Glutathione-dependent activities of *Trypanosoma cruzi* p52 makes it a new member of the thiol:disulphide oxidoreductase family

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Trypanothione:glutathione disulphide thioltransferase of *Trypanosoma cruzi* (p52) is a key enzyme in the regulation of the intracellular thiol–disulphide redox balance by reducing glutathione disulphide. Here we show that p52, like other disulphide oxidoreductases possessing the CXXC active site motif, catalyses the reduction of low-molecular-mass disulphides (hydroxyethyl-disulphide) as well as protein disulphides (insulin). However, p52 seems to be a poor oxidase under physiological conditions as evidenced by its very low rate for oxidative renaturation of reduced ribonuclease A. Like thioltransferase and protein disulphide isomerase, p52 was found to possess a glutathione-dependent dehydroascorbate reductase activity. The kinetic parameters were in the same range as those determined for mammalian dehydroascorbate reductases. A catalytic mechanism taking into account both trypanothione- and glutathione-dependent reduction reactions was proposed. This newly characterized enzyme is specific for the parasite and provides a new target for specific chemotherapy.

INTRODUCTION

Because most parasites have decreased metabolic capabilities and take advantage of the host metabolism, they are highly dependent on a few limited pathways for their survival. A greater knowledge of the enzyme systems lacking in or specific for the parasite might reveal numerous proteins potentially crucial for its maintenance in the host environment and is essential for the development of efficient chemotherapies against parasitic diseases.

The principal antioxidative thiol of eukaryotes is glutathione (GSH), which participates in several enzyme-mediated and non-enzymic redox processes such as thiol–disulphide exchange reactions, peroxide reduction and free radical scavenging. Trypanosomatidae do not possess the classical redox system based on GSH and the flavoprotein NADPH-dependent GSH reductase, which reduces glutathione disulphide (GSSG). They produce an analogous thiol, the N^1,N^8-bis(glutathionyl)-spermidine named trypanothione [$T(SH)_2$], which like GSH is important in the prevention of oxidative stress by oxidation into its disulphide form [$T(S)_2$] [1]. Regeneration of the reduced form is ensured by a specific flavoprotein, the NADPH-dependent trypanothione reductase (TR). Thus the redox system based on the couple $T(SH)_2$–TR represents a potential target for the development of new trypanocidal drugs. However, the existence of significant GSH concentrations in Trypanosomatidae (evaluated to be approx. 20% of total thiol) suggests enzymic processes based on GSH [2].

We previously reported the isolation of a 52 kDa $T(SH)_2$:GSSG thioltransferase (p52) from *Trypanosoma cruzi*, representing the missing link between the GSH-based metabolism present in the host and the specific $T(SH)_2$-based metabolism of the parasite. This enzyme could participate in the regeneration of GSH in *T. cruzi* [3]. p52 seemed to be localized in multivesicular structures (reservosomes) of parasites and to belong to excretion/secretion products [4,5]. The sequence deduced from its cDNA was reported to display several features of a GSH S-transferase and to contain two homologous domains [4]. In the N-terminal one, a CPFC motif can be found that is replaced by a SPFS sequence in the C-terminal domain.

Several proteins involved in thiol–disulphide redox reactions exhibit the same CXXC motif in their active site and have been grouped together in a structurally conserved family based on a thioredoxin (Trx) fold [6]. Trx-like proteins divide into two closely structurally related sub-families with different functional properties: compared with the major cellular thiol, GSH, some are more reducing whereas others are more oxidizing. In the first case, proteins such as Trx, and as thioltransferase also named glutaredoxin (Grx), might be involved in the regulation of the reducing properties of the cytoplasm. Their reduction has to be ensured by a stronger reductant, NADPH, via flavoenzymes, respectively Trx reductase and GSH reductase/GSH redox system. In contrast, protein disulphide isomerase (PDI) and DsbA (periplasmic protein thiol:disulphide oxidoreductase from *Escherichia coli*) are present in subcellular compartments where oxidant properties are required for the formation of disulphide bonds, i.e. eukaryotic endoplasmic reticulum and bacterial periplasm. GSSG is abundant in endoplasmic reticulum and PDI uses it for disulphide oxidation, but it is absent from *Escherichia coli* periplasm, where DsbA requires an additional membrane protein, DsbB, for its reduction [7,8]. Interestingly, both Grx and PDI displayed a GSH-dependent dehydroascorbate reductase (DHAR) activity, and dehydroascorbate (DHA) has been proposed as an essential primary oxidant in protein disulphide bond

Abbreviations used: DHA, dehydroascorbate; DHAR, DHA reductase; DsbA, DsbB, periplasmic protein thiol:disulphide oxidoreductase from *Escherichia coli*; DTT, dithiothreitol; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulphide; HED, 2-hydroxyethyl disulphide; PDI, protein disulphide isomerase; TR, trypanothione reductase; Trx, thioredoxin; $T(SH)_2$, trypanothione; $T(S)_2$, trypanothione disulphide.

