Purification and characterization of a trypanothione-glutathione thioltransferase from *Trypanosoma cruzi*

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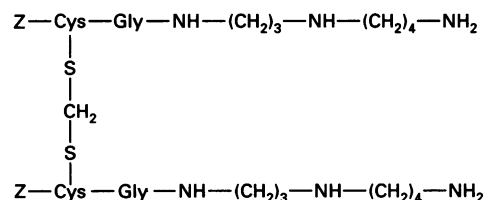
Although trypanothione [T(S)$_2$] is the major thiol component in trypanosomatidae, significant amounts of glutathione are present in *Trypanosoma cruzi*. This could be explained by the existence of enzymes using glutathione or both glutathione and T(S)$_2$ as cofactors. To assess these hypotheses, a cytosolic fraction of *T. cruzi* epimastigotes was subjected to affinity chromatography columns using as ligands either *S*-hexylglutathione or a non-reducible analogue of trypanothione disulphide. A similar protein of 52 kDa was eluted in both cases. Its partial amino acid sequence indicated that it was identical with the protein encoded by the TcAc2 cDNA previously described [Schoneck, Plumas-Marty, Taibi et al. (1994) Biol. Cell **80**, 1–10]. This protein showed no significant glutathione transferase activity but surprisingly catalysed the thiol–disulphide exchange between dihydrotrypanothione and glutathione disulphide. The kinetic parameters were in the same range as those determined for trypanothione reductase toward its natural substrate. This trypanothione–glutathione thioltransferase provides a new target for a specific chemotherapy against Chagas' disease and may constitute a link between the glutathione-based metabolism of the host and the trypanothione-based metabolism of the parasite.

INTRODUCTION

Unlike most prokaryotic and eukaryotic cells, in which the major thiol component involved in both enzyme-mediated and non-enzymic redox processes is glutathione (GSH), trypanosomatidae rely on a glutathione–spermidine conjugate, N^1N^8-bis-(glutathionyl)spermidine named dihydrotrypanothione [T(SH)$_2$] [1]. Trypanothione [T(S)$_2$] is maintained in its reduced form by trypanothione reductase (TR; EC 1.6.4.8.), an NADPH-dependent flavoprotein unique to trypanosomatidae and closely related to its counterpart glutathione reductase (GR; EC 1.6.4.2.) [2]. The bifunctional structure of T(SH)$_2$ confers on this compound a greater reactivity than glutathione in thiol–disulphide exchange reactions [3]. In the same study, the non-enzymic formation of S-conjugates was shown to be easier for T(SH)$_2$ than for GSH. However, the existence of a trypanothione transferase catalysing the formation of trypanothione S-conjugates has already been detected in trypanosomatidae [4].

Although TR does not reduce glutathione disulphide (GSSG) and GR is absent from these organisms, significant quantities of GSH are found and are evaluated to be about 20 % of total thiol [1]. The existence of an enzymic activity related to glutathione, which could be different from those implicated in the biosynthesis of T(S)$_2$, might account for such an amount of GSH. In particular, it has been demonstrated that glutathione is an important factor in the resistance of *Trypanosoma cruzi* to drugs [5]. To investigate the possibility that trypanosomatidae could rely on GSH, we turned our attention on glutathione-dependent proteins such as glutathione transferases (GST; EC 2.5.1.18.). Several reports have described the purification of GSTs from different origins by affinity chromatography methods, using glutathione or *S*-hexyl-glutathione as ligands [6,7]. Recently, three *T. cruzi* glutathione-binding proteins have been isolated using a glutathione–agarose matrix [8]. However, sequencing of cDNA clones corresponding to these proteins revealed that they were homologous to mammalian elongation factor subunits γ and β [9,10]. These observations raised an important question about the validity of the glutathione–agarose matrix as a support to isolate parasite proteins which specifically interact with glutathione. Therefore, we chose the *S*-hexyl glutathione matrix to perform affinity chromatography. In order to investigate possible relationships between glutathione and T(S)$_2$ pathways into parasites, we also developed affinity chromatography using a non-reducible analogue of trypanothione disulphide as a ligand (the djenkolic derivative described previously [11]). Starting from a cytosolic fraction of *T. cruzi* epimastigotes, a similar protein of 52 kDa was eluted from the two types of affinity columns. Its partial amino acid sequence indicated that it was identical with the protein encoded by the TcAc2 cDNA previously described [12]. Although TcAc2 showed some homology with genes of various stress proteins and some glutathione transferases, our protein did not possess significant glutathione or trypanothione transferase activity. However, a new and unexpected enzymic activity, using both T(SH)$_2$ and GSSG, was observed and characterized (see Scheme 1).



