH)_2\) by GSSG consists in two elementary steps (Scheme 1, reactions 1 and 3). Reaction 1 is first-order in GSSG and Grx1(SH)_2, whereas reaction 3 is 0 order in GSSG. In principle either reaction could be responsible for the change in Trp^18 fluorescence as we have seen that all assayed modifications on Cys^21 lead to an important decrease in emission. What we see in the time course of oxidation is a two-phase reaction consisting in a very fast transient (Fig. 2A, inset) and a first-order decay in emission that is also first order on GSSG (Fig. 2B). Therefore, reaction 1 is either rate-limiting or it
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The plausible biological relevance of the oxidation of Grx1 to a monothiol mechanism requires binding and activation of a second GSH molecule that, in canonical class I Grx, is glutathionylated at Cys21. This clearly indicates that the internal disulfide of Grx1 is more stable than its glutathionylated form and, hence, that reaction 3 is faster than reactions 1 and −3 under the conditions assayed. As will be discussed below in detail, Grx1 lacks residues involved in binding and activation of a second GSH molecule that, in canonical class I Grx, will attack the mixed disulfide with GSH releasing reduced protein and GSSG (reaction 2). This may explain the inability of the trypanosomal enzyme to operate via a monothiol mechanism and to employ the adjacent Cys24 to complete the oxidative phase of its catalytic cycle with GSH (reaction 3). The plausible biological relevance of the oxidation of Grx1 to a disulfide is provided below.

**Efficient reduction of Grx1S2 requires a two-electron reductant**

Reduction of Grx1S2 by GSH consists of two consecutive thiol-disulfide exchange reactions (Scheme 1, reactions −3 and 2). The lower limit for their rate constants can be estimated using the empirical Brønsted relationship obtained with low molecular weight thiols in the absence of enzyme catalysis (Equation 4) (6).

\[
\log k_{\text{app}} = 5.22 + 0.50 \log pK_{\text{nuc}} - 0.27 pK_a^\text{nuc} - 0.73 pK_a^\text{thiol}
\]

Where \( pK_{\text{nuc}} \) is the pK of the nucleophilic attacking thiol, \( pK_a^\text{nuc} \) is the pK of the electrophilic thiol whose sulfur is being attacked in the disulfide, and \( pK_a^\text{thiol} \) is the pK of the leaving group thiol. It is important to note that Equation 4 has been modified from the original to yield rate constants in units of \( M^{-1} \text{s}^{-1} \). The thiols involved in the reactions are GSH and the two active-site cysteines of Grx1, Cys21 and Cys24. In reaction −3, GSH acts as nucleophile (\( pK_a^\text{nuc} = 8.94 \) (44)) attacking on the electrophilic center of Cys21 sulfur (\( pK_a^\text{thiol} = 5.15 \)), being Cys24 (\( pK_a^\text{thiol} = 8.5 \)) the leaving group. Therefore, according to Equation 4 the lower limit for this reaction is 1.4 \( M^{-1} \text{s}^{-1} \) (\( k_{\text{app}} \) at pH 7.0). In reaction −3, the GSH-binding site is available, therefore catalysis can be expected to be important. In fact, the rate-limiting step in the catalysis of Grx as thiol transferase is reaction 2, as evidenced by the pH profile of the activity (53).

In reaction 2, a second GSH molecule (nuc) attacks the sulfur of the bound GSH (c) and Cys21 is the leaving group. The lower kinetic limit for this reaction is \( k_{\text{app}} = 37.5 M^{-1} \text{s}^{-1} \), coincident with the value experimentally determined here (39 \( M^{-1} \text{s}^{-1} \), Table 2) and previously reported (28).

For human Grx1 the rate constant of reaction 2 has been measured as \( 3.7 \times 10^4 M^{-1} \text{s}^{-1} \) at pH 9.5 (53). That value, although 3 orders of magnitude faster than the rate constant determined for Grx1, is also very similar to the value calculated using Equation 4, considering that the human enzyme has a very acidic N-terminal cysteine (\( pK_a = 3.5 \)), calculated \( k_{\text{app}} = 4.1 \times 10^4 M^{-1} \text{s}^{-1} \) at pH 9.5 and 597 \( M^{-1} \text{s}^{-1} \) at pH 7.0). Thus, the reduction of glutathionylated Grx (reaction 2) by a second GSH molecule, the preferred reaction in most class I Grx, is relatively fast even in the absence of catalysis, because it is favored by the usually very low \( pK_a \) of the N-terminal cysteine that acts as leaving group (Table 1).