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formation by regenerating oxidized PDI [9,10]. On the basis of experimental work, a catalytic mechanism for Grx has been proposed, but that of DHAR has not yet been elucidated [11,12].

Trx and Grx also serve as cofactors in enzymes not directly related to GSH metabolism: T7RNA polymerase and ribonucleotide reductase. They have been also shown to be implicated in diverse cellular events through their redox activity as transcription factor regulators. For example, a Grx homologue from vaccinia virus was proposed to establish a role in virus–host relation via stabilization of virions in oxidizing environments and activation of the viral transcription system to initiate gene expression [13]. Recently, a 58 kDa protein, shown to display a thiol-dependent reductase activity in the insulin degradation assay, was reported to be secreted from the cell and involved in oncogenic transformation [14].

In *T. cruzi*, no Trx or Grx has been detected until now. Recently, the cloning and cDNA sequence analysis from *T. brucei* have revealed a structure analogous to PDI, but the protein has not yet been isolated and characterized [15]. As it has been suggested that the TR system substitutes for both the Trx and Grx systems [2], it was interesting to prove experimentally that p52, which possesses the CPFC motif, belongs to the Trx family. Moreover, a GSH-dependent DHAR activity has also been reported in *T. cruzi* epimastigotes and trypomastigotes [16]; it was then straightforward to test p52 for a GSH- or $T(SH)_2$-dependent DHAR activity.

Here we present the characterization of new reducing properties for p52 through its GSH-dependent disulphide reductase (Grx and insulin disulphide reductase) and DHAR activities. A general mechanism taking in account both $T(SH)_2$-and GSH-dependent activities of the enzyme is proposed. These features define it as a potential target for the search of a chemotherapy against Chagas disease.

EXPERIMENTAL

Materials

GSH, GSSG and dehydroascorbic acid were purchased from Aldrich, and 2-hydroxyethyl disulphide (HED) from Fluka. $T(SH)_2$ was synthesized as described previously [17]. A double-beam Uvikon 930 spectrophotometer (Kontron Instruments) was used for spectrophotometric measurements. GSH reductase (EC 1.6.4.2) from bovine intestinal mucosa was a product from Boehringer. In accordance with the described procedures [3], epimastigote cultures were established with the Y strain of *T. cruzi* and p52 was purified as a homogeneous enzyme then stored at 4 °C after freeze-drying.

Thioltransferase assay

This method was adapted from the thioltransferase assay described in [18]. The thioltransferase activity was coupled with GSH reductase, which reduced GSSG with NADPH as a cofactor. The rate was determined at room temperature (23 °C) by following the disappearance of NADPH at 340 nm. The enzymic reaction was performed in 500 μl of 50 mM Tris/HCl buffer, pH 7.0, containing 5 mM EDTA, 300 μM NADPH, 1 mM GSH and 1 unit/ml GSH reductase. The buffer was left for few minutes until A_{340} had stabilized, to ensure that no GSSG was present. HED (final concentration 0.75 mM) was added to both sample and reference cuvettes. p52 (0.74–5.55 μg) initiated the enzymic reaction. The reaction rate was linear for more than 2 min. A blank was measured without HED in the presence of p52.

DHAR activity

DHA reduction was measured by a direct spectrophotometric assay, monitoring the increase in A_{265} due to the formation of ascorbate from DHA [19]. The test was performed in a final volume of 500 μl of 0.1 M sodium phosphate, pH 7, containing GSH (0.3–5 mM) at 25 °C. p52 (1.11 μg) was added and the enzymic reaction was initiated by the addition of DHA (0.2–2.4 mM). Initial rates were obtained during the first minute after the addition of DHA to the GSH-containing medium. The reaction rate was linear for up to 2 min at 25 °C. A blank without enzyme was run for each analysis and subtracted from the readings. Various potential reductants were tested in place of GSH, either at 265 nm in the conditions described above for 0.5 mM lipoic acid, 0.1 mM dithiothreitol (DTT) or 1 mM 2-mercaptoethanol, or at 340 nm for 0.1 mM NADPH.

