SUPPLEMENTARY MATERIAL 2

Gamma-glutamylcysteine synthetase and tryparedoxin 1 exert high control on the antioxidant system in *Trypanosoma cruzi* contributing to drug resistance and infectivity.

by

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Redox Biology, 2019.

SUPPLEMENTARY MATERIAL 2. Construction of the kinetic models for $T(SH)_2$ synthesis and $T(SH)_2$-dependent peroxide reduction.

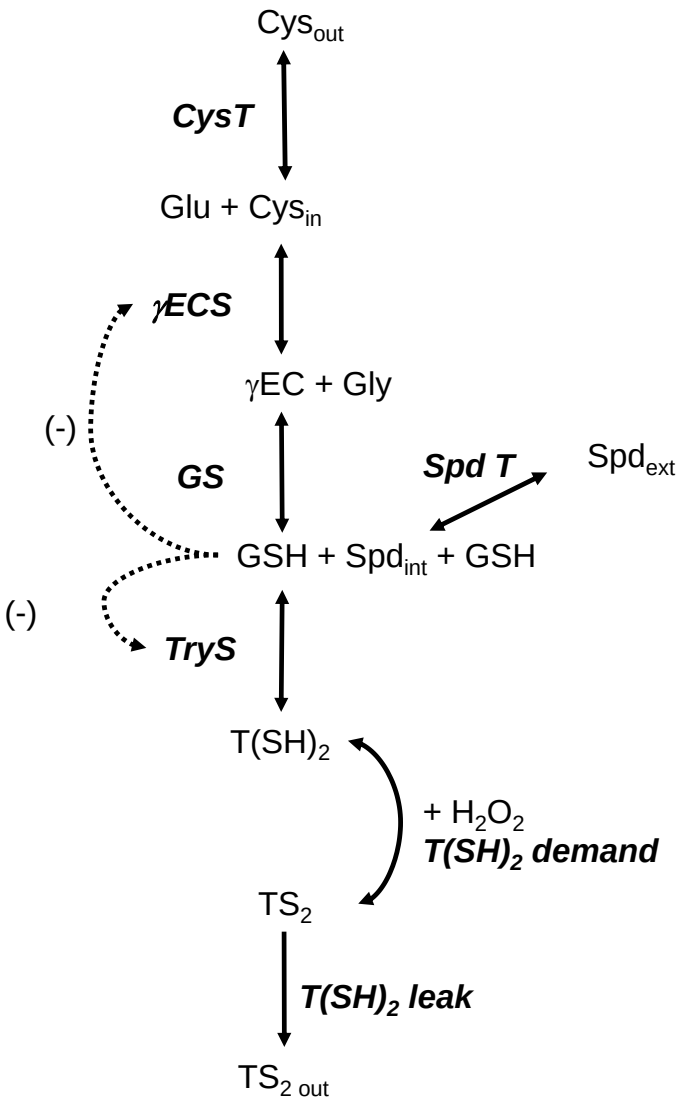

Fig. S2.1 Reactions included in the $T(SH)_2$ synthesis pathway kinetic model

CysT, Cys transport; Cys_{out}, external cysteine; Cys_{in} internal Cys; SpdT, spermidine transport; Spd_{out}, external spermidine; Spd_{in}, internal spermidine; TS_2, oxidized trypanothione; TS_{2out}, excreted oxidized trypanothione. All other abbreviations are shown in Fig. 1.

Table S2.1 Summary of the kinetic parameter values for the $T(SH)_2$ synthesis model