For the trypanosomal Grx1, both GSH-dependent reductive steps are quite slow indicating that this monothiol is not the preferred reductant. Reduction of glutathionylated Grx by a monothiol mechanism requires binding and activation of a second GSH molecule in the active site of Grx. A recent study proposed the presence of a GSH-activator site in class I Grx that is formed by highly conserved basic residues: a Lys or Arg 3 residues upstream of the N-terminal active site Cys and an Arg and/or Lys at the N terminus of the loop containing the strictly conserved cis-Pro present in all Trx-fold proteins (34). Interestingly, in Grx1 none of these positions is occupied by positively charged residues, with Trp replacing the Lys/Arg nearby the active site of classical Grx and a Glu replacing the Lys/Arg at the P-loop of classical Grx (Fig. S2). Under such a molecular scenario, the stabilization of the glutathionylated intermediate at Cys21 as well as activation of a second GSH molecule is clearly not favored in Grx1.
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In contrast to GSH, T(SH)$_2$ proved to be an excellent reductant of Grx1S$_2$ ($k \sim 5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). Such performance is not strictly related to a particular selectivity of the trypanosomal protein. In fact, EcGrx1 has been shown to use T(SH)$_2$ with higher efficiency than GSH in redox assays with diverse oxidized proteins (28). The physicochemical properties of the low molecular weight dithiol are likely responsible for making it a good reductant. The mean pH$_K$ of the Cys from T(SH)$_2$ was estimated to be 7.4, which is significantly lower than that of GSH (pH$_{K_a}$ 8.94 (44)), likely due to the absence of one carboxylate and to the basic character of the N$_\alpha$ group of spermidine. Thus, in contrast to GSH, thiol-disulfide exchange using T(SH)$_2$ as reductant requires less activation of its thiol groups because at physiological pH half of them will be in the thiolate form. Additionally, reduction of the trypanothionylated form of Grx will be solved by the intramolecular nucleophilic attack by the second Cys of T(SH)$_2$. Nevertheless, the rate constant of T(SH)$_2$ with Grx1 needs to involve binding and specific activation of the nucleophilic attack because the measured rate constant is nearly 6 orders of magnitude higher than the one calculated with equation 4.

In summary, the sluggishness of Grx1 reduction by GSH makes the dithiol mechanism much more efficient in the reductive as it bypasses reaction 2 when using the much faster two-electron reductant T(SH)$_2$ (Scheme 1, reaction 4). This slow reduction of Grx1 by GSH has a physiological significance as discussed below.

**Biological implications derived from Grx1 kinetic behavior and physicochemical properties**

In most organisms, GR is extremely fast and efficient in the reduction of GSSG (e.g. $k_{cat}/K_m$ is $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for EcGR (54)). Organisms lacking GR evolved other possibilities, like a specialized form of thioredoxin reductase in fruit flies (55) or a Grx-domain (with GSSG reductase activity) fused to a canonical thioredoxin reductase in platyhelminth parasites (56). Indeed, any dithiol protein with $E^{\prime\prime} < -240 \text{ mV/NHE}$ could perform the reduction given the right concentration and conditions. Although many Trx have reduction potentials near $-270 \text{ mV/NHE}$ (47), they are too slow to reduce GSSG (rate constants $\sim 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (57–60)), with the exception of the thioredoxin system of *Mycobacterium tuberculosis* ($k_{cat}/K_m = 6.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (59)). Trx from *T. brucei* is one of the slowest reported, reducing GSSG with a $k = 23 \text{ M}^{-1} \text{ s}^{-1}$ (61). On the other hand, TXN from *Trypanosoma cruzi* was recently reported to reduce GSSG with a rate constant of $1.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in a coupled assay with trypanothione reductase (62). We obtained the same rate constant using *T. brucei* TXN and measuring the rate constant by directly monitoring the reaction through TXN fluorescence (Fig. S7).