Scheme 1 Djenkolic analogue of trypanothione disulphide

Abbreviations used: T(SH)$_2$, dihydrotrypanothione; T(S)$_2$, trypanothione; BNPS-skatole, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole; PVDF, polyvinylidene difluoride; CDNB, 1-chloro-2,4-dinitrobenzene; ENP, 1,2-epoxy(3-*p*-nitrophenoxy)propane; TFA, trifluoroacetic acid; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; TR, trypanothione reductase; GR, glutathione reductase.

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EXPERIMENTAL

Materials

S-Hexylglutathione and *S*-hexylglutathione–agarose were obtained from Sigma. The djenkolic analogue of trypanothione disulphide [*NN'*-bis(benzyloxycarbonyl)-L-djenkolic-glycyl-N^8 spermidine] was synthesized as described [11]. The ligand was attached by the amine group of the spermidine to the activated CH-Sepharose 4B from Pharmacia. Pre-stained protein molecular-mass standards for SDS/PAGE were from GIBCO-BRL. Sephadex G-100 (Superfine) and the calibration kit with standard proteins for gel filtration were obtained from Pharmacia-LKB. $T(SH)_2$ was synthesized as described [13]. GSSG was purchased from Aldrich. A double-beam Uvikon 930 spectrophotometer (Kontron Instruments) was used for spectrophotometric measurements. For sequencing, sequencing-grade trypsin was obtained from Boehringer Mannheim, poly-(vinylidene difluoride) (PVDF) membrane from Millipore, formic acid from Merck, CNBr from Aldrich, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole (BNPS-skatole) from Pierce and *N*-acetylated tyrosine from Rexim. Antibodies against two synthetic peptides from the TcAc2 sequence coupled to ovalbumin (Ps1: P^{41}QWYKELNPRETVPTLQVDG^{60} and Ps2: I^{65}ESDLISRYIDRISSP^{80}), against a fusion protein between TcAc2 and the 26 kDa GST from *Schistosoma japonicum* (TcAc2-SjGST26), against ovalbumin, and against the GST from *S. japonicum* (SjGST26) were all raised in mice as described previously [12]. GR from bovine intestinal mucosa was from Sigma. TR from *T. cruzi* was isolated from the SG5 *Escherichia coli* strain carrying the overproducing expression vector pIBITczTR as described previously [14].

Parasite culture

The Y strain of *T. cruzi* was used [15]. Epimastigotes were grown at 28 °C in GLSH medium (0.1% glucose/0.45% hydrolysed lactalbumin/15% bovine haemoglobin extract) supplemented with 10% decomplemented fetal calf serum (Boehringer) [16].

Protein purification

All the following steps were carried out at 4 °C. Two litres of 5-day-culture epimastigotes were harvested from the culture medium by centrifugation at 2000 ***g*** for 20 min and washed three times in Hanks–Wallace balanced salt solution. The pellet was frozen in liquid nitrogen and thawed twice. It was then sonicated for 6 min at 150 W and 0.5 s intervals in the column buffer [20 mM Hepes (pH 7.25)/1 mM EDTA/0.15 M KCl] supplemented with phenylmethanesulphonyl fluoride to a 0.5 mM final concentration. The homogenate was clarified by centrifugation at 105000 ***g*** for 1 h. The supernatant was diluted to obtain a protein concentration of 2 mg/ml. It was then loaded onto a 5 ml *S*-hexylglutathione–agarose column (Sigma) previously equilibrated with the column buffer for one night. After extensive washing with 300 ml of the same buffer, elution was performed using 20 ml of 2.5 mM *S*-hexylglutathione in the column buffer. Protein concentration was determined by the method of Bradford [17] with BSA as a standard (Bio-Rad). The eluate was concentrated by centrifugation through an Amicon Centriprep 10 concentrator to a final concentration of approx. 300 μg/ml. SDS/PAGE was carried out as described by Laemmli [18], using a 13% polyacrylamide gel, and proteins were visualized by silver staining.