	Reaction coded in Gepasi/Copasi	*Kinetic parameters*		Comments	Reaction mechanism
CysT	Cysout = Cysin	Vm_f	0.002(0.004)	a	Uni-uni reversible
		Km_{Cysout}	0.05	b	
		K_{eq}	20	c	
		Km_{Cysin}	0.65	d	
γECS	Glu + Cysin = gEC	Vm_f	0.00044(0.0012)	e	Bi-uni random with GSH inhibition
		α	1	f	
		Km_{Glu}	0.13	f	
		Km_{Cys}	0.21	f	
		K_{eq}	5597	f	
		Ki_{GSH}	1.6	f	
		Km_{gEC}	0.43	f	
		β	0.03	f	
GS	gEC + Gly = GSH	Vm_f	0.0086	f	Bi-uni random
		α	1	f	
		Km_{gEC}	0.04	f	
		Km_{Gly}	1.2	f	
		K_{eq}	5597	f	
		Km_{GSH}	12.6	f	
SpdT	Spdext = Spdint	Vm_f	0.0058	f	Uni-uni reversible
		Km_{Spdext}	0.00084	f	
		K_{eq}	1000	f	
		Km_{Spdint}	10	f	
TryS	GSH + Spdint + GSH = TSH	Vm_f	0.0012 (0.002)	g	Tri-uni random with competitive inhibition by GSH
		α	1	h	
		Km_{GSH}	1.6	i	
		Km_{Spdint}	0.86	f	
		Km_{GSH}	1.6	i	
		K_{eq}	5597	f	
		Ki_{GSH}	7.3	i	
		Km_{TSH}	1	h	
		β	1	h	
TSH demand	TSH + H2O2 = TS2	Vm_f	0.005	j	Bi-uni ordered reversible
		Km_{TSH}	0.003	k	
		$Km_{peroxide}$	0.008	l	
		K_{eq}	100	m	
		Km_{TS2}	10	n	
TS2 leak	TS2-> TS2out	k_1	0.002	o	Mass action irreversible

Km and *Ki* in mM; *Vm* in µmol/min x mg of cellular protein. The CysT, γECS and TryS *Vm* values in parentheses were parameterized to simulate the increases in thiol contents in the Cys supplementation experiments.

[a]*Vm* parameterized to reach the physiological intracellular Cys concentrations.

[b]*Km* reported in [34].

[c]*Keq* parameterized to maintain Cys concentrations within the physiological interval. Cysteine transport in *T. cruzi* depends on extracellular pH [34].

[d]*Kp* obtained from adjusting CysT kinetic data reported by [34] to substrate inhibition $v = \frac{Vm*S}{Km+S+\frac{S^2}{Ki}}$.

[e]*Vm* parameterized: the maximum *ex vivo* flux of $T(SH)_2$ synthesis attained in the OE-TryS parasites (0.3 nmoles/min x mg cell protein) was assumed to be near the *Vm* of γECS considering negligible control by GS.

[f] Kinetic values used in our previously reported model [26].

[g]*Vm* parameterized: the maximum *ex vivo* flux of $T(SH)_2$ synthesis attained in the OE-γECS parasites (1.1-2.4 nmoles/min x mg cell protein; Table S1.2 in SM1) was assumed to be near the *Vm* of TryS, assuming negligible control of GS [26].

[h] arbitrary values.

[i]*Km* and *Ki* values previously reported for TryS using cell soluble protein of the OE-TryS parasites [29].

[j] maximum *ex vivo* flux of the $T(SH)_2$-dependent peroxide reduction system using H_2O_2 reported by our group [14].

[k]*Km* for $T(SH)_2$ of the complete reconstituted pathway [14].

[l] average *Km* for CumOOH, tert-butOOH and H_2O_2 of recombinant TXNPx [14].

[m] and [n], arbitrary values.

[o] reaction included to allow free variation in TS_2 (and consequently in $T(SH)_2$), with a parameterized *k* value.

Table S2.2. Initial and fixed (F) metabolite concentrations used in the kinetic model of $T(SH)_2$ synthesis

(mM)	mM
Cys out	0.04 **F** (0.14 **F***)
Cysin	1
Glu	9 **F**
gEC	0.15
Gly	9 **F**
GSH	1
Spdext	0.0011**F**
Spdint	2.5
TSH	0.01
TS2	0.00001
TS2out	0.00001**F**
H2O2	0.001 **F**

* Fixed value of Cys_{out} for the simulation in Cys supplementation experiments

Rate equations used in the T(SH)$_2$ synthesis kinetic model

In all equations, K_x are the binding constants for the respective ligands and α and β are the factors by which the binding of one substrate changes the affinity for the co-substrates.