Another candidate for T(SH)$_2$-dependent reduction of GSSG in *T. cruzi* and *Leishmania* are the GSH transferase TCS2 (63) and a thiol-dependent reductase ($k = 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) (64), respectively. However, because both proteins are excreted/secreted to the extracellular medium, their GSSG-reductase activity does not likely contribute to the intracellular GSH homeostasis in the parasites. Finally, the spontaneous reaction between T(SH)$_2$ and GSSG has been proposed as responsible for maintaining the reduced pool of GSH (65). However, a rate constant of $0.31 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7 was calculated using Equation 4 and it is too slow to have any biological relevance on the reduction of GSSG (Fig. 8A).

Kinetic simulations, based on rate constants obtained in this work and spanning a range of physiological concentrations of substrates and intracellular T(SH)$_2$/TS$_2$ ratios, suggested that Grx1 and TXN work concertedly to maintain GSH reduced at the expense of T(SH)$_2$. According to the model, the main role of TXN is to operate as an efficient backup of Grx1 when cells face oxidative stress. The putative role of Grx1 as GSSG/T(SH)$_2$ oxidoreductase has recently been challenged by a study showing that the overall redox state of GSH/GSSG in a *T. brucei* cell line lacking Grx1 (and also Grx2) is similar to that of the WT cell line (66), these observations support our experimental evidence that TXN can efficiently take over this function.

Glutaredoxins are efficient and specific catalysts of the (de)glutathionylation of proteins. A recent study shows that Grx1 does not contribute to the overall protein S-thiolation of *T. brucei* (i.e. the level of protein-bound GSH was almost identical in WT and Grx1-deficient cells (67)). This is likely explained by the fact that Grx1 is rapidly oxidized to its disulfide by GSSG and that the glutathionylated intermediate could only be detected at low concentrations of GSSG. Nonetheless, Grx1 may eventually participate in the glutathionylation of specific proteins when the GSH/GSSG is high. Such a mechanism may prove important to fine-tune the activity of proteins under physiological conditions. The reverse reaction, namely the reduction of mixed disulfides of GSH with proteins, is the primary function of glutaredoxins and Grx1 is not the exception (28). In the infective form of *T. brucei*, Grx1 accounts for about 40–50% of the glutathionylase activity measured in cell extracts (27), which was then confirmed by the slow reversion of protein S-thiolation in Grx1-KO parasites exposed to diamide (67). The current evidence shows that Grx1 does not function as a major protein oxidoreductase in the protection against oxidative stress (66, 67) but as key redox regulator of the activity of yet unknown partner(s) involved in parasite thermotolerance (27).

The obligatory dithiol mechanism used by Grx1 in thiol-disulfide exchange reactions raises interesting questions about the biochemical context in which such atypical behavior may prove useful. For a monothiol mechanism, the reduction of GSH-mixed disulfides by Grx yields GSSG, reduced target protein, and Grx(SH)$_2$ as products (reactions 1 and 2). As shown here, this is not the preferred mechanism employed by Grx1. Instead, operating via a dithiol mechanism the products are GSH, the reduced target protein, and Grx1S$_2$. This may prove useful for organisms lacking GR, as trypanosomatids, because GSH but not GSSG is produced by the reaction, and Grx1S$_2$ is then efficiently reduced by T(SH)$_2$. In contrast, in the absence of a GR, the steady accumulation of GSSG by the monothiol mechanism would gradually inactivate Grx1, and protein deglutathionylation would be compromised if the GSH/GSSG ratio is not restored. Although GSSG can be reduced by TXN, it is important to recall that this reaction is 3 orders of magnitude slower than that catalyzed by a GR. Thus the monothiol mecha-
anism represents a dead-end path for GR-deficient cells. This led us to speculate that the dithiol mechanism evolved by the trypanosomal Grx reflects an evolutionary adaptation to overcome a kinetic bottleneck with potentially harmful consequences for the parasite. The major change undergone by Grx1 was the loss of the GSH-activator site conserved in almost all class I Grx (Fig. S2). Point mutations substituted the otherwise highly conserved residues of the GSH-activator site and abolished the capacity of the trypanosomal protein to operate with GSH using a monothiol mechanism. Adding value to this evolutionary hypothesis, all class I Grx from trypanosomatids lack the residues conforming the GSH-activator site and, therefore, we here propose are obligated to catalyze thiol-disulfide exchange reactions using a dithiol mechanism (Fig. S2). From an evolutionary perspective, although T. brucei can fully dispense of Grx1 activity, the protein probably played an essential role in an ancient trypanosomatid, during the establishment of a redox metabolism dependent on T(SH)₂. Indeed, this process involved not only the loss of the genes encoding for GR and thioredoxin reductase but also the adaptation (mutation) of the active site of several redox proteins to use T(SH)₂ as substrate (25). Thus, the nowadays superfluous GSSG reductase and protein de(glutathionylase) activity of Grx1 may have contributed to a smooth transition from a GSH to a T(SH)₂-dependent metabolism.