GSH reductase-coupled insulin reductase assay

The capacity of enzymes to reduce the native disulphide bonds of insulin was monitored in a spectrophotometric assay based on the following reactions:

$$\text{Insulin-}(S)_2 + \text{E-}(SH)_2 \rightarrow \text{insulin-}(SH)_2 + \text{E}(S)_2 \quad (1)$$

$$\text{E-}(S)_2 + 2\text{GSH} \rightarrow \text{E-}(SH)_2 + \text{GSSG} \quad (2)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad (3)$$

Insulin (15 μM) was preincubated at 37 °C in 0.2 M sodium phosphate/5 mM EDTA/120 μM NADPH/8 mM GSH/1 unit/ml GSH reductase (pH 7.5) until A_{340} was stable, i.e. approx. 2 min. The reaction was started by the addition of 1 μM PDI, p52 and DsbA or Trx (final volume of reaction mixture 1 ml). The rate of reduction of insulin disulphides is directly proportional to the decrease in A_{340} with time.

Catalysis of ribonuclease A refolding

The PDI-catalysed folding of RNase A was assayed in accordance with [20], with minor modifications. The protein was denatured at 10 mg/ml in 0.1 M Tris/acetate (pH 8.0)/0.1 M DTT/6 M guanidinium chloride, then dialysed against 0.1 M acetic acid/3 M guanidinium chloride (pH 3.5) to remove DTT. The formation of active RNase from reduced and denatured enzyme was monitored continuously as the increase in A_{296} of the CMP formed by hydrolysis of cCMP in the presence of the refolded enzyme. The reaction mixture contained 0.1 M Tris/HCl, pH 8.0, 1 mM GSH, 0.2 mM GSSG and 1 μM PDI or p52. Interestingly, the residual 30 mM guanidinium chloride had no effect on the PDI activity. The concentration of active RNase at any time (E_t in μM) can be calculated from:

$$E_t = \frac{\partial A_{296}/\partial t\{K_m(\text{cCMP})(1 + [\text{CMP}]_t/K_i(\text{CMP})) + [\text{cCMP}]_t\}}{k_{cat}[\text{cCMP}]_t} \quad (4)$$

to take into account the competitive inhibition of CMP on the hydrolysis of cCMP. The following constants were used in eqn. (4), in accordance with [20]: K_m(cCMP), 8 mM; K_i(CMP), 2.1 mM; catalytic-centre activity ('turnover number') for fully active RNase (k_{cat}), 14.3 min^{-1}. The concentrations of cCMP and CMP at time t, $[cCMP]_t$ and $[CMP]_t$, were deduced from A_{296} by using 0.19 and 0.38 $mM^{-1} \cdot cm^{-1}$ respectively as the molar absorption coefficients for cCMP and CMP. $\partial A_{296}/\partial t$ is the first derivative of absorbance against time.

$T(SH)_2$:GSSG thioltransferase activity

The $T(SH)_2$:GSSG thioltransferase activity was measured directly by capillary zone electrophoresis. The enzymic reaction

was conducted at room temperature in 20 μl of 100 mM phosphate buffer, pH 7.5, containing 250 μM GSSG, 250 μM $T(SH)_2$ and 0.8 μg of p52. The reaction was started by the addition of $T(SH)_2$ at a final concentration of 250 μM and stopped in due time by addition of 5 μl of 1 M HCl (final pH 1.5–2). The reaction mixture was found to be stable for more than 1 day; it was directly injected (3 nl) into the capillary zone electrophoresis apparatus. $T(SH)_2$ and $T(S)_2$ were analysed on an Applied Biosystems model 270A-HT capillary electrophoresis system under the following conditions: field, 27 kV; capillary length, 50 cm; capillary diameter, 50 μm fused silica. The separation was performed at 30 °C with 20 mM sodium citrate, pH 2.5, as buffer. Detection was monitored at 200 nm. Studies of the optimum pH for thioltransferase activity were performed with either 50 mM Tris/HCl buffer (pH 9.0–6.5) or 100 mM phosphate buffer (pH 8.0–5.0). A blank without enzyme was measured at each pH and subtracted from the data obtained in presence of the enzyme.

