Compared Reactivities of Trypanothione and Glutathione in Conjugation Reactions

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In order to compare the non-enzymatic capacities of the xenobiotic conjugation of trypanothione (a spermidine-glutathione conjugate unique to kinetoplastidae) and glutathione, the reactivity of their respective thiols was investigated. The acido-basic properties of both compounds and their nucleophilicity toward Ellman’s reagent and 1-chloro-2,4-dinitrobenzene were studied. Our results show that although glutathione is a better nucleophile than trypanothione, the latter is more reactive because it is more ionized in a large pH range. This pH range likely includes the pH to which such conjugation reactions are expected to happen in vivo. Thus, the better conjugation capacity of trypanothione could make it the cornerstone for the xenobiotic detoxication of trypanosomatidae.

Keywords trypanothione; glutathione; xenobiotic detoxication; thiol reactivities; Trypanosoma cruzi

Glutathione S-conjugates play an essential role in the physiological mechanisms involved in the survival of the cell. They are the products of the nucleophilic addition of glutathione on a wide variety of electrophiles catalyzed by glutathione S-transferases (GST) (EC 2.5.1.18), and represent the most important pathway for the detoxication of endogeneous and xenobiotic electrophilic substances. These glutathione S-conjugates are less reactive and more polar than the initial electrophilic molecules and can therefore be more easily eliminated. 5) Some glutathione S-conjugates serve as mediators, for example cysteine leukotrienes (LTC4),2) whose chemical structure represents a convenient form for export via the ATP-dependent glutathione S-conjugate export pump.3,4) These conjugates can also be formed in a reaction which is chemically reversible under physiological conditions and can serve in vivo as transporters for biochemically important electrophilic compounds. 5)

All organisms contain at least one low molecular weight thiol in high amounts available for this conjugate formation. The far more common compound is glutathione, but some organisms, such as kinetoplastidae, have analogs of glutathione, and instead use trypanothione (N1,N8-bis(glutathionyl)spermidine) (Fig. 1). This unusual dithiol (T(SH)2) is essential for reducing glutathione disulfide (GSSG). 5) Indeed, trypanosomatidae lack glutathione reductase (EC 1.6.4.2) and possess instead trypanothione reductase (EC 1.6.4.8), an enzyme unique to these organisms, which regenerates T(SH)2 from trypanothione disulfide (T(S)). Therefore T(SH)2 can represent the main parasite molecule in defense against reactive oxygen species. 7,8) Its potential role in xenobiotic detoxication has been suspected for a long time.

In 1981 the purification of a protein having a low GST activity was described in Trypanosoma cruzi. 9) However, we could not detect any significant GST or trypanothione S-transferase activity in fresh lysates of T. cruzi. The non-enzymatic capacities of the conjugation of trypanothione were thus studied and compared to those of glutathione. The experimental conditions were chosen from the physiological data known on T. cruzi. On the one hand, given that the reactivity of thiols for conjugation reactions is strongly related to their acido-basic properties, the ionization profiles of T(SH)2 versus GSH were determined. On the other hand, since these conjugation reactions proceed through a S-S2 mechanism, two electrophiles were used: Ellman’s reagent10) and 1-chloro-2,4-dinitrobenzene (CDNB). These reagents are used, respectively, to study thiol-disulfide exchange and to evaluate glutathione S-transferase activities.11)

Experimental

Materials CDNB, Ellman’s reagent (Ell-S-S-Ell: 5,5’-dithiobis(2-nitrobenzoic acid)) and GSH were obtained from Aldrich. T(S) was synthetized according to Fauchet et al.10) pH was determined using an Orion Research model 601 A pH meter, and UV spectra were measured using a Unikron 930 spectrophotometer (Kontron instruments). Glutathione reductase (GR) from bovine intestinal mucosa was purchased from Sigma. Trypanothione reductase (TR) was purified as previously described.13)

Spectrophotometric Titration of GSH and T(SH)2. The study was carried out according to the protocol of Benesch.14) The buffer used was a mixture of orthophosphoric and boric acid at a concentration of 0.02 M in each acid. The pHs were adjusted with NaOH. All the pH measurements and the corresponding spectrophotometric readings were made at 28 °C. For spectrophotometric measurements, 10 µl of a 20 mM solution of thiol was added to 1 ml of the buffer solution. The absorption spectrum was determined immediately after mixing, using the corresponding buffer as a blank.