Experimental procedures

Chemicals

Chemical reagents were of analytical grade and purchased from Sigma, AppliChem, or DorWill. Trypanothione (T(SH)₂) and glutathionylspermidine (GSP) disulfide were obtained from Bachem AG, Switzerland. Unless otherwise indicated all kinetic experiments were performed in a buffer of constant ionic strength (68) consisting in Tris (30 mM), MES (15 mM), acetic acid (15 mM), 120 mM NaCl, and 0.1 mM diethylenetriamine pentaacetate (TMA buffer).

Proteins

HSA (Sigma A1653) was delipidated as previously described (69). GSH reductase from Saccharomyces cerevisiae (ScGR) was purchased from Sigma (G3664). E. coli Grx1 (EcGrx1) was a kind donation of IMCO Corp. Ltd. AB (Sweden).

Protein expression and purification

The expression vector encoding for the wildtype (WT) form of T. brucei Grx1 (accession number XP828228, TriTryp Tb927.11.1370), namely pET-trx1b TbGrx1, was kindly provided by Dr. L. Krauth-Siegel, Heidelberg University, Germany (28). The cysteine to serine mutants C24S and C78S of Grx1, was kindly provided by IMCO Corp. Ltd. AB (Sweden). The labeled protein was expressed and purified as described above and the buffer exchanged to 50 mM sodium phosphate, pH 7.2, 150 mM NaCl (buffer B). Fractions containing the protein of interest were collected, concentrated, and stored in aliquots at −80 °C after addition of 10% glycerol. Due to the cloning strategy, all tag-free versions of Grx1 contain an N-terminal GAME sequence instead of Met¹ yielding a calculated molecular weight of 10,833.49. ¹⁵N uniformly labeled Grx1 or the corresponding C24S or C78S mutants were grown at 37 °C and 220 rpm in 2YT medium supplemented with 50 μg/ml of kanamycin and induced with 200 μM isopropyl 1-thio-β-d-galactopyranoside when A₆₀₀～1.0. After incubation at 20 °C for 16 h, cells were harvested by centrifugation (4,000 × g, 10 min), resuspended in 50 mM sodium phosphate, pH 7.8, 300 mM NaCl (buffer A) plus 1 mM phenylmethylsulfonyl fluoride, 40 μg/ml of 1-chloro-3-tosyl-ami-do-7-amino-2-heptanone, 150 mM pepstatin, 4 mM cysatin, 0.1 mg/ml of aprotinin, and 1 mg/ml of lysozyme and disrupted by sonication. The lysate was treated with DNase (10 μg/ml, Life Technologies) and 10 mM MgCl₂ and centrifuged at 20,000 × g for 1 h. The supernatant was filtered (0.45 μm, Millipore) and loaded onto a HisTrap column (GE Healthcare) equilibrated with buffer A. After washing the column with 20 mM imidazole in buffer A, the recombinant proteins were eluted with a linear gradient up to 500 mM imidazole in the same buffer. Fractions containing the fusion protein (as assessed by SDS-PAGE) were pooled and treated with 5 mM DTT, 2 mM EDTA, and His-tagged 3C-type tobacco etch virus protease (prepared as described in Ref. 29) at a 70:1 protein:protease ratio (in mg) for 2 h at room temperature. The sample was then concentrated by ultrafiltration (Vivaspin, 5 kDa molecular weight cut-off), buffer exchanged with a HiPrep® column coupled to an AKTA-FPLC system (GE Healthcare) equilibrated in buffer A and applied again onto a HisTrap® pre-equilibrated in buffer A. Tag-free WT Grx1 or its Cys-to-Ser mutants was collected in the flow-through, treated with 5 mM DTT and 2 mM EDTA during 30 min at room temperature, and concentrated by ultrafiltration before loading it onto a preparative size exclusion column (HiLoad 26/60 Superdex 75 prep grade column, GE) equilibrated with 100 mM sodium phosphate, pH 7.4, with 150 mM NaCl (buffer B). Fractions containing the protein of interest were collected, concentrated, and stored in aliquots at −80 °C after addition of 10% glycerol. Due to the cloning strategy, all tag-free versions of Grx1 contain an N-terminal GAME sequence instead of Met¹ yielding a calculated molecular weight of 10,833.49. ¹⁵N uniformly labeled Grx1 for NMR was prepared as previously reported (31) by growing E. coli BL21(DE3)-transformed cells in M9 minimal medium containing 1 g/liter of ¹⁵NH₄Cl as the only nitrogen source. The labeled protein was expressed and purified as described above and the buffer exchanged to 50 mM sodium phosphate, pH 7.2, 150 mM NaCl (buffer C) with 10 mM DTT in H₂O/D₂O (95:5, v/v) and the protein concentrated by ultrafiltration. T. brucei tryparedoxin (TXN) and monothiol glutaredoxin 1 C104S mutant (Tb1CGrx1 C104S) were obtained as previously described (70, 71), respectively.