Partial sequencing

Digestion of P52 with trypsin and isolation of tryptic fragments

Denatured P52 (500 pmol) was digested for 24 h in 20 mM Hepes (pH 7.25)/0.15 M KCl/1 mM EDTA using an enzyme ratio of approx. 1:20 (w/w). The tryptic peptides were purified by reverse-phase HPLC (Applied Biosystems 130A HPLC system, Vydac 200HSB C_{18} column, 25 × 0.1 cm) at 30 °C using 0.50% trifluoroacetic acid (TFA) in water (A) and 0.45% TFA in 75% acetonitrile (B) with a flow rate of 50 μl/min. A non-linear gradient was performed (4.5% B, 20 min; from 4.5% B to reach 40% B, 140 min; 40% B, 10 min; from 40% B to reach 60% B, 40 min; 60% B, 10 min; from 60% B to reach 75% B, 10 min).

Specific chemical cleavages of electroblotted P52

After SDS/PAGE, the P52 spots electroblotted onto PVDF membrane [19] were chemically cleaved. A first spot was treated with 70% formic acid for 72 h at 37 °C [20] and a second one by CNBr vapours (15 mg of CNBr in 30 μl of 70% TFA [21]). Finally, a third spot was treated with a 100-fold excess of BNPS-skatole and *N*-acetylated tyrosine in 75% acetic acid for 68 h at room temperature in the dark [22].

Amino acid sequence analysis

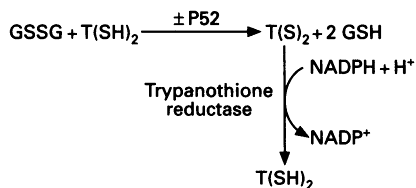
Automatic Edman degradation of peptides generated by tryptic proteolysis and chemical cleavages of electroblotted P52 were performed using an Applied Biosystems 470A sequencer equipped with an on-line HPLC phenylthiohydantoin-amino acid analyser, a data acquisition unit and a processing unit.

Western blotting

Proteins were subjected to SDS/PAGE (13% acrylamide) before electrotransfer to nitrocellulose in a Bio-Rad Transblot apparatus. For immunodetection, nitrocellulose paper was probed with the antisera raised against the different antigens and the immunocomplexes were detected with anti-(mouse IgG) antibody conjugated to horseradish peroxidase (Pasteur Diagnostics, France). The immunoblots were developed with 3.4 mM 4-chloro-1-naphthol and 0.03% hydrogen peroxide in 20% ethanol (v/v).

Expression of P52 according to the pH of the medium

Cultures of epimastigotes were sown at 1×10^6 parasites/ml. On day 1, HCl was added to obtain the desired pH in the medium. On day 2, epimastigotes were harvested as described previously and washed three times in Hanks–Wallace solution. Parasites were then stirred overnight at 4 °C in a lysis buffer [10 mM Tris/1% Nonidet P.40 (NP40)/2 mM EDTA/5 mM iodoacetamine/1 mM α-toluenesulphonyl fluoride/10 μg/ml L-1-*p*-tosylamino-2-phenylethylchloromethyl ketone/100 units/ml aprotinin). After centrifugation at 2000 ***g***, protein concentrations were determined according to Bradford [17]. Amounts of P52 in lysates were evaluated by enzyme-linked immunosorbent assay (ELISA). Microtitration plates were coated overnight at 4 °C with 2 μg of lysate proteins in 0.05 M carbonate–bicarbonate buffer, pH 9.6. After saturation with 1% casein in PBS (w/v) for 1 h at room temperature, 200 μl of diluted anti-TcAc2 sera (1:50, 1:100, 1:200 and 1:1000) were added into wells for 2 h at 37 °C. After



Scheme 2 Trypanothione-glutathione thioltransferase assay

three washes with PBS-Tween 1‰ (w/v), wells were incubated at 37 °C for 2 h with 200 μl of peroxidase conjugated anti-(rabbit IgG) (Diagnostics Pasteur, France) diluted to 1:5000 in PBS-casein. After three washes with PBS-Tween, the presence of antibodies was detected with 200 μl of 0.4 mg/ml *o*-phenylenediamine, 0.8‰ H_2O_2 diluted in 0.1 M citrate–phosphate buffer, pH 5.5, for 15 min. The enzymic reaction was stopped with 50 μl of 4 M H_2SO_4. The absorbance was read at 490 nm using an MR 600 microplate reader (Dynatech).

