Evidence for the co-existence of glutathione reductase and trypanothione reductase in the non-trypanosomatid Euglenozoa: *Euglena gracilis Z.*

Françoise Montrichard[a,*], Fabienne Le Guen[a], Danielle L. Laval-Martin[a], Elisabeth Davioud-Charvet[b]

[a] *G.B.B.M.V.-EA917, UFR Sciences, 2 boulevard Lavoisier, 49045 Angers, France*
[b] *Institut de Biologie-Institut Pasteur de Lille, URA CNRS 1309, Université Lille II, 1 rue du Pr. Calmette, BP 447, 59021 Lille cedex, France*

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Abstract Two NADPH-dependent disulfide reductases, glutathione reductase and trypanothione reductase, were shown to be present in *Euglena gracilis*, purified to homogeneity and characterized. The glutathione reductase (M_r 50 kDa) displays a high specificity towards glutathione disulfide with a K_M of 54 μM. The amino acid sequences of two peptides derived from the trypanothione reductase (M_r 54 kDa) show a high level of identity (81% and 64%) with sequences of trypanothione reductases from trypanosomatids. The trypanothione reductase is able to efficiently reduce trypanothione disulfide (K_M 30.5 μM) and glutathionylspermidine disulfide (K_M 90.6 μM) but not glutathione disulfide, nor *Escherichia coli* thioredoxin disulfide, nor 5,5′-dithiobis(2-nitrobenzoate) (DTNB). These results demonstrate for the first time (i) the existence of trypanothione reductase in a non-trypanosomatid organism and (ii) the co-existence of trypanothione reductase and glutathione reductase in *E. gracilis*.

Key words: Trypanothione reductase; Glutathione reductase; Thioredoxin reductase; Trypanosomatid; *Euglena gracilis*

1. Introduction

Euglena gracilis is a phytoflagellated protozoan which belongs to the euglenoid subphyllum of Euglenozoa [1]. *E. gracilis* has been reported to possess the two classical redox systems based on glutathione/glutathione reductase (EC 1.6.4.2, GR) and thioredoxin/thioredoxin reductase (EC 1.6.4.5, TrxR) to maintain an intracellular reducing environment [2,3]. The other subphyllum of Euglenozoa is constituted by protozoan parasites: the kinetoplastida. Among them, the trypanosomatids differ from all known eucaryotic and procaryotic organisms in their specific redox system based on a glutathione-spermidine conjugate, the N^1-N^8-bis-(glutathionyl)spermidine (trivially named trypanothione) and an NADPH-dependent trypanothione reductase (EC 1.6.4.8, TR) [4]. The TR replaces the GR which is absent from these parasites. Moreover, as TrxR has not been detected yet in trypanosomatids, the TR system was suggested to substitute for the TrxR system found in most other cells (see for a review [5]). TRs, GRs and TrxRs are related enzymes which belong to the same family of dimeric flavoprotein disulfide oxidoreductases that also includes lipoamide dehydrogenases and mercuric ion reductases [6–12].

During the purification of NAD^+ kinase from *Euglena gracilis* Z. (manuscript in preparation), a 54-kDa contaminant protein was co-purified with NAD^+ kinase at all steps of the purification procedure. The sequences of two peptides derived from this protein, compared with sequences from data banks, were shown to be very similar to sequences of TRs from trypanosomatids. In view of the phylogenetic relationship between euglenoids and kinetoplastida, it was of interest to study the pattern of disulfide reductases of *E. gracilis*. In the present study, we report the purification and characterization of two NADPH-dependent disulfide reductases from *E. gracilis*, the previously reported glutathione reductase, and the unexpected trypanothione reductase.