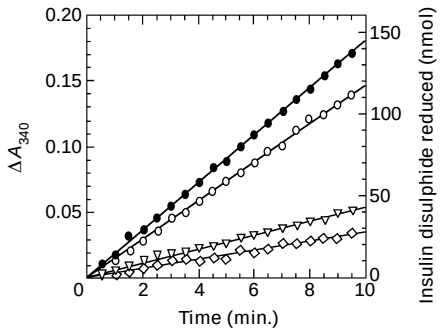


Figure 1 Enzymic reduction of insulin disulphides

The reaction was followed with a GSH reductase-coupled assay (see the Experimental section) and monitored continuously after the addition to the assay cuvette of 1 μM enzyme: p52 (●), PDI (○), DsbA (▽) or Trx (◇). The spontaneous reduction of insulin by GSH was negligible but was corrected at each time point.

RESULTS

Thioltransferase activity of p52

The identity between catalytic centres of pig, vaccinia virus and rice Grx species and p52 (CPFC) led us to investigate whether p52 exhibited a Grx-type activity (Table 1). The GSH:disulphide thioltransferase assay described by Holmgren [18] was used for this purpose. p52 showed a significant activity. The rate of the uncatalysed reaction was negligible. Moreover, no reaction occurred in the assay performed without HED to measure a possible NADPH oxidase activity. Under our experimental conditions (0.7 mM HED and 1 mM GSH), the reaction rate was a linear function of p52 concentration up to 8.5 μg/ml (results not shown). The k_{cat} of the enzymic reaction, 522 min^{-1}, was estimated from the slope of this plot. The assay was adapted to replace GSH with the parasitic $T(SH)_2$, already known as a substrate of p52 through its $T(SH)_2$:GSSG thioltransferase activity. At pH 7 the non-enzymic HED reduction by $T(SH)_2$ was instantaneous and complete: no difference was observed in the absence or presence of the enzyme. p52 did not catalyse thiol–disulphide exchange between $T(SH)_2$ and HED. Indeed, the thioltransferase activity of p52 seemed to be specifically GSH-dependent.

DHAR activity

In mammals, Grx has been shown to display a GSH-dependent DHAR activity. The common GSH:disulphide thioltransferase activity of Grx and p52 induced us to examine whether p52 could also catalyse the reduction of DHA. DHA reduction was measured by the direct spectrophotometric assay described by Stahl et al. [19]. With GSH as hydrogen donor, p52 reduced DHA with Michaelis–Menten-type kinetics for both substrates. The kinetic parameters of the DHAR activity were: K_m 0.67 ± 0.14 mM (S.D.) for DHA in the presence of 5 mM GSH (V_{max} 15.28 ± 1.32 μM/min); K_m 2.6 ± 0.5 mM for GSH in the presence of 1.5 mM DHA (V_{max} 7.59 ± 0.70 μM/min). The ratios k_{cat}/K_m for DHA and GSH were 1.8×10^4 and 2.3×10^3 $s^{-1} \cdot M^{-1}$ respectively. The reaction was inhibited by *S*-hexylglutathione with an IC_{50} of 205 μM at pH 7.5 and an IC_{50} of 340 μM at pH 7 in the presence of 2 mM GSH and an excess of DHA (1.5 mM). Other hydrogen donors such as NADPH and the thiols DTT, lipoate and 2-mercaptoethanol were tested for DHAR activity. The dithiols $T(SH)_2$, DTT and lipoate efficiently reduced DHA in a non-enzymic way. At pH 7, DHAR activity from *T. cruzi* displayed a strict requirement for GSH as hydrogen donor.

Table 1 Primary structures of active sites of p52 and thioltransferases from different species

Site/species	Sequence			
p52, N-terminus	**F**	KDRL	**C** PF **C**	QRVLITAK
p52, C-terminus	**F**	VNKY	*S* PF *S*	DRPRLAC
Grx, pig	**F**	IKPT	**C** PF **C**	RKTQEL
Grx, rice	**F**	IKPT	**C** PF **C**	VRVKKL
Grx, vaccinia virus	**F**	VKYT	**C** PF **C**	RNALDIL
Grx, human	**F**	IKPT	**C** PY **C**	RRAQEI
Grx, rabbit	**F**	IKPT	**C** PY **C**	RKTQEIL
Grx1, *E. coli*	**F**	GRSG	**C** PY **C**	VRAKDL