Kinetics Reaction with Ellman’s Reagent: Ellman’s reaction was studied according to Wilson’s protocol15): 0.04 M sodium acetate buffer, pH 4.7, 1 M KCl, 40 µM Ellman’s reagent, and 0.25 to 1 mM thiol

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Fig. 1. Structure of Reduced Trypanothione T(SH)2

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concentration. The reaction was studied at a pH value far below their 
$pK_a$ so that the reaction could proceed at a measurable rate. The thiol 
centration were verified by titration with Ellman's reagent in 0.1 M 
phosphate buffer, pH 8. The reaction was studied spectrophotometrically 
at 412 nm and 28 °C for 1 min. The pH of the solution was measured 
after each run.

Addition on CDNB: The reaction was studied under the following 
conditions: 0.1 M phosphate buffer, pH 6.8 and 7.8, 250 μM CDNB, and 
0.1 to 1 mm thiol concentration. The formation of the conjugate 
CDNB-thiol was followed at 340 nm and 28 °C for 2 min. The molecular 
excitation coefficients used for the conjugates were $ε = 9600$ cm$^{-1}$ M$^{-1}$ 
for GSH$^{11}$ and $ε = 9900$ cm$^{-1}$ M$^{-1}$ for T(SH)$_2$ per thiol group, 
respectively.

Results

Ionization Profiles of T(SH)$_2$ and GSH It has been 
established that thiols react predominantly as thiolates and 
that the nucleophilicity of a thiolate anion depends 
on its basicity.$^{11}$ As GSH and T(SH)$_2$ possess numerous 
ionizable functions, a direct study of their acido-basic 
properties is difficult. The method of Benesch$^{14}$ allowed 
us to quantify the percentage of thiolate according to the 
pH. For pHs between 5.5 and 9.5, T(SH)$_2$ (Fig. 2, curve 
A) is more ionized than GSH (Fig. 2, curve B). For pHs 
less than 5.5, neither T(SH)$_2$ nor GSH are  sufficiently 
ionized to possess a significant reactivity. For pHs above 
9.5, both species are almost completely ionized; therefore, 
the thiol reactivity in nucleophilic addition depends no 
more on thiolate quantity but on their intrinsic nucleo-
philicity.

At pH 7.4, close to the physiological value, the 
percentage of ionization of thiolate function is 1% for 
glutathione and 15% for trypanothione. Given the di-thiol 
structure of T(SH)$_2$, the quantity of thiolate is therefore 
three-fold higher for T(SH)$_2$ than for GSH. This ratio 
decreases from pH 5.5 to 9.5.

Kinetics of Reduction of Ellman's Reagent The 
reduction of disulphide bonds is known to proceed through 
the thiolate ion.$^{11}$ The thiol-disulphide exchange with 
Ellman's reagent (Chart 1) enabled us to easily follow 
this kind of reaction using spectrophotometry. Previous 
studies$^{15,16}$ indicate that thiolate-disulphide exchange is a 
mechanistically simple S2 displacement reaction.

For monothiols, it is shown that $k_2 < k_1$. Thus, for 
reaction times of less than 1 min, any contribution to the 
formation of Ell-S$^-$ from (iii) in Chart 1 can be neglected. The rate expression of Ell-S$^-$ formation is:

\[
v = \frac{d[\text{Ell-S}^-]}{dt} = k_1[\text{RS}^-][\text{Ell-S-El}]
\]

(1)

The large excess of thiol enables us to write $[\text{RS}^-] = 
[\text{RS}^-]_0$ Equation 1 simplifies to:

\[
v = \frac{d[\text{Ell-S}^-]}{dt} = k_1[\text{Ell-S-El}]_0 - [\text{Ell-S}^-]_0
\]

(2)

with $k_1[\text{Ell-S-El}]_0 = k_1 \frac{K_a}{K_a + [\text{H}_2\text{O}]} ([\text{RS}^-] + [\text{RS}]_0$)

For dithiols, the reaction (iii) is essentially in-
tramolecular. In a dilute solution, the intermolecular 
thiol-disulphide exchange does not compete with the 
intramolecular reaction. A rate equation of the same form

\[
v = \frac{d[\text{Ell-S}^-]}{dt} = 2k_2[S^-][\text{Ell-S-El}]
\]