Protein reduction and oxidation

To obtain dithiol Grx1 (Grx1(SH)₂), the protein was reduced with excess DTT (10 mM) for 30 min at room temperature, DTT was removed by gel filtration using a PD10 column (GE Healthcare). The active site disulfide form of Grx1 (Grx1S₂) was obtained by oxidation with an excess of H₂O₂ (1 mM) for 30 min, followed by gel filtration to remove the remaining H₂O₂. The product of H₂O₂-treated Grx1 was analyzed by MS and no fur-
ther modifications were identified. Protein and thiol concentrations were assessed in Grx1(SH)2 and GrxS2. Quantitative oxidation of the active site Cys was considered when a ratio of [RSH]/[protein] = 1, meaning no oxidation of Cys was detected.

**Thiol and protein quantitation**

Thiol concentration was determined by chromogenic disulfide reduction using 4,4’-dithiopyridine (Acros Organics), \( \varepsilon_{224} = 21,400 \text{ M}^{-1} \text{cm}^{-1} \) (72). Protein concentrations were measured at 280 nm using the following molar extinction coefficients: 11,460 \( \text{M}^{-1} \text{cm}^{-1} \) for Grx1, 35,300 \( \text{M}^{-1} \text{cm}^{-1} \) for HSA, 29,500 \( \text{M}^{-1} \text{cm}^{-1} \) for TbTXN, 10,000 \( \text{M}^{-1} \text{cm}^{-1} \) for EcGrx1, and 15,470 \( \text{M}^{-1} \text{cm}^{-1} \) for TbICGrx1 C104S.

**Preparation of mixed GSH disulfides**

Different stock solutions of the heterodisulfide between GSH and 2-mercaptoethanol (GSSEtOH) were prepared by mixing excess HED (10 mM in water) with the desired concentration of GSH in Tris/MES/acetic buffer, pH 7.1, and incubating at room temperature for 2 h. Under these conditions and according to the reported rate constants for the reaction (6), the conversion of GSH to GSSEtOH is nearly quantitative (>99%) with a very small (< 0.5%) GSSG contamination.

HSA glutathionylated in Cys (HSA-SSG) was prepared by oxidation of the reduced protein (HSA-SH) with GSSG. Briefly, a solution of delipidated HSA (1.22 mM) was reduced with 10 mM 2-mercaptoethanol for 2 h at room temperature, excess 2-mercaptoethanol was removed by gel filtration using a PD10 column and the resulting HSA was mixed with 5 mM GSSG overnight at room temperature. Thiol concentration in the protein fraction after gel filtration was measured before and after the treatment, in a typical preparation the resulting albumin fraction after gel filtration was measured before and after oxidation of the active site Cys was considered when a ratio of [RSH]/[protein] = 1, meaning no oxidation of Cys was detected.