Enzymic reduction of insulin disulphides

The second mammalian protein disulphide oxidoreductase also known for its DHAR activity was PDI. Therefore we checked whether p52, in the same way as PDI, could not only reduce small disulphides but also protein disulphides. Protein disulphide oxidoreductases are able to attack hidden disulphide bonds within folded protein structures, whereas simple thiols such as GSH and even DTT either do not do so, or only very slowly. We measured the reducing capacity of p52 towards insulin disulphides in the presence of excess GSH, i.e. GSH:insulin thioltransferase activity. The GSH consumption necessary for active site regeneration was followed spectrophotometrically with a GSH reductase-coupled assay (Figure 1). p52 behaves as a very efficient disulphide reductase, k_{cat} 14.5 min^{-1}, better than the other previously characterized enzymes PDI, DsbA and Trx, with respective k_{cat} values of 11.8, 4.35 and 2.9 min^{-1} respectively under the conditions used in the assay. It has to be noted that Trx is a very efficient reductase only when associated with Trx reductase to catalyse the NADPH-dependent reduction of Trx-S_2 into Trx-$(SH)_2$ [21]. In the same manner, DsbA works efficiently when DsbB is present for its reduction [6] and spontaneously reduces only poorly when GSH is provided as sole donor (E. Quéméneur, unpublished work). Therefore in our experiment the reductions by GSH of E-$(S)_2$ to E-$(SH)_2$ were certainly rate-limiting in the Trx and DsbA reactions. In contrast,

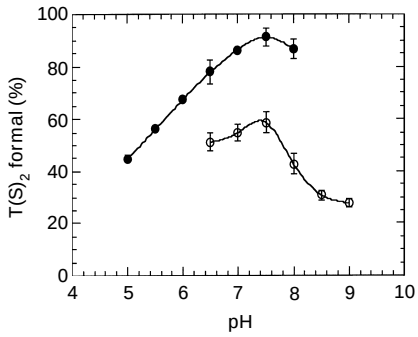


Figure 2 Optimum buffer and pH for $T(SH)_2$:GSSG thioltransferase activity

A direct assay by capillary zone electrophoresis was performed to study the optimum pH and the influence of the buffer on $T(SH)_2$:GSSG thioltransferase activity (see the Experimental section). Thioltransferase activity was measured in the pH range 5.0–8.0 in phosphate buffer (●) and in the pH range 6.5–9.0 in Tris buffer (○). A blank without enzyme was measured at each pH and subtracted from the data obtained in presence of the enzyme. Enzyme activity is expressed as the percentage of $T(S)_2$ formed in 5 min; the larger value obtained was set to 100% activity to allow comparisons between assays. These results are representative of three experiments.

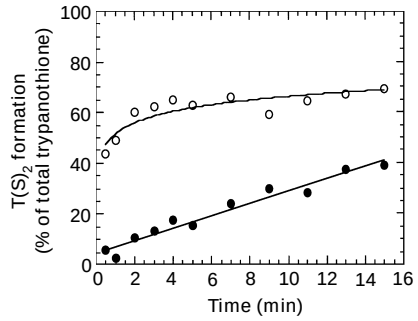


Figure 3 Kinetics of the thiol–disulphide exchange reaction catalysed or not by p52

The percentage $T(S)_2$ of the total $T(SH)_2$ was measured by the direct capillary electrophoresis assay. Both enzymic (○) and non-enzymic (●) reactions were performed at room temperature, in 20 μl of 100 mM phosphate buffer, pH 7.5. Both reactions were initiated by adding $T(SH)_2$ (final concentration 250 μM) to the medium containing 250 μM GSSG. The enzymic reaction was initiated by adding 0.8 μg of p52. The reactions were stopped by decreasing the pH medium to pH 1.5 with HCl.

p52 and PDI are able to use GSH efficiently, possibly owing to the presence of a GSH-binding site close to the enzyme active site.

Catalysis of disulphide formation in a protein substrate

We looked for the protein disulphide oxidase and isomerase properties of p52 through its ability to promote the folding of RNase A in the presence of a mixture of GSSG and GSH. Under the conditions tested, p52 is 1/25 as active as PDI, their k_{cat} values being respectively 0.255 and 5.02 μmol of active RNase/min per μmol of enzyme. However, with a k_{cat} of 0.153 min^{-1}, p52 is only approx. 1.7-fold more active than Trx, which was also previously described as a catalyst of RNase oxidation [22]. Under our conditions, p52 accelerates approx. 20-fold the reaction compared with the non-assisted one, which has a k_{cat} of 0.011 min^{-1}. Therefore although p52 seems to be a very good GSSG-dependent oxidant for small substrates such as $T(SH)_2$, it is rather poor towards larger substrates.