2. Materials and methods

2.1. Chemicals and enzyme assays

All products were purchased from Sigma except trypanothione disulfide ($T(S)_2$) and glutathionylspermidine disulfide ($Gspd(S)_2$) which were purchased from Bachem. GR, TR, TrxR activities were assayed as described in [2], [13] and [14], respectively. One unit of TR or GR activity corresponds to 1 μmol of $T(S)_2$ or glutathione disulfide (GSSG) reduced per min. Pure enzymes (as judged from a silver stained SDS-PAGE) were used for the determination of kinetic constants. Protein contents were determined [15] with bovine serum albumin as a standard.

2.2. Cell culture and soluble extract preparation

E. gracilis Z. cells were grown in darkness in the presence of 2 mM $AlCl_3$ as previously described [16]. After 7 days, when the culture reached the stationary phase of growth, cells were harvested by centrifugation (500 × *g*, 5 min, 4°C). They were then disrupted by freezing at −20°C, thawing and subsequent homogenization by 10 strokes in a Potter homogenizer in the following extraction buffer: 10 mM Tris-HCl, pH 7.5, 5 mM EDTA and 5 mM 2-mercaptoethanol (5 ml per g of cells). Finally, the cell homogenate was centrifuged (40 000 × *g*, 120 min, 4°C) and the resulting soluble extract was either immediately used or stored at −20°C.

2.3. Purification of GR and TR

For GR and TR purification, 650 ml of soluble extract was first applied on a 10-ml 2′,5′-ADP-agarose column (Sigma) previously equilibrated with extraction buffer. After a 50-ml wash with the same buffer, bound proteins, among them GR and TR, were eluted by extraction buffer containing 0.5 M NaCl. Proteins were then 6-fold concentrated by precipitation with ammonium sulfate at 50% saturation, supplemented with 0.005% bromophenol blue, and submitted to a preparative electrophoresis in a polyacrylamide gel cast in the Prep Cell apparatus from Bio-Rad (500 μg of proteins were loaded per cm^2). The gel was composed of a separating gel (10% acrylamide/0.27% bisacrylamide in 175 mM Tris-HCl, pH 8.5 and 10% glycerol)

*Corresponding author. Fax: (33) (2) 41 733 352.
E-mail: francoise.montrichard@univ-angers.fr

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); GR, glutathione reductase; GSSG, glutathione disulfide; $Gspd(S)_2$, glutathionylspermidine disulfide; TR, trypanothione reductase; $Trx(S)_2$, thioredoxin disulfide; TrxR, thioredoxin reductase; $T(S)_2$, trypanothione disulfide

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Peptide 1	*E. gracilis Z*	1	K	M	G	A	T	I	R	D	F	Y	T	T	I	G	V	H	16	References
TR	*C.fasciculata*	446	.	.	.	.	K	.	S	.	.	.	N	.	.	.	.	.	.	461	[8]
	L.donovani	446	.	.	.	.	K	.	S	.	.	.	H	S	.	.	.	.	.	461	[11]
	T.brucei	446	R	L	N	.	K	.	S	.	.	.	N	.	.	.	.	.	.	461	[8]
	T.congolense	446	.	L	N	.	K	.	S	.	.	.	N	.	.	.	.	.	.	461	[6]
	T.cruzi	445	.	L	N	.	K	.	S	.	V	.	N	.	.	.	.	.	.	460	[7]
GR	*E.coli*	424	.	.	.	.	.	K	K	.	.	D	N	.	V	A	I	.		439	[20]
	M.musculus	440	.	.	.	.	.	K	A	.	.	D	N	.	V	A	I	.		455	[21]
	Human	452	.	.	.	.	.	K	A	.	.	D	N	.	V	A	I	.		467	[22]