**Preparation of fluorescein-labeled GSH disulfide**

GSH disulfide labeled with carboxyfluorescein (FGSSGF) was synthesized from 3 mM 5/6-carboxyfluorescein succinimidyl ester (NHS fluorescein, Thermo Fisher) and 6 mM GSSG in borate buffer, pH 8.5. The reaction was allowed to proceed for 1 h at room temperature. The product mixture was reduced with 20 mM DTT for 30 min, acidified with TFA, and separated in a 500 mg of C18 disposable extraction column (Bakerbond spe, J.T. Baker) previously activated with 2 ml of acetonitrile and equilibrated with 0.1% TFA in water. After loading the sample, the column was washed three times with 1 ml of 0.1% TFA and the labeled GSH (FGSH) was then eluted with 50% acetonitrile. The eluate was alkalinized to pH 8 and oxidized with 5 mM \( \text{H}_2\text{O}_2 \) for 1 h. Finally, FGSSGF was purified with the same chromatographic protocol used for FGSH. The purity of the FGSSGF was assessed by RP HPLC (Agilent Eclipse Plus C18, 100 × 4.6 mm column) before and after reduction with 50 mM DTT to rule out contamination with FGSH.

**Kinetics assays**

Unless otherwise specified, all kinetic experiments were conducted using a wide-range buffer solution of constant ionic strength (I = 0.15) independent of the pH as proposed by Ellis and Morrison (68): TMA buffer, was used in the pH range of 3.5 to 9.0.

The reaction of Grx1(SH)2 (0.2–1.0 \( \mu \text{M} \)) initial concentration) with oxidants was monitored by the change in intrinsic fluorescence of the protein under pseudo first-order conditions with the oxidant in excess. The fastest reactions (\( t < 60 \text{ s} \)) were monitored in a SX20 stopped-flow spectrometer (Applied Photophysics) using an excitation wavelength (\( \lambda_{\text{ex}} \)) of 280 nm and an emission cutoff filter \( \lambda_{\text{em}} > 320 \text{ nm} \); intermediate reactions (300 s > \( t > 10 \text{ s} \)) were followed in a Cary Eclipse spectrofluorimeter (Agilent) using a RX2000 rapid mixing stopped flow unit (Applied Photophysics) (\( \lambda_{\text{ex}}, \lambda_{\text{em}} = 280, 350 \text{ nm} \)), respectively; and the slowest reactions (\( t > 100 \text{ s} \)) were studied using a Variscan Flash plate reader (Thermo) (\( \lambda_{\text{ex}}, \lambda_{\text{em}} = 280, 350 \text{ nm} \), respectively). In most cases, Grx(SH)2 reacted with excess oxidant. The initial concentrations of oxidants were chosen according to the reaction rate, thus GSSG and GSSEtOH and TbICGrx1 Cys(SH)2–SSG were used in the range from 2.5 to 100 \( \mu \text{M} \), whereas cysteamine, cystine methyl ester, HED, and \( \text{H}_2\text{O}_2 \) were in the range of 1 to 15 \( \mu \text{M} \). Time courses were fitted to a first-order function and the values of the reaction rate constants were obtained using Gepasi 3.30 (74).

Exceptionally, the reactions of Grx1(SH)2 with HSA-SSG and with FGSSGF were performed under pseudo first-order conditions with Grx1(SH)2 in excess over the disulfide. This experimental design avoids interference of the higher fluorescence of HSA or fluorescein, respectively, with the fluorescent signal of Grx1. The concentrations used were 400 \( \text{nM} \) HSA-SSG (4–10 \( \mu \text{M} \) Grx1(SH)2) and 36 \( \text{nM} \) FGSSGF (0.4–4 \( \mu \text{M} \) Grx1(SH)2) and the reactions were monitored as indicated above.

Reduction of Grx1S2 by T(SH)2 was studied under pseudo first-order conditions with T(SH)2 in excess in the stopped flow. The reaction was monitored as indicated previously and the data fitted to a double exponential function to estimate the rate constants. The data were also fitted to a system of two consecutive reactions using Gepasi 3.30 (74).