$T(SH)_2$:GSSG thioltransferase activity

Previous studies have shown that p52 catalysed the thiol–disulphide exchange between $T(SH)_2$ and GSSG and not the reverse reaction between GSH and $T(S)_2$ [3]. Now two novel enzymic activities of p52, thioltransferase and DHAR, have been characterized and found to be strictly GSH-dependent. In both cases no catalysis occurred when $T(SH)_2$ was used as the hydrogen donor in the presence of p52. This paradox led us to re-examine the already described $T(SH)_2$:GSSG thioltransferase activity of p52. Our first experiments [3] used a coupled assay with TR. To avoid any shift due to the use of TR, which regenerated $T(SH)_2$, a direct assay with capillary electrophoresis was developed. This assay confirmed the $T(SH)_2$:GSSG thioltransferase activity of p52 and enabled us to determine the optimal buffer and pH. Thioltransferase activity was measured in the pH range 5.0–8.0 in phosphate buffer and 6.5–9.0 in Tris buffer. The enzymic activity had an optimum at pH 7.5 in the two buffers tested. A higher activity was obtained in phosphate buffer (Figure 2). A kinetic study of the enzymic and non-enzymic reactions was performed in phosphate buffer, pH 7.5 (Figure 3), in the presence of 10-fold more enzyme than in the spectrophotometric assay previously described [3]. It is noteworthy that the catalysed reaction rate is linear for up to 2 min with one-tenth of the amount of the enzyme [3]. The enzymic reaction rapidly reached a maximum at 60–70% transformation of the initial $T(SH)_2$. Two hypotheses can be proposed to explain this observation: either enzyme substrates rapidly became limiting factors, or reaction products were competitors of the reaction. This will be discussed below.

DISCUSSION

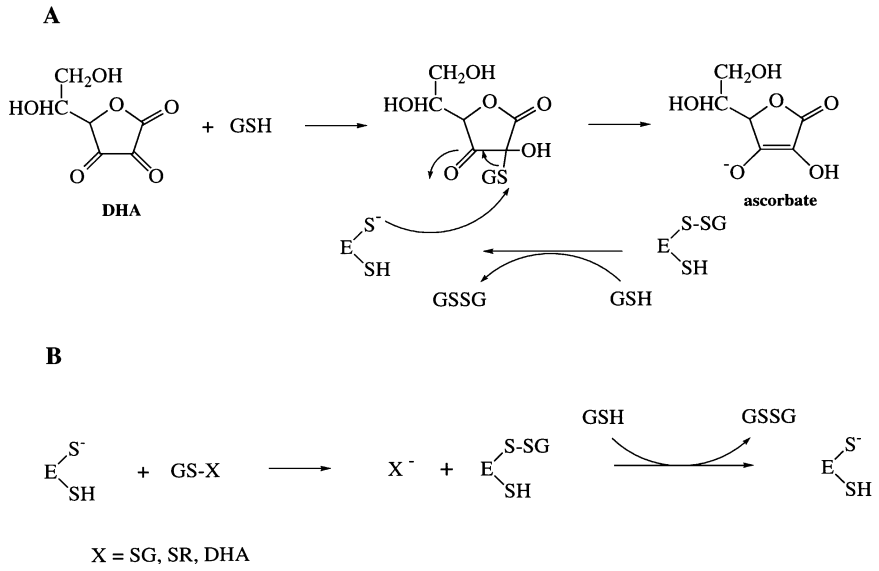
All data reported here confirm experimentally that p52 is a new member of the Trx-like oxidoreductase family, whose CPFC sequence is probably involved in the oxidoreduction process. In its catalytic properties p52 shares many features with Grx and Trx. However, the described properties of p52 indicate that it reduces not only low-weight disulphides, like Grx already known for its broad substrate specificity [23], but also protein disulphides, as do Trx and PDI. Neither Trx nor PDI have been shown to display activity with regard to HED, nor Grx to reduce insulin disulphides [8]. Moreover, Grx and Trx are small proteins of 10–12 kDa, whereas p52 is a homodimer of 52 kDa per subunit [3]. Nevertheless several atypical oxidoreductases in the 20–28 kDa range, showing both Grx and insulin-reduction activities, have already been isolated from bacteria, cyanobacteria and in chloroplasts of unicellular green algae or of higher plants [24,25], indicating that molecular mass might not be the most important characteristic for such an activity.