Peptide 2	*E. gracilis Z*	1	K	V	V	T	D	H	G	T	G	Y	V	L	G	V	14	References
TR	*C.fasciculata*	414	R	I	.	.	N	.	A	D	.	E	.	.	.	.	427	[8]
	L.donovani	414	R	I	I	.	N	E	S	N	.	E	.	.	.	.	427	[11]
	T.brucei	414	.	I	.	.	N	.	S	D	.	T	.	.	.	.	427	[8]
	T.congolense	414	.	I	I	.	.	.	.	D	.	T	.	V	.	.	427	[6]
	T.cruzi	406	.	I	I	.	N	.	S	D	.	T	.	.	.	.	419	[7]
GR	*E.coli*	392	.	L	.	C	V	G	S	E	E	K	I	V	.	I	405	[20]
	M.musculus	408	.	M	.	C	A	N	K	E	E	K	.	V	.	I	421	[21]
	Human	420	.	M	.	C	A	N	K	E	E	K	.	V	.	I	433	[22]

Fig. 1. Sequence alignments of the peptides 1 and 2, derived from the *E. gracilis* 54-kDa protein, with regions of the sequences of TR and GR. Identical residues are indicated by (·).

and a stacking gel (4.5% acrylamide/0.12% bisacrylamide in 133 mM Tris-HCl, pH 6.8) to which 0.17% TEMED and 0.13% ammonium persulfate were added for polymerization. Electrophoresis was conducted in a 25 mM Tris, 192 mM glycine, pH 8.6, electrophoresis buffer at 20 mA. The elution was performed at a 0.25 ml/min flow rate with electrophoresis buffer. Collection of 1.5 ml fractions started after the dye front had reached the bottom of the gel, and were carried on until the elution of $\mu > 0.30$ proteins was achieved. TR and GR activities were assayed and protein contents determined in all the collected fractions. Finally, fractions displaying TR activity were pooled and submitted to anion exchange chromatography on a Resource-Q column (1 ml, Pharmacia) previously equilibrated with extraction buffer. After the column was washed with 25 ml of the same buffer, bound proteins were eluted by a 100-ml linear gradient of NaCl (0–1 M) at a 0.3-ml/min flow rate, and 1-ml fractions were collected. TR was eluted at about 0.37 M NaCl.

2.4. Analytical polyacrylamide gels and Western blotting

For the detection of TR and GR activities in gels, proteins (500 μg per cm^2) were separated by native-PAGE performed as above, but within slab gels instead of preparative gels. After electrophoresis, TR and GR activities were revealed in the gels as described in [17] in the presence of 1 mM $T(S)_2$ or 4 mM GSSG, respectively.

For protein detection or Western blotting, proteins (1–5 μg per well) were resolved by 10% SDS-PAGE according to Laemmli [18], then either stained in the gels with silver or Coomassie blue R250, or electroblotted onto PVDF membranes. The membranes were probed with a 1:50 dilution of antibodies raised against either the peptide 131–152 or the peptide 249–275 of TR of *Trypanosoma cruzi* [19] using the peroxidase method.

2.5. Internal peptide sequence determinations

The determination of the N-terminal amino acid sequences of two internal peptides derived from the 54-kDa protein (TR), after proteinase K hydrolysis, were performed at the 'Microséquençage des protéines' Laboratory (Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France).

3. Results

3.1. Evidence for both TR and GR activities in E. gracilis

The N-terminal amino acid sequences of two internal peptides derived from the 54-kDa protein which was purified with the NAD^+ kinase-enriched fraction were determined. Both peptides showed high similarity with sequences of TRs from the trypanosomatids *Crithidia fasciculata*, *Leishmania donovani* and *Trypanosoma* species (Fig. 1; see also [20–22]). For peptide 1, the highest score was 81% identity with the K446–H461 sequence of TR from *C. fasciculata* and, for peptide 2, 64% identity with the K414–V427 sequence of TR from *T. brucei* or *T. congolense*. Peptides 1 and 2 also displayed homologies, albeit to a lesser extent (56% and 14–21% identity, respectively), with regions of GRs from *E. coli*, mouse and man (Fig. 1).