Gel filtration of native P52

Purified P52 was applied to a Sephadex G-100 column (24 × 0.4 cm) equilibrated with 0.1 M Tris, pH 8/1 M NaCl. The following proteins of the low-molecular-mass calibration kit (Pharmacia) were used as standards: ribonuclease A (bovine pancreas, 13.7 kDa), chymotrypsinogen A (bovine pancreas, 25 kDa), ovalbumin (hen egg, 43 kDa), BSA (67 kDa). In addition, native GR from bovine intestinal mucosa (104 kDa) was used as a control for homodimerization. Proteins were eluted with the buffer used for the equilibration at a flow rate of 0.01 ml/min.

Enzyme assays

Transferase assay

The transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically at 340 nm with a final volume of 500 μl of 0.1 M sodium phosphate, pH 6.5, at 28 °C. The concentrations of GSH or $T(SH)_2$ and CDNB were 1 mM. A molar absorption coefficient of 9600 $M^{-1} \cdot cm^{-1}$ was used [23].

The GST activity with 1,2-epoxy(3-*p*-nitrophenoxy)propane (ENP) was measured by spectrophotometry at 360 nm using the phosphate buffer described above. The concentrations of GSH and ENP were 10 mM and 0.5 mM respectively. The molar absorption coefficient used was 510 $M^{-1} \cdot cm^{-1}$ [24].

Trypanothione–glutathione thioltransferase assay

The different steps were performed at 28 °C, using 500 μl microcuvettes and the thermostated double-beam spectrophotometer described above. The activity of P52 was measured using $T(SH)_2$ and GSSG as substrates. The enzymic assay buffer was 20 mM Hepes (pH 7.25), 0.15 M KCl and 1 mM EDTA. First, P52 (3 μg/ml) was mixed with GSSG diluted in the buffer and $T(SH)_2$ was then added to start the enzymic reaction. $T(S)_2$ produced during the linear phase was titrated by enzymic reduction with the TR (0.8 μg/ml) from *T. cruzi* (Scheme 2) and 300 μM NADPH as cofactor, as described by Krauth-Siegel et al. [2]. Initial velocities of $T(S)_2$ reduction were measured following NADPH oxidation by the absorbance decrease at 340 nm, and enabled us to determine the initial $T(S)_2$ concentration using a standard curve with known concentrations of this compound.

RESULTS

Purification of P52

The supernatant obtained from a culture of 2.5×10^{10} parasites by centrifugation at 105000 ***g*** contained 95 mg of proteins. It was allowed to pass through the affinity gels. As shown by SDS/PAGE and silver staining (Figure 1), a unique protein was obtained in one step from the *S*-hexylglutathione–agarose column using 2.5 mM *S*-hexylglutathione as eluent. The overall yield for that protein was 0.2 % of the proteins obtained in the supernatant (200 μg). The molecular-mass markers indicated that under denaturing conditions, the molecular mass of this protein was approx. 52 kDa. In the case of the $T(S)_2$ analogue column, the elution performed with 4.5 mM Djenkolic derivative gave two proteins of 52 and 54 kDa respectively, with a total yield of < 0.01 %.

Partial sequencing of P52

Partial sequencing of P52, the protein eluted from *S*-hexylglutathione–agarose, was performed. Direct sequencing was not possible, indicating that P52 was N-terminal blocked. Using enzymic and specific chemical methods, the sequences of five tryptic peptides and five peptides generated by CNBr, BNPS-skatole and Asp–Pro bond cleavages were determined. Comparison of the sequences obtained from the tryptic fragments with other proteins available in databases revealed 98 % identity with the protein encoded by TcAc2, a cDNA of *T. cruzi* showing homology with some GSTs [12]. All the peptides which were sequenced could be localized in the deduced amino acid sequence from the TcAc2 gene. These results showed that, out of 151 residues identified, only four were different from the sequence deduced from TcAc2. Indeed, Val^{113}, Thr^{275}, Thr^{278} and Leu^{428} in the TcAc2 sequence were replaced by Ala, Ala, Ala and Phe respectively (Figure 2). In all cases, the mutation could be justified by a single base change in the corresponding codon. No amino acid between Asn^{127} and Thr^{129} could be identified by Edman degradation.