CysT and SpdT

The rate equation was Michaelis-Menten reversible (Haldane) [62].

$$v = \frac{\frac{Vm}{Ks}\left(S \cdot \frac{P}{Keq}\right)}{1 + \frac{S}{Ks} + \frac{P}{Kp}}$$

For CysT, S=Cys_{out} and P=Cys_{in}. For SpdT, S=Spd_{ext} and P=Spd_{int}.

γECS

Bi-uni random reversible with GSH inhibition

$$v = \frac{\frac{Vm}{\alpha \cdot Ka \cdot Kb} \cdot \left(A \cdot B \cdot \frac{P}{Keq}\right)}{1 + \frac{A}{Ka} + \frac{B}{Kb} + \frac{A \cdot B}{\alpha \cdot Ka \cdot Kb} + \frac{In}{Ki} + \frac{P}{Kp} + \frac{B \cdot In}{\beta \cdot Ki \cdot Kb}}$$

A=Glu, B=Cys_{in}, P =γEC and In= GSH. *Ki* of GSH *versus* Glu.

GS

Bi-uni random reversible

$$v = \frac{\frac{Vm}{\alpha \cdot Ka \cdot Kb} \cdot \left(A \cdot B \cdot \frac{P}{Keq}\right)}{1 + \frac{A}{Ka} + \frac{B}{Kb} + \frac{P}{Kp} + \frac{A \cdot B}{\alpha \cdot Ka \cdot Kb}}$$

A=γEC, B=Gly and P=GSH.

TryS

Tri-uni random with competitive inhibition by GSH against GSH

$$v = \frac{\frac{Vm}{\alpha \cdot Ka \cdot Kb \cdot Kc}\left(A \cdot B \cdot C \cdot \frac{P}{Keq}\right)}{1 + \frac{A}{Ka} + \frac{B}{Kb} + \frac{C}{Kc} + \frac{A \cdot B}{\alpha \cdot Ka \cdot Kb} + \frac{A \cdot C}{\alpha \cdot Ka \cdot Kc} + \frac{B \cdot C}{\alpha \cdot Kb \cdot Kc} + \frac{A \cdot B \cdot C}{\alpha \cdot Ka \cdot Kb \cdot Kc} + \frac{I}{Ki} + \frac{I \cdot B}{\alpha \cdot \beta \cdot Ki \cdot Kb} + \frac{A \cdot B \cdot I}{\alpha \cdot \beta \cdot Ka \cdot Kb \cdot Ki} + \frac{P}{Kp}}$$

A= GSH, B= Spd, C is a second GSH ligated after glutathionyl-spermidine formation, P is $T(SH)_2$ and I is GSH as substrate inhibitor.

TSH demand

Bi-uni ordered reversible

$$v = \frac{\frac{Vm}{Ka \cdot Kb} \cdot \left(A \cdot B \cdot \frac{P}{Keq}\right)}{1 + \frac{A}{Ka} + \frac{P}{Kp} + \frac{A \cdot B}{Ka \cdot Kb}}$$

A= $T(SH)_2$, B= peroxide (H_2O_2, CumOOH, t-butOOH), P= TS_2.

TS2 leak

Reaction that allows free variation of $T(SH)_2$ during modeling. Without this reaction TS_2 has to be fixed, which cancels $T(SH)_2$ variation.

Mass action irreversible

v= *k1** [substrate]