To test whether the 54-kDa protein of *E. gracilis* corresponded to TR or GR, soluble proteins were separated by

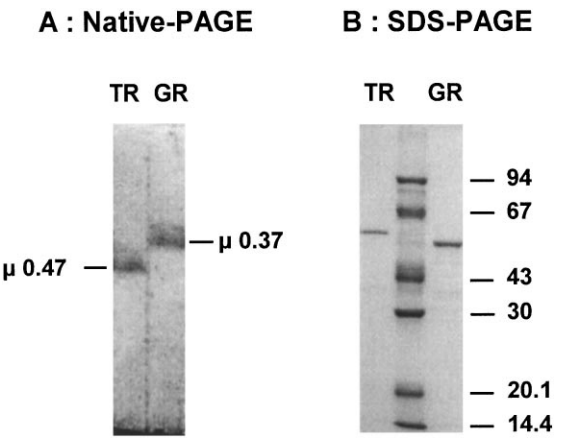

Fig. 2. TR and GR in native- and SDS-PAGE. A: Proteins from soluble extracts were separated by a 10% native-PAGE. Then, the gel was cut into two lanes, which were stained for either TR or GR activity by incubation in mixtures containing DTNB, NADPH and either $T(S)_2$ or GSSG, respectively. B: TR fraction from anion exchange chromatography (1 μg) and GR fraction from preparative electrophoresis (2.5 μg) were submitted to a 10% SDS-PAGE and the gel was subsequently stained with Coomassie blue. The M_rs of TR and GR were then determined by comparison with those of standard proteins (central lane) indicated in kDa at the right of the figure.

Table 1
Purification of GR and TR from 150 g of *E. gracilis* cells

	GR				TR			
	Total activity	Total protein	Specific activity	Purification factor	Total activity	Total protein	Specific activity	Purification factor
Soluble proteins	110	448	0.25	1	30	448	0.067	1
2′,5′-ADP-agarose	106	2.65	40	163	28	2.65	10.6	158
Native electrophoresis	91	0.24	379	1544	11	0.28	89.9	695
Resource-Q	–	–	–	–	7.4	0.03	272	4091

The total activities are expressed in U, total protein in mg, and specific activity in U mg^{-1}.

native-PAGE and two lanes of the gel were stained for either TR or GR activity (Fig. 2A). Only a single yellow band was revealed in each lane after 10 min of reaction, bands still corresponding to different electrophoretic mobilities: $\mu = 0.47$ with $T(S)_2$ or $\mu = 0.37$ with GSSG. These results demonstrated the presence of two disulfide reductases in *E. gracilis*: the previously reported GR [2] and TR. It is noteworthy that no protein was able to directly reduce 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and that TR activity was not revealed in the mixture containing GSSG, nor GR activity in the mixture containing $T(S)_2$.

3.2. Purification of GR and TR from E. gracilis

The purification of GR and TR from soluble extract is summarized in Table 1. In the soluble extract, the specific GR activity (0.246 U mg^{-1}) is nearly 4-fold higher than that of TR (0.067 U mg^{-1}). Fig. 3 shows that TR and GR were resolved during the step of preparative electrophoresis. TR was first eluted from the preparative gel (fractions 48–60) and was clearly separated from GR which was eluted later (fractions 70–98). Peaks of TR and GR activities exactly coincided with two consecutive peaks of proteins eluted from the gel. Mutually exclusive specificities of both enzymes were confirmed. The most active GR fractions, obtained from the preparative electrophoresis, displayed only a single protein band of 50 kDa in 10% SDS-PAGE (Fig. 2B) and band of $\mu = 0.37$ in 10% native-PAGE which displayed GR activity (results not shown). Thus, the 50-kDa protein corresponds to GR purified to homogeneity. The most active TR fractions, obtained from preparative electrophoresis, displayed two proteins of 54 kDa and 35 kDa corresponding to TR and a contaminant, respectively. The latter was readily eliminated by an anion exchange chromatography (Fig. 2B). GR and TR, isolated by the two- or three-steps procedure described here, were purified 1544-fold with a specific activity of 379 U mg^{-1} and 4091-fold, with a specific activity of 272 U mg^{-1}, respectively (Table 1).