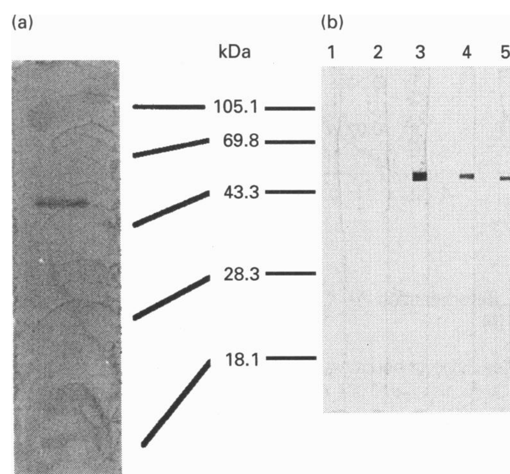


Figure 1 Detection of P52 and immunoblotting

(**a**) P52 was subjected to SDS/PAGE and revealed by silver-staining. (**b**) Lanes 1 and 2, immunoblotting with antibodies raised against ovalbumin and SjGST26 respectively. Lane 3, immunoblotting with antibody raised against the fused protein TcAc2–SjGST26; lanes 4 and 5, antibodies raised against Ps1 and Ps2 respectively, two synthetic peptides derived from the TcAc2 sequence.

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TcAc2   1  MKALKLFKDR LCPFCQRVLI TAKEKRVTLE EVEVPLGDDM PQWYKELNPR
p52
               CNBr                        Trypsin-1        BNPS-
TcAc2  51  ETVPTLQVDG KKCMIESDLI SRYIDRISSP ANALMGSSPY QRHRVEFFLG
p52
           skatole 1
TcAc2 101  EIGDLVKAYF GLVRDPFNEE KRKSVDNNTA YIEDIIAEHQ GDGPYFLDDT
p52                  A
               Trypsin-2      HCOOH cleavage
TcAc2 151  FSMAEVMVVP FLACFRPVLS YYCGYDIFHN APRLKKMYVT SMQRTTVKET
p52

TcAc2 201  ISKPEEYIIG FKSKVPKSHV TWSLAPGYVL FVNKYSPFSD RPRLACALKN
p52
                          Trypsin-3   BNPS-skatole 2
TcAc2 251  IDLPMLEIDL KQLPPWFRWF NQRETVPTLL TPQGTYVHES QLIVHYLDDG
p52                                 A  A
                                Trypsin-4
TcAc2 301  FPEHGPALLP KDADGSYHVR FVESNVDYFM DAMYSFIKDP KNMNAKEEFD
p52

TcAc2 351  WAAGELEKLL AEHQFGEGPF FGGATMNAAD VSLVPMLVHL KACTPELTEG
p52
              BNPS-skatole 3
TcAc2 401  QDLLANYKLL AAAAEAGLTS EAGKKVFLSL SEYSSIYKTF LRPSS
p52                                     F
                                      Trypsin-5
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Figure 2 Sequencing of P52

Partial amino acid sequence of P52 compared with the amino acid sequence deduced from the TcAc2 gene. The sequences determined by microsequencing are underlined. All of them are localized in the deduced amino acid sequence of the TcAc2 gene. Methods to generate fragments of P52 are designated by Trypsin-1–5, BNPS-skatole 1–3, CNBr and HCOOH cleavage.

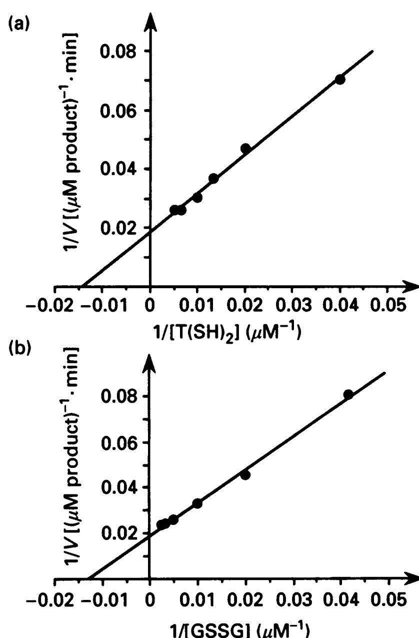

Figure 3 Determination of K_m and $V_{max.}$ for $T(SH)_2$ (a) and glutathione disulphide (b)

The $T(S)_2$ formed during the reaction was quantified by reduction with TR. Enzymic assays were performed at 28 °C, using a 20 mM Hepes buffer, pH 7.25, 0.15 M KCl and 1 mM EDTA. Enzyme concentrations in P52 and TR were 3 and 0.8 μg/ml respectively. The kinetic parameters for $T(SH)_2$ and GSSG were determined in the presence of 200 μM GSSG and 200 μM $T(SH)_2$ respectively. (**a**) K_m, 67 μM; $V_{max.}$, 53 μM · min^{-1}. (**b**) K_m, 76 μM; $V_{max.}$, 53 μM · min^{-1}.