At pH 7.5, p52 seemed to be more reducing than PDI but less than Trx. Thus in *T. cruzi* p52 could be considered to be a physiological reducing agent equivalent to the Trx or Grx system. PDI, which has nearly the same size as p52 and is also a homodimeric protein, possesses two Trx domains, both containing the CXXC motif. One common hypothesis is that this duplication might be responsible for the efficient isomerase activity of PDI. In p52 the mutation of the Cys residues into Ser in the C-terminal sequence might partly explain the low isomerase activity, but without justifying the role of the SPFS motif found in the C-term domain of p52. However, the localization of p52 could be compatible with a role in disulphide bond formation, which commonly occurs in extracytoplasmic compartments [5]. In this perspective, the capacity of p52 to refold reduced denatured RNase has been assayed under the optimal experimental conditions determined for PDI, at a physiological pH

Table 2 Kinetic parameters for glutathione-dependent DHAR

Parameters were determined with either (1) constant GSH (5.0 mM) or DHA (1.5 mM) at pH 7.0 and 25 °C, or (2) constant GSH (3.0 mM) or DHA (1.5 mM) at pH 6.85 and 30 °C. All data were corrected for non-enzymic reactions between DHA and GSH.

		DHA		GSH		
Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	Reference
p52 (1)	717	0.67	1.8×10^4	2.6	2.3×10^3	This study
Grx (2)	374	0.26	2.4×10^4	3.4	1.8×10^3	[10]
PDI (2)	16	2.80	1.0×10^2	2.9	0.9×10^2	[10]



Scheme 1 Proposed mechanism for the DHAR activity of p52 (A) and general mechanism proposed for p52 reductase activities

value, which also corresponds to the optimal pH for the $T(SH)_2$:GSSG thioltransferase activity of p52. Disulphide bond formation and rearrangement are reversible thiol–disulphide exchange reactions and are known to be thermodynamically and kinetically affected by the redox state of the environment [22]. Until now no information has been available on pH and redox state in trypanosomes' compartments. The presence of both GSH and $T(SH)_2$ in reduced and oxidized states might induce a redox environment different from that found in endoplasmic reticulum. Although p52 has no relevant isomerase activity in the buffer used for PDI activity, such an activity cannot be ruled out under physiological conditions.

GSH-dependent DHAR was first described in plants several decades ago [26,27], then recently in mammals, insects and protozoans. For many years there has been controversy over the existence of an enzymic reduction of DHA to ascorbic acid by cellular GSH in mammals because it is known that DHA is quickly reduced by hydrogen donors such as GSH and lipoic acid [28–33]. Recently, the controversy was settled by a report of the isolation and characterization of a cytosolic GSH-dependent DHAR from rat liver [34]. Moreover, other proteins such as Grx and PDI have also been shown to display substantial DHAR activity involving the direct catalytic reduction of DHA by GSH [9]. The similarities summarized in Table 2 in the described features of the characterized enzyme p52, and those of Grx and PDI, should be pointed out, i.e. the specific requirement for GSH and the comparable kinetic parameters for DHA and GSH [10]. The presence of GSH-dependent DHAR activity was also recently reported in *T. cruzi* epimastigotes and trypomastigotes [16]. DHA reduction was measured in crude homogenates by coupled assay in the presence of NADPH/GSH reductase and by direct spectrophotometric assay. As noted by Clark et al. [16], values obtained in the direct assay were reported to be one-fifth of those obtained in the coupled assay. However, it should be mentioned that this DHAR activity was measured from a non-dialysed cell extract, which is known to contain a considerable amount of endogenous substrates as low-molecular-mass thiols. Approx. 30 % of these non-protein thiols correspond to $T(SH)_2$ [35]. We checked that $T(SH)_2$ was able to reduce DHA efficiently by a redox reaction at pH 7. Treatment by boiling of an epimastigote cell extract at 100 °C for 3 min did not abolish the rate of DHA reduction by GSH, but caused a slight (25 %) decrease (results not shown). In fact, DHAR activity measured from a non-dialysed cell extract could be partly due to non-enzymic reaction between DHA and endogenous $T(SH)_2$ or another thiol. The DHAR activity of p52 alone does not completely reflect the observed rate of DHA metabolism reported by Clark et al. [16].