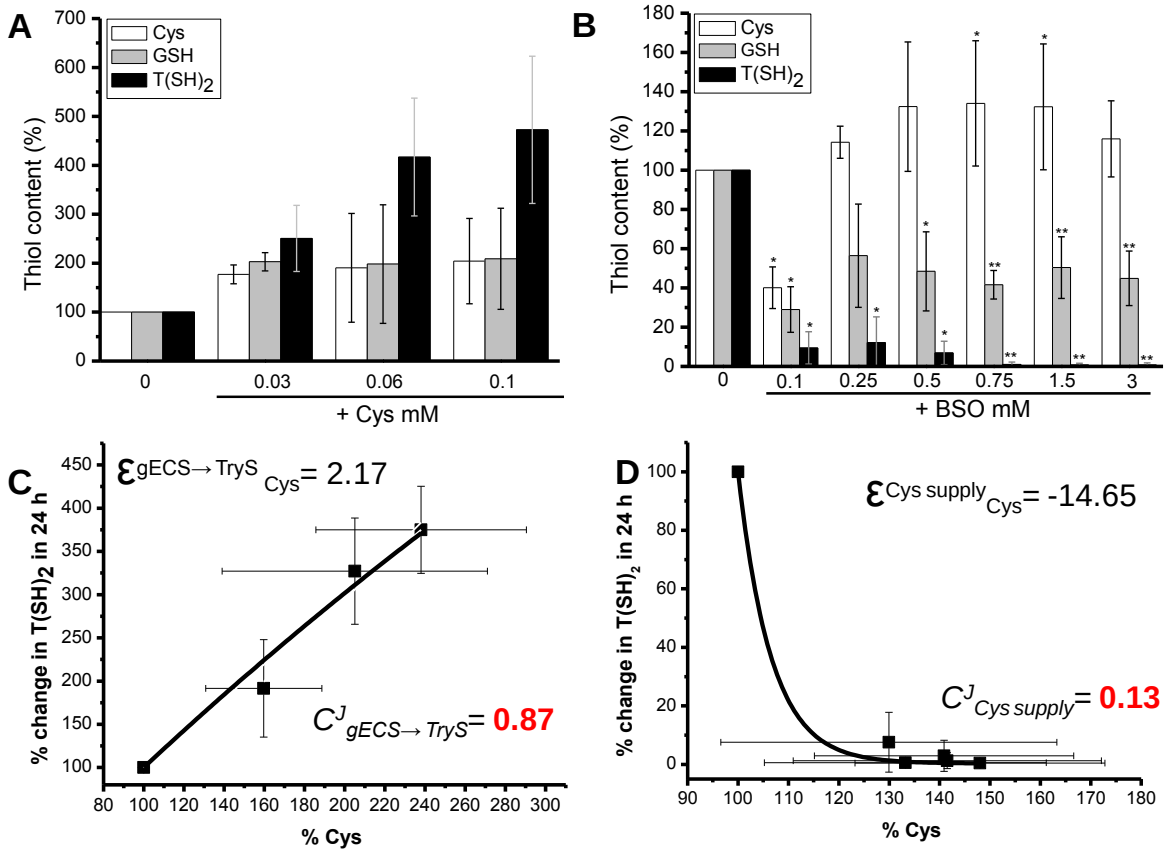

Fig. S2.2 Elasticity analysis of the $T(SH)_2$ synthesis pathway.

Panels A and B data replotted from [29].

(A) Parasites were supplemented with the indicated concentrations of Cys. After 24 h, the cells were harvested and the intracellular thiol contents were determined by HPLC. 100% thiol was equivalent to Cys, 4.2 ± 1.8; GSH, 6.9 ± 1; $T(SH)_2$, 4.3 ± 1.6 nmol/mg cell protein (n=5).

(B) The contents of Cys, GSH and $T(SH)_2$ were determined by HPLC in parasites treated with DL-(*S,R*)-BSO for 24 h. 100% thiol contents were Cys 4.8 ± 1, GSH 5.7 ± 2.2 and $T(SH)_2$ 3.1 ± 0.9 nmol/mg protein (n=17). *p < 0.05, **p < 0.01 *vs*. mock

(C and D) Elasticity coefficients determination. The % of $T(SH)_2$ change in 24 h *vs.* % of internal Cys were plotted from the two sets of experiments performed in parallel in panels A and B. The elasticity coefficient of the group of reactions that consume Cys ($\varepsilon^{consumer}_{Cys}$; *i.e.* γECS, GS and TryS) and the one that supply it ($\varepsilon^{supplier}_{Cys}$: *i.e* CysT) are calculated from the slope of the tangent (derivative) at the point of 100% Cys. From the elasticity coefficients values, the C^J_{ai} are calculated by solving the two-equations system derived from the summation theorem ($C^J_{supplier}$+ $C^J_{consumer}$=1) and the connectivity theorem ($C^J_{supplier}$ x $\varepsilon^{supplier}_{Cys}$ + $C^J_{consumer}$ x $\varepsilon^{consumer}_{Cys}$ =0) of MCA [23, 24]. The resulting C^J_{ai} values are shown in red.