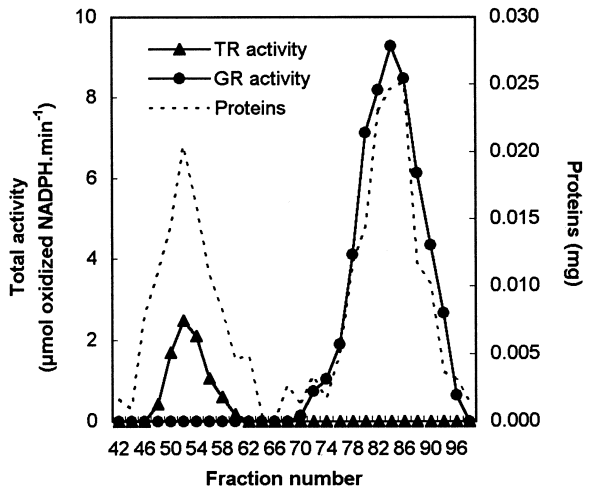

Fig. 3. Separation of TR and GR by preparative PAGE. TR and GR enriched fraction from 2′,5′-ADP-agarose was submitted to preparative electrophoresis in native conditions. Then, TR and GR activities were assayed and protein contents determined in the collected fractions.

3.3. Disulfide substrate specificities of GR and TR

In order to determine the specificities of GR and TR towards several putative disulfide substrates, the activities of both pure enzymes were assayed in the presence of either 200 μM GSSG, 60 μM $T(S)_2$, 60 μM thioredoxin disulfide ($Trx(S)_2$) from *E. coli*, 60 μM $Trx(S)_2$ from *Spirulina* or 2 mM DTNB, and 200 μM NADPH (results not shown). Among them, only GSSG and $T(S)_2$ were found to act as substrates for GR and TR, respectively. A K_M of 54 ± 4.2 μM with a k_{cat} of 19 000 min^{-1} were calculated for GR measured in the presence of 10–300 μM GSSG and a K_M of 30.5 ± 5.5 μM with a k_{cat} of 17 000 min^{-1} for TR, in the presence of 30–180 μM $T(S)_2$ and 200 μM NADPH. In addition, TR was also found to reduce $Gspd(S)_2$ with a K_M of 90.6 ± 5.3 μM and a k_{cat} of 14 500 min^{-1}, in the presence of 30–225 μM $Gspd(S)_2$ and 270 μM NADPH. Both enzyme activities were strictly dependent on NADPH since no activity was found when NADPH was replaced with NADH.

3.4. Western blot analysis

On Western blots, antibodies raised against two peptides derived from *T. cruzi* TR did not cross-react with the TR isolated from *E. gracilis*, while they clearly detected the TR of *T. cruzi*, used as a positive control (results not shown).

4. Discussion

In the present paper, two NADPH-dependent disulfide reductases were evidenced in *E. gracilis* and purified to homogeneity: the already reported GR and a novel one, the TR. The procedure established for their purification, which included a highly resolving step of preparative electrophoresis, was rapid and allowed the isolation of GR in just 2 steps and of TR in 3 steps. The purification factor, specific activity, K_M and k_{cat}/K_M of the pure GR calculated in the presence of GSSG are similar to those found for GRs from different organisms and GR from *E. gracilis*, which has been isolated for the first time by Shigeoka et al. in 1987 [2] (Table 2). However, while GR purified by Shigeoka et al. was shown to have an unusual M_r of 40 kDa per subunit, GR of our preparation

Table 2
Purification factors, kinetic parameters and molecular weights of TR and GR purified from *E. gracilis*: comparison with purification factors, kinetic parameters and molecular weights of TR purified from trypanosomatids and with those of GR from other organisms