Reduction of Grx1S2 by GSH was performed in the presence of ScGR and NADPH to ensure the absence of contaminating GSG (e.g. our freshly prepared GSH stock solutions contained ~0.5% GSSG). Thus, GSH free from GSSG was prepared by incubating different concentrations of GSH with 0.4 units/ml of GR and 10 \( \mu \text{M} \) NADPH during 10 min. Briefly, a mixture containing GR (0.4 units/ml), NADPH (10 \( \mu \text{M} \)), and GSH (0–100 \( \mu \text{M} \)) in buffer, pH 7.0, was incubated for 10 min at 25 °C, then Grx1S2 (2 \( \mu \text{M} \)) was added and fluorescence was emission monitored during 20 min in an ISS ChronosFD spectrofluorimeter.
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\[ pK_a \] measurement

The \( pK_a \) of the N-terminal cysteine of Grx1(SH)\(_2\) was determined by three independent techniques: (i) pH dependence of the tryptophan fluorescence, (ii) pH dependence of the second-order rate constant of GSSG reduction, and (iii) rate of alkylation with mBBr (71). In all cases the measured variable was fitted to Equation 5 at pH < 7.5.

\[
y = A \left( \frac{[H^+]^2}{K_a + [H^+]} \right) + B \frac{K_a}{K_a + [H^+]} \tag{Eq. 5}\]

Where \( A \) and \( B \) are the parameters characteristic of the protonated or ionized cysteine, respectively, in the protein.

NMR

The titration with GSH was performed on 550 \( \mu \)l of 100 \( \mu \)M \(^{15}\)N-labeled Grx1S\(_2\) solutions in 50 mM phosphate buffer, 50 mM NaCl at pH 7.0 in H\(_2\)O/D\(_2\)O, 10:1.

Grx1S\(_2\) was prepared by adding a stoichiometric amount of \( \text{H}_2\text{O}_2 \) to fully reduced Grx1. The mixture was left to react overnight at room temperature yielding Grx1 with a Cys\(^{21}\)–Cys\(^{24}\) disulfide and a free thiol at Cys\(^{28}\).

To titrate \(^{15}\)N-labeled Grx1S\(_2\), GSH (6.1 mM in 50 mM phosphate buffer, pH 7.0) was added in 2-\( \mu \)L aliquots and a \(^{15}\)N-SOFAST-HMQC (75) was recorded after each addition. \(^{15}\)N-SOFAST-HMQC experiments were collected on a Bruker DFX 600 MHz spectrometer with a room temperature probe, at 298 K. Each experiment was acquired with 32 scans, 1024 and 128 increments in 1\(^{2}\)H dimension, and 128 increments and \(^{15}\)N dimension, respectively. A recovery delay of 200 ms was used before each scan.

Mass spectra

The MS of Grx1(SH)\(_2\) (17 \( \mu \)M in 20 mM ammonium acetate) was determined before and after reaction with 1 or 6 eq of GSSG. Grx1S\(_2\) (17 \( \mu \)M in 20 mM ammonium acetate) was measured before and after reaction with 1 or 10 eq of GSH. The spectra were recorded on a Xevo G2 from Waters, using electrospray ionization. Prior to injection, samples were diluted in H\(_2\)O:CH\(_3\)CN, 50:50, with 0.1% formic acid.

Glutathionylation of Grx1 with fluorescein-labeled GSH

Grx1 WT, C24S, and C78S were reduced and total protein and thiol content was measured to verify each had the expected number of reduced cysteines. The Grx1 variants were reacted with a substoichiometric concentration of fluorescein-tagged GSH disulfide ([Grx] = 9 \( \mu \)M, [FGGSGF] = 7.65 \( \mu \)M) at pH 6.85 in TMA buffer. The reaction products were separated using two Hitrap columns in series in a HPLC (Agilent 1260 Infinity) while monitoring absorbance at 280 and 495 nm. At 1.66 and 4.66 min, coincident with the maxima at 280 and 495 nm, the UV-visible spectra were taken using the Diode Array detector of the chromatograph.

Kinetic simulation of GSSG reduction

The reduction of GSSG was simulated using Gepasi 3.30 (74) in a system containing T(SH)\(_2\), Grx1(SH)\(_2\), and TXN as possible reductants under the conditions described in Table 4.

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