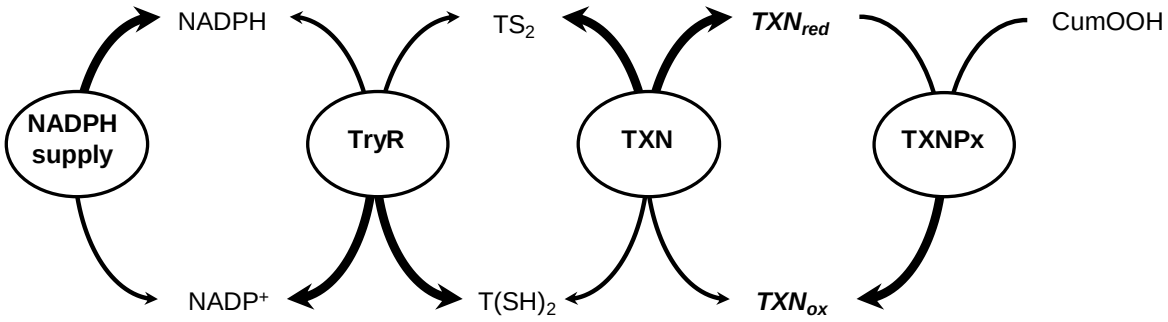

Fig. S2.3 Reactions included in the peroxide reduction pathway kinetic model.

All reactions are reversible except TXNPx. The bolder arrows indicate the predominant electron flux.

Table S2.3 Summary of the kinetic parameter values for the peroxide reduction model

	Reaction coded in Gepasi/Copasi	*Kinetic parameters*		comments	Reaction mechanism
TryR	NADPH + TS2 = NADP + TSH	Vm_f	0.264	a	Bi-bi ordered reversible
		Km_{NADPH}	0.009	a	
		Km_{TS2}	0.023	a	
		K_{eq}	3.5 e7	b	
		Km_{NADP}	0.011	c	
		Km_{TSH}	10	d	
TXN	TSH + TXNox = TS2 + TXNred	Vm_f	0.088	e	Bi-bi ping pong reversible
		Km_{TSH}	0.092	f	
		Km_{TXNox}	0.0006 - 0.001	g	
		K_{eq}	1000	d	
		Vmr	0.00088	h	
		Km_{TS2}	1	d	
		Km_{TXNred}	1	d	
		Ki_a	1	d	
		Ki_q	1	d	
TXNPx	CumOOH + TXNred-> TXNox	Vm_f	0.179	e	Bi-bi ping pong irreversible
		Km_{cumOOH}	0.011	e	
		Km_{TXNred}	0.0006	e	
NADPH supply	NADP = NADPH	k_1	10	i	Mass action reversible
		k_2	1.5	i	

Km and *Ki* in mM; *Vm* in µmol/min*mg of cellular protein.

[a] value reported in [26].

[b] value calculated reported in Table S6 in [26].

[c] value taken from Table S7 in [26].

[d] arbitrary value

[e] values reported in Table 1 [14].

[f] using the *Km* for $T(SH)_2$ of the recombinant (0.092 mM) or the complete reconstituted pathway (0.003 mM) [14] gave similar simulation results.

[g] parameterized in the interval of 0.0006 to 0.001, the first value is equal to the *Km* for TXN of TXNPx reported in [14].

[h] arbitrary value corresponding to 1/100 of the *Vmf*

[i] parameterized values.

Table S2.4. Initial and fixed (F) metabolite concentrations used in the kinetic model of peroxide reduction

metabolite	mM
TSH	0.45
TS2	0.016
NADPH	0.16
NADP	0.025
TXNox	1.e-5**
TXNred	1.e-4*
CumOOH	0.1 **F**

*TXN concentration used in the pathway reconstitution [14]. **arbitrary value considering that only 1/10 of TXN is in its oxidized state. F means fixed concentration.

Rate equations

In all equations, *Ka, Kb, Kp* and *Kq* are the respective binding constants for the ligands.