		Purification factor	Specific activity (U mg^{-1})	K_M $T(S)_2$ (μM)	K_M GSSG (μM)	k_{cat} (min^{-1})	Subunit M_r (kDa)	k_{cat}/K_M (M^{-1} s^{-1})	References
TR	*C. fasciculata*	1404	295	53	NS[a]	31 000	53.8	9.7×10^6	[23]
	L. donovani	–	113	36	NS	10 700	52	5.0×10^6	[24]
	T. congolense	–	–	31	NS	9600	54	5.2×10^6	[25][b]
	T. cruzi	2367	284	45	NS	14 200	50	5.3×10^6	[13]
	E. gracilis	**4091**	**272**	**30.5**	**NS**	**17 000**	**54**	$\mathbf{9.3\times10^6}$	**This work**
GR	*E. coli*	–	–	NS	61–70	44 000	48.7	6.2×10^6	[5,26]
	R. rubrum	1159	102	–	90	–	54.5	–	[27]
	S. maxima	1300	238	–	120	–	47	–	[28]
	Human	–	240	NS	65	12 600	52.5	3.2×10^6	[13,29–31]
	E. gracilis	1715	223	–	50	–	40	–	[2]
		1544	**379**	**NS**	**54**	**19 000**	**50**	$\mathbf{5.9\times10^6}$	**This work**

[a]Non-substrate.
[b]TR overexpressed in *E. coli*.

was found to have a higher M_r (50 kDa). Such a discrepancy could be due to either (i) a partial proteolysis of the preparation of Shigeoka et al. since 50 kDa is closer to the molecular weight generally found for GRs or (ii) the existence of several GR isoforms in *E. gracilis* which may be differently expressed depending on the culture conditions.

The subunit molecular weight of TR from *E. gracilis* is similar to that of TRs from trypanosomatids (Table 2) and, interestingly, the sequence of peptide 1 (Fig. 1) is nearly identical to one of the most highly conserved regions found in TRs [7,8,11,24], a region which corresponds to a part of the active site containing the reactive histidine residue (H 460 or 461 depending on the origin of TR). *E. gracilis* TR may therefore be structurally elated to TRs from trypanosomatids although it did not react with two antibodies raised against specific *T. cruzi* TR peptides. TR was shown to display a high specificity towards $T(S)_2$ and can use $Gspd(S)_2$ as a substrate but not GSSG, like the TRs from trypanosomatids ([13,31] and Table 2), nor DTNB, nor $Trx(S)_2$ from *E. coli* – two alternative substrates for large TrxRs [14,32]. The purification factor, specific activity, K_M and k_{cat}/K_M of the pure TR, calculated in the presence of $T(S)_2$, are also similar to those generally found for TR isolated from different trypanosomatids (Table 2). All these results demonstrate for the first time (i) the existence of TR in *E. gracilis* showing that the trypanothione/TR system is not unique to trypanosomatids, this result being consistent with the recent finding of the presence of trypanothione in *Entamoeba histolytica* [33], and (ii) the coexistence of TR and GR in *E. gracilis*, while these two enzymes were thought to be mutually exclusive. In addition, TR may correspond to the protein of *E. gracilis* partially purified by Munivalli et al. in 1975 [3] which, without further evidence, they designated TrxR, when the existence of trypanothione had not yet been discovered, nor DTNB described as an alternative substrate for TrxR. Moreover, we were unable to detect any TrxR activity in *E. gracilis* extracts using DTNB as substrate (Fig. 2A) suggesting that TrxR is absent from this organism.

The TR in trypanosomatids has been described to replace the GR found in other organisms. However, the present study shows that TR and GR co-exist in *E. gracilis*. This could be linked to the peculiar route of evolution of this organism [34] and suggests that the common ancestor of both trypanosomatids and *Euglena* possessed the TR gene, and that the GR gene has been recruited later by *Euglena*, possibly with the engulfment of the chlorophytic endosymbiont. The comparison of the amino acid sequences of TR and GR from *E. gracilis* with those from other organisms would therefore be useful to draw their respective evolutionary origins. More investigations are also required to study the subcellular localization of TR and GR in *E. gracilis* and their respective functions by using specific inhibitors.

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