Immunological studies of relationships betwwen P52 and TcAc2 gene product

In order to further confirm that P52 was identical with the TcAc2 gene product, we checked whether these two proteins showed an immuno-cross-reactivity. By immunoblotting, P52 was revealed by antibodies against the fused protein TcAc2–SjGST26 (Figure 1b, lane 3) and by antibodies raised against Ps1 and Ps2, two synthetic peptides derived from the TcAc2 sequence given in the Experimental section (Figure 1, lanes 4 and 5). Neither antisera obtained by immunization with ovalbumin alone (Figure 1, lane 1) or SjGST26 alone (Figure 1, lane 2) nor pre-immune sera or control sera (adjuvant alone) were able to reveal P52. In addition, P52 was not detected by antibodies against TR [25] (results not shown). Immunoblotting also enabled the proteins eluted from the $T(S)_2$ analogue column to be identified. The 54 kDa protein was identified both by immunoblotting and enzymic activity as being TR, the second (52 kDa) as being TcAc2.

We further examined the expression of P52 in relation to the pH of the culture medium. Amounts of P52 in lysates were evaluated by ELISA (results not shown). The best results were obtained with anti-TcAc2 diluted 1:100. The expression of P52 was maximal at pH 4.2 and decreased when the pH of the culture medium increased. At pH 8, the level of P52 represented only 65% of the quantity measured at pH 4.2. A control test using immunoblotting confirmed the results obtained.

Furthermore, gel filtration of native P52 through a Sephadex G-100 column performed under non-denaturing conditions showed that this protein is a homodimer. Indeed, using molecular-mass standards and GR as a control of homodimerization, its elution pattern was identical with that of GR, indicating a molecular mass of 104 kDa.

Characterization of a P52 dependent-trypanothione–glutathione thioltransferase activity

Transferase assays

Using CDNB and ENP, no glutathione transferase activity was found. The rate of the conjugation reaction between $T(SH)_2$ and CDNB was increased in the presence of P52. However, this rate increase remained very limited: the addition of 3 μg of P52 only increased 1.5-fold the non-enzymic reaction rate. Moreover, no kinetic parameters could be determined.

Trypanothione–glutathione thioltransferase activity

The trypanothione–glutathione thioltransferase activity of P52 was assayed. P52 was able to reduce GSSG using $T(SH)_2$ following typical Michaelis–Menten kinetics. The kinetic parameters for $T(SH)_2$ in the presence of 200 mM GSSG (K_m, 67±2 mM; $k_{cat.}$, 30.6 s^{-1}) and for GSSG in the presence of 200 mM $T(SH)_2$ (K_m, 76±4 mM; $k_{cat.}$ 31 s^{-1}) were determined (Figures 3a and 3b respectively). The ratio $k_{cat.}/K_m$ for $T(SH)_2$ and GSSG were 4.57×10^5 and 4.03×10^5 M$^{-1}\cdot$s^{-1} respectively. Both values showed that P52 was relatively efficient at catalysing this redox reaction. The reaction was inhibited in the presence of *S*-hexylglutathione. In the presence of an excess of GSSG (200 μM), at 70 μM $T(SH)_2$ an IC_{50} of 450 μM was found. In the presence of an excess of $T(SH)_2$ (280 μM), at 50 μM GSSG we found an IC_{50} of 115 μM.

P52 did not catalyse the reverse reaction, i.e. the reduction of $T(S)_2$ by GSH. It was also unable to catalyse the reduction of vasopressin by $T(SH)_2$.