(3)

with $[S^-] = [\text{SRS}^-] + [\text{SRSH}]$

The factor of 2 in Eq. 3 reflects the assumption that the 
reaction (ii) is rate limiting and the production of a second 
equivalent of Ell-S$^-$ by reaction (iii) follows rapidly, once 
the intermediate disulfide HSR-S-S-El is formed. "SRSH" 
and "SRS" are supposed to be equally reactive, so only 
the $pK_a$ for HSR-S-S-El was taken into account. In 
these conditions, estimates of $k_1$ and $pK_a$ are higher than
the real values. Equation 3 can be written in a similar manner to the equation for monothiols Eq. 2:
\[ e^{-d[Ell-S^+] \over dt} = k_{1,obs}([Ell-S-Ell][Ell-S^-]) \]
Equations 2 and 4 can be integrated in:
\[ \ln\left(\frac{[Ell-S-S-Ell][Ell-S^-]}{([Ell-S-Ell][Ell-S^-])_0}\right) = k_{1,obs}t \]
The \( k_{1,obs} \) values are obtained by plotting the logarithmic expression Eq. 5 versus \( t \), and are then plotted versus the ratio of total thiol concentration on \([H_2O^+]\) to obtain the product \( k_1K_a \). The reaction of RS\(^-\) with Ellman's reagent is well correlated by a Brønsted-type equation, i.e. the reactivity is increased as the pKa of the parent thiol increases (log \( k_1 \) is proportional to the pKa, and the proportionality coefficient is named \( \beta_{\text{max}} \)). The Brønsted correlation established by Wilson\(^{15}\) \( \beta_{\text{max}} = 0.49 \) enabled us to obtain the \( k_1 \) and pKa values from the product \( k_1K_a \). In the case of trypanothione, although the molecule is not symmetrical, both thiolate functions were considered equivalent. The pKa obtained in this study does not correspond to the microscopic pKa value of one given ionized species. It only gives an account of the macroscopic reactivity of \( \text{T(SH)}_2 \).

The \( k_1 \) and pKa values obtained from the first experiments on GSH are \( 2.0 \times 10^5 \) s\(^{-1}\) and 8.7, respectively (Table IA). This pKa is compatible with the value obtained by titration (pKa 8.83) and the estimate of Bruce (pKa 8.7).\(^{17}\) It is 0.4 unit less than the value of pH obtained previously for 50% ionization. The pKa value obtained for \( \text{T(SH)}_2 \) was 7.4. This value is 0.3 unit less than the pH value obtained for 25% ionization of \( \text{T(SH)}_2 \) in the ionization study. Although GS\(^-\) is intrinsically a better nucleophile than \( S(S)_2 \), trypanothione is so ionized that its resulting reactivity is higher. Therefore, the \( k_{1,obs} \) (or \( k_1[RS^-]_0 \)), or the reaction rates, are better parameters than \( k_1 \) for characterizing the reactivity of a thiol, as both take into account the amount of thiolate form (Table IB).

The observed kinetics of Ellman's reaction are not particular: most reactions of thioldyes with electrophiles are well correlated by Brønsted type equations, and \( \beta_{\text{max}} \) values are generally low (inferior to 0.5—0.7 in aqueous medium).\(^{15}\) Thus, at pHs around 7, \( \text{T(SH)}_2 \) would react better than GSH on most electrophiles.

**Reaction on CDNB** The substitution of thioldyes to CDNB (Chart 2) was found to be a second order reaction (Sw2 type). Its kinetic law is analogous to the law obtained in the case of the reaction with Ellman's reagent Eq. 1, and can be written as follows:
\[ e^{-d[P] \over dt} = k_1[CDNB][RS^-] \]

with \( P: \) thiol—CDNB conjugate

Reaction rates were found to be sufficiently low to consider that the CDNB concentration is constant for 2 min. As previously, we obtained the constant value \( k_1 \) [CDNB] by plotting \( \ln([RS^-]_0-[P]) \) versus \( t \). In the case of trypanothione, both thiolate functions were supposed to react in an independent way. Subsequently, the concentration used was the thiol concentration (twice the trypanothione concentration). The \( \varepsilon \) was measured and found to be equal to 9900 cm\(^{-1}\)M\(^{-1}\) per thiol group (\( \varepsilon = 9600 \) cm\(^{-1}\)M\(^{-1}\) for GSH\(^{11}\)). The similarity between these \( \varepsilon \) values is compatible with the hypothesis that each thiol of trypanothione reacts independently.