TryR

Bi bi ordered reversible kinetics similar to that used in [26]

$$v = \frac{\frac{\mathrm{Vm}}{Ka \cdot Kb} \cdot \left(\mathrm{A} \cdot \mathrm{B} - \frac{\mathrm{P} \cdot \mathrm{Q}}{\mathrm{Keq}}\right)}{1 + \frac{\mathrm{A}}{Ka} + \frac{\mathrm{A} \cdot \mathrm{B}}{Ka \cdot Kb} + \frac{\mathrm{P} \cdot \mathrm{Q}}{Kp \cdot Kq} + \frac{\mathrm{Q}}{Kq}}$$

A = NADPH; B= TS2; P = NADP; Q= TSH,

TXN equation

The oxidation/reduction of TXN was considered as a bi-bi ping-pong reversible kinetics assuming that TXN_{ox} is first reduced by $T(SH)_2$, releasing TS_2 and TXN remaining in a modified state as TXN_{red} to further donate the electrons to TXNPx.

$$v = \frac{\mathrm{Vmf} \cdot \left([\mathrm{A}] \cdot [\mathrm{B}] - \frac{[\mathrm{P}] \cdot [\mathrm{Q}]}{\mathrm{Keq}}\right)}{[\mathrm{A}] \cdot [\mathrm{B}] + Kb \cdot [\mathrm{A}] + Ka \cdot [\mathrm{B}] \cdot \left(1 + \frac{[\mathrm{Q}]}{\mathrm{Kiq}}\right) + \frac{\mathrm{Vmf}}{\mathrm{Vmr} \cdot \mathrm{Keq}} \cdot \left(Kq \cdot [\mathrm{P}] \cdot \left(1 + \frac{[\mathrm{A}]}{Kia}\right) + [\mathrm{Q}] \cdot (Kp + [\mathrm{P}])\right)}$$

A= TSH, B= TXNox, P= TS2, Q= TXNred. *Kia* and *Kiq* are the dissociation constants of the first substrate and the last product.

The TXN reaction was also modeled with mass action reversible kinetics

v= k_1 [TXN_{ox}] *[TSH] - k_2 [TXN_{red}] *[TS2],

for which it was necessary to parameterized the *k* values (Fig. S2.4). The values that closely simulated the experimental *ex vivo* and *in vitro* fluxes were for TXN k_1= 160, k_2 = 1 and for the NADPH supply reaction k_1= 0.04 and k_2= 0.5.

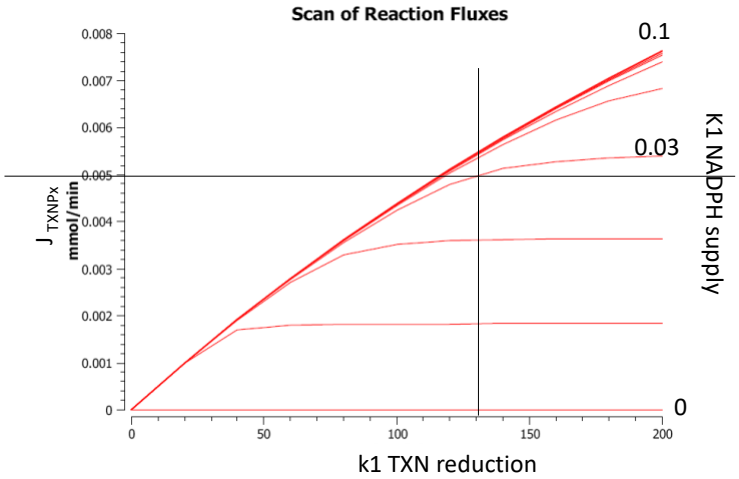

Fig. S2.4 Parameterization of the *k* values of TXN and NADPH supply reactions
A simultaneous parameter scan was performed for the *k* values or the two reactions to attain a similar flux to that of the reconstituted pathway.

TXNPx equation

A bi-bi ping-pong irreversible kinetics was considered assuming that TXNPx accepts and donates the electrons from TXN in a two-step fashion.

$$v = \frac{Vm \cdot [A] \cdot [B]}{Kb \cdot [A] + Ka \cdot [B] + [A] \cdot [B]}$$

A= CumOOH, B= TXNred.

NADPH supply

Mass action reversible kinetics

$$v = k_1 \cdot [S] - k_2 \cdot [P]$$

S= NADP and P is NADPH. k_1 and k_2 had arbitrary values.