DISCUSSION

The aim of this work was to investigate whether some enzymes in trypanosomatidae could use glutathione as a substrate. One hypothesis was that a part of the metabolism of trypanosomatidae

could be based on glutathione, which would explain the significant amount of this compound present in these parasites. The other hypothesis was the existence of enzymes which could use both glutathione and $T(S)_2$ as a cofactor, unlike TR which exhibits a strict substrate specificity.

P52, the TcAc2 gene product, was the only protein which could be detected in the retained fractions of both affinity columns. It has been previously shown that TcAc2 consists of two structurally homologous domains, indicating that the protein could have evolved from a single domain by a process of gene duplication and fusion. Moreover, both domains shared significant homologies with a number of small stress proteins and with GSTs mainly from the θ class [12]. Cytosolic GSTs have a monomer size of 24–28 kDa. They usually exist as either homo- or hetero-dimer complexes of about 50 kDa due to the non-covalent association of two monomers in an inverted orientation [26]. Therefore, our initial hypothesis was that P52 could represent a new class of GSTs in which the two domains were covalently linked as a single-chain protein. The protein would play the same role as the usual non-covalent dimeric complex. However, using CDNB, the most polyvalent substrate for the different classes of GSTs, and ENP, a substrate for the θ class of GSTs [24], we were not able to demonstrate any significant GR or TR activity.

The determination of the molecular mass of native P52 under non-denaturing conditions indicated a mass of 104 kDa, which was not in the usual range of GST masses but could correspond to a homodimeric complex. This led us to suppose that, after gene duplication, the two domains of the protein could have evolved towards different binding specificities, one retaining the capacity to bind glutathione, the second evolving to bind $T(S)_2$. The covalent link between the two domains might allow accurate positioning of the two substrates. Following this new hypothesis, the possibility that P52 could catalyse the thiol–disulphide exchange between $T(SH)_2$ and GSSG was envisaged. Until now, this reaction was thought to happen non-enzymically. Indeed, trypanosomatidae do not possess GR to regenerate GSH from its oxidized form whereas GSH is at least involved in the biosynthesis of $T(S)_2$. Thus, they depend on $T(SH)_2$, and consequently on TR to maintain glutathione in its reduced form. Although the thiol groups of $T(SH)_2$ are fairly reactive [3] themselves in a non-enzymic thiol–disulphide exchange with GSSG, P52 was able to catalyse with efficiency this reaction between the two compounds. The turnover for both substrates was very close and suggested that 1 mol of $T(SH)_2$ was used to reduce 1 mol of GSSG. In addition, the kinetic parameters of P52 for $T(S)_2$ were of the same order as those of TR, the only kinetically characterized enzyme using $T(S)_2$ (K_m, 51 μM; $k_{cat.}$, 225 s^{-1} and $k_{cat.}/K_m$, 4.69×10^6 $M^{-1} \cdot s^{-1}$). Several other observations argued for the specificity of this activity: (1) the reaction was inhibited by *S*-hexylglutathione; (2) the reverse reaction, the reduction of $T(S)_2$ by GSH, did not occur under our conditions; and (3) no activity was observed using another disulphide-containing peptide such as vasopressin instead of GSSG.

Our results showed that P52 expression was increased as the pH of the medium for the culture of parasites decreased: the acidity of the medium induced production of the protein. It has previously been shown that the expression of the TcAc2 gene product was effectively increased as epimastigotes entered the stationary phase [12]. This might also be correlated with the pH of the medium during the stationary phase, which is approximatively equal to 6. Induction of proteins homologous to P52 includes such different stresses as heat shock, plant hormones, oxidative stress and infection by pathogens. Their common feature is involvement in the stress response. Acidification of the environment of the parasite also constitutes a factor of stress and may induce various behaviours [12]. This suggests a role of P52 in protection mechanisms against stress.

In conclusion, we have described for the first time the isolation and the characterization of a new trypanothione–glutathione thioltransferase activity. It may constitute a link between the glutathione-based metabolism of the host and the $T(S)_2$-based metabolism of the parasite and thus provides a new insight into the metabolism of the parasite. In *T. cruzi*, it can complement downstream the TR to fight against oxidative stress by generating thiol scavengers. It can also complement upstream the metabolism of $T(S)_2$ by maintaining glutathione in a reduced state. Reduced glutathione is actually the substrate of glutathionylspermidine synthetase which catalyses the first step of $T(S)_2$ biosynthesis [27]. In addition to TR, it provides a new target for a specific chemotherapy against Chagas' disease.

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