Whereas thioltransferase and DHAR activities were thought to take place at the same active centre, CXXC, the mechanism of the GSH-dependent DHAR catalysis of thioltransferase has not yet been clearly established [12]. The possibility that p52 could be directly reduced by GSH or $T(SH)_2$ corroborates the kinetics observed for $T(SH)_2$:GSSG thioltransferase activity. After 2–4 min the GSH produced competes with $T(SH)_2$ for the reduction of p52, leading to an equilibrium in which nearly 70 %

of the $T(SH)_2$ is oxidized (Figure 3). Therefore, by supposing the formation of a reduced enzyme intermediate $E(SH)_2$, we propose a mechanism taking into account both GSH and $T(SH)_2$ reduction reactions (Scheme 1). In DHA reduction, DHA would first react with GSH leading to a glutathionyl-thiohemiketal intermediate. The nucleophilic thiolate of the reduced enzyme would then react on the glutathionyl-thiohemiketal to form the GSH mixed disulphide enzyme and liberate ascorbate by an intramolecular pathway. A second GSH molecule would then attack the mixed disulphide to yield GSSG and regenerate the reduced enzyme. This mechanism differs slightly from the mechanism proposed by Yang and Wells [12], who supposed the formation of a thiohemiketal by the early reaction of the enzyme thiolate on DHA. In the presence of $T(SH)_2$, the fact that no enzymic catalysis occurred in DHA reduction is not surprising because the trypanothionyl-thiohemiketal is probably involved in a favoured intramolecular attack of the free thiolate RS-T-SH to liberate ascorbate. The lifetime of this intermediate is supposed to be too short for an attack by the nucleophilic thiolate of the reduced enzyme. This greater rate has already been observed with dithiols as lipoate in the reduction of DHA [30]. In thioltransferase activity, mixed disulphides GS-SR have been reported to be good substrates of Grx and suggested to be the competent substrate of the enzyme [11,23]. In all reduction reactions catalysed by p52, we think that the motif preferentially recognized by the reduced enzyme is the GS-X entity, respectively the thiohemiketal GS-DHA, GS-S-CH_2CH_2OH and GSSG for the $T(SH)_2$:GSSG thioltransferase activity. For the latter activity, $T(SH)_2$ would play the role of a physiological endogenous reductant of enzyme disulphides in *T. cruzi*, as with DTT *in vitro*, although the redox potentials of $T(SH)_2$ and DTT are quite different ($E'_0 = -0.242$ and -0.330 V respectively). Indeed, reduction of GSSG was achieved enzymically when substituting DTT for $T(SH)_2$ in the $T(SH)_2$:GSSG thioltransferase assay (results not shown). Moreover, previous studies have shown that p52 did not catalyse the exchange reaction between $T(SH)_2$ and vasopressin disulphide [3]. Another argument that corroborates GS-X recognition by p52 is the fact that the enzyme binds exclusively to the *S*-hexylglutathione-agarose matrix, but not to *S*-glutathione-agarose nor to NH-glutathione-agarose [36]. In the insulin degradation assay p52 was able to reduce the three disulphide bridges, which are buried in the protein structure and link the two polypeptide chains of insulin [37]. In this case the formation of a mixed disulphide between insulin and GSH is unlikely because GSH is unable to reduce insulin non-enzymically. GSH probably reduces the active disulphide centre of the enzyme, which can then reduce the insulin disulphide bonds. This would be consistent with an active site protruding from the rest of the molecule as can be observed in the three-dimensional structure of Trx and Grx [38,39].

Similarly to Trx and Grx proteins, p52 is thought to be a protein with multifunctional activities. Furthermore p52 was recently reported to induce T-cell unresponsiveness through specific interaction with cysteine and GSH, in agreement with the observed immunosuppression in Chagas disease [40]. Because of a possible involvement of Trx in lymphocyte activation [41], we suggest that p52, through its Trx- and Grx-like activities, could be a regulatory factor of redox potential both in the parasite and in the host cell and exert its effects on the T-cell response. Moreover the rapid reduction of DHA by DHAR activity of p52 might protect the trypanosomal membranes from damage in the host environment. By their catalytic properties, both TR/NADPH and p52 might represent the complete electron transport chain helping to maintain ascorbate levels in the parasite against oxidative stress. Taking into account the reducing properties of p52 and its atypical molecular mass, we think that p52 is a promising new target for chemotherapy against Chagas disease.

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