To evaluate the evolution of the rates according to the pH, two kinetic analyses were performed at pH 6.8 and 7.8. This study confirms that the quantity of thioldyes is a major factor: when the pH of the solution decreases, the difference between the resulting reactivities of GSH and \( \text{T(SH)}_2 \) increases (Table II). At pH 7.8, the reaction rate is three-fold higher with \( \text{T(SH)}_2 \) than with GSH (9.35 and 3.12 \( \mu \)M min\(^{-1}\) respectively). At pH 6.8, it becomes nearly five-fold higher (2.18 and 0.46 \( \mu \)M min\(^{-1}\) respectively).
Discussion

For numerous organisms, low molecular weight thiols (particularly glutathione or in the case of kinetoplastidae, trypanothione) are key molecules for the physiological defense against oxidative stress and xenobiotic detoxication. Their overriding feature is the presence of the thiol group and its inherent reactivity.

We studied the non-enzymatic reaction of GSH and T(SH)$_2$ on two different electrophilic centres: Ellman's reagent and CDNB. The results obtained are valid for pHs between 6 and 9.5, where the percentage of ionized thiol is higher for T(SH)$_2$ than for GSH. This pH range likely includes the pH of the compartments of T. cruzi in which such conjugation reactions are expected to happen. In both cases, around physiological pH and at 28°C (the temperature of proliferation for epimastigote forms), we observed that the formation of trypanothione S-conjugates is faster than the formation of glutathione S-conjugates. With electrophiles like CDNB, when pH decreases until 5.5, T(SH)$_2$ still reacts when GSH is almost no longer reactive. Although GSH is intrinsically a better nucleophile than T(SH)$_2$, the better percentage of ionization of T(SH)$_2$ at physiological pH makes it more reactive. Spectrophotometric studies have recently shown that GST is activated by deprotonation when it is complexed with GST. The high amount of T(SH)$_2$ present in trypanosomes (1 to 2 mM estimated from Fairlamb et al.)$^9$ makes its non-enzymatic conjugation reaction extremely effective with many electrophiles. This might be an explanation for the low activity of GST$^{9,19}$ or the absence of activity$^{20}$ detected in fresh lysates of T. cruzi: the non-enzymatic formation of trypanothione S-conjugates might be efficient enough to eliminate xenobiotics. As for the existence of a trypanothione S-transferase, some activity has been detected in some trypanosomatidae (Crithidia fasciculata, T. brucei brucei) and in Leishmania donovani; nevertheless, no such activity has been found in T. cruzi.$^{21}$

In the case of the reaction with Ellman's reagent, the dithiol structure of T(SH)$_2$ results in a kinetic mechanism different from that obtained with GSH. The last equilibrium which leads to the release of T(S)$^+$ and a thiolate anion becomes intramolecular, and consequently much faster than in the case of GSH (Chart 1). In dilute media, which may occur in some organelles, such a structure would be more efficient than GSH to reduce RSSR compounds in their corresponding thiols. Moreover, the presence of a protonated amine in the spermidine bridge could eventually assist in the release of the thiolate anion.$^{11}$

Recent works enlightened other non-enzymatic biological roles of trypanothione. Carnieri et al.$^8$ showed that the peroxidase activity observed in T. cruzi$^{22}$ was due to non-enzymatic reactions of endogenous reduced thiols (in particular T(SH)$_2$) with peroxides. Also, Awad et al.$^{23}$ found that T(SH)$_2$ played an important role in protecting DNA against irradiation by OH$^-$ scavenging and H atom donation for chemical repair or restitution processes.

The presence of trypanothione in trypanosomes could have been selected to improve many biological pathways based on glutathione in other species. In all protective means reported here, the non-enzymatic trypanothione-based system seems more efficient than the glutathione one. Thus, in trypanosomes the xenobiotic detoxication and the system of defense against oxidative stress would be highly dependent on the amount of T(SH)$_2$ present in the cell. This makes the enzymes involved in the trypanothione metabolism (trypanothione reductase, glutathionyl spermidine synthase, trypanothione synthase)$^{24}$ good targets for a specific chemotherapy against trypanosomiosis.

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References and Notes

20) a) B. Pumias-Marty, C. Verguette, M. Loyens, P. Velge, A. Taibi, M.-F. Cesbron, A. Capron, M. A. Ouaissi, Parasitol., 104, 1 (1992);
   b) This study, data not shown.