COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Rapid reduction of pentavalent antimony by trypanothione: potential relevance to antimonial activation†

Siucheong Yan,[a] Iris L. K. Wong,[b] Larry M. C. Chow[b] and Hongzhe Sun*[a]

[a] *Department of Chemistry and Open Laboratory of Chemical Biology, University of Hong Kong, Pokfulam Road, Hong Kong, P. R.. China. E-mail: hsun@hkucc.hku.hk; Fax: (+852) 2857 1586*

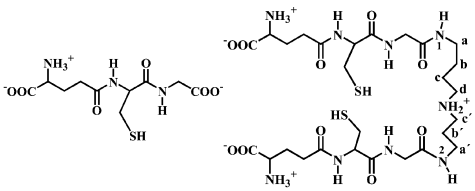
[b] *Department of Applied Biology and Chemical Technology and Central Laboratory of IMTDDS, The Hong Kong Polytechnic University, Kowloon, Hong Kong, P. R.. China*

Received (in Cambridge, UK) 18th October 2002, Accepted 27th November 2002
First published as an Advance Article on the web 18th December 2002

The dithiol trypanothione can reduce an antiparasitic pentavalent antimony agent to trivalent rapidly; this reduction process is both pH and temperature dependent and trypanothione may therefore play an important role in the activation of the drug.

Several pentavalent antimony (Sb^V) compounds (Pentostam® and Glucantime®) have been widely used clinically for the treatment of *Leishmaniasis* for several decades. Despite extensive use of these compounds, the mechanism of antileishmanial action still remains unknown. It has been suggested that Sb^V is a prodrug and is bioreduced to Sb^{III}, the active form of the drug. To support this hypothesis, the anti-leishmanial activity of Sb^V has been found to be dependent on its reduction to the trivalent form (Sb^{III}) inside parasites.[1] The greater susceptibility of intracellular amastigotes to Sb^V compared with promastigotes, suggests that the reductive activation of the drug is catalyzed either by the host macrophage or the intracellular amastigote itself. Arsenate (As^V), an analogue of antimony(v), is known to be reduced by arsenate reductase (ArsC) in *Staphylococus aureus* and in *E. coli*. A mechanism for this catalysis has recently being proposed by several groups.[2] Sb^V can not be reduced by this enzyme,[3] and as yet no analogous reductase enzyme has been identified in *Leishmania*. This suggests that the reduction of Sb^V may be mediated by different mechanism(s).

Low molecular mass (weight) thiols such as glutathione have been reported to reduce antimony(v) (and arsenate) but this process is thought to be too slow to be biologically significant.[2c,4,5] In this communication, we report the rapid reduction of Sb^V by trypanothione ($T(SH)_2$).



GSH(γ- L- Glu-L-Cys-Gly) $T(SH)_2$

Trypanothione is the most abundant (> 80%) low molecular mass (weight) thiol inside *Leishmania* species.[6–8] Together with trypanothione reductase (TR),† $T(SH)_2$ provides an intracellular reducing environment against oxidative stress, which is crucial for the survival of the parasite.[9,10]

Freshly prepared Sb^V gluconate (the active component of Pentostam) was found to be stable up to 24 h in 10 mM phosphate buffer, pH 6.4 (310 K) in the absence of trypanothione, based on the UV–vis measurements using bromopyrogallol red (BPR) to monitor the formation of Sb^{III} (data not shown). Reduced trypanothione ($T(SH)_2$) was obtained by the reduction of the trypanothione disulfide [T(S–S)] followed by HPLC purification.[11,†] $T(SH)_2$ and freshly prepared Sb^V gluconate were incubated in a 1 mM : 1 mM ratio at pH 6.4 and 310 K under an atmosphere of nitrogen. After 24 hours, the Sb^{III} content was measured to be 0.52 mM, determined by UV-vis titration using BPR as an indicator. A similar result (0.49 mM) was obtained when the mixture was incubated at pH 6.4 but at the slightly lower temperature of 298 K. These results indicate the stoichiometry of Sb^V : $T(SH)_2$ to be 1 : 2 (*vide infra*).

The kinetics of Sb^V reduction by $T(SH)_2$ were then investigated at different temperatures and pH values. ^{1}H NMR was used for this characterization, so that the site of the reduction could be evaluated.† This method relies on the differences in chemical shifts of β protons of Cys residues of trypanothione in the disulfide, dithiol and metal-bound forms (Fig. 1).[12] Solutions of $T(SH)_2$ and freshly prepared Sb^V gluconate (both buffered by 10 mM phosphate pH 6.4 in D_2O) were first exposed to nitrogen (99.9%) for 5 min prior to mixing (molar ratio 2 mM : 1 mM). The ^{1}H NMR spectrum of the solution was acquired periodically during the incubation at 310 K (Fig. 1).

The major change observed in the ^{1}H NMR spectrum was the gradual decrease in intensity of the resonance at δ 2.98, which finally disappeared after 12 h (Figs. 1 and 2). This resonance was previously assigned to the β protons of Cys residues of $T(SH)_2$.[12] The rate of the decrease in intensity of this resonance was calculated to be k = 4.42 M^{-1} min^{-1}. Other resonances remained essentially unchanged during the incubation period. The disappearance of the β protons of Cys (δ 2.98) was due to the oxidation of the trypanothione (and probably also to the complexation of $T(SH)_2$ to the resulting Sb^{III} species). The rate of the decrease in intensity of this resonance is equivalent to the rate of Sb^V reduction, since the formation of the Sb^{III}-trypanothione complex is rapid (within several minutes).[12] At 298 K, the decrease in intensity of the β protons of Cys was much slower (k = 1.24 M^{-1} min^{-1}, Table 1). It was noted that three sets of new resonances appeared at δ 3.05, 3.24 and 3.34 during the incubation, which overlapped with the resonance corresponding to the spermidine moiety of $T(SH)_2$ (Fig. 1). The

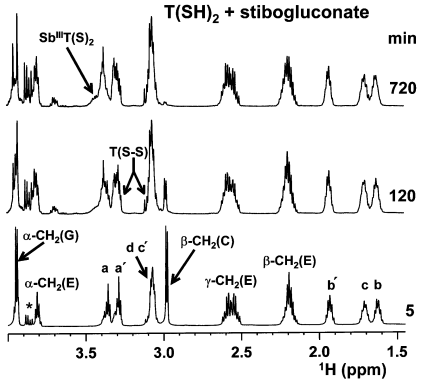


Fig. 1 600 MHz ^{1}H NMR spectra of $T(SH)_2$ after addition of sodium stibogluconate (2 mM : 1 mM) at pH 6.4 and 310 K, observed at 5, 120 and 720 min. Note the decrease in intensity of the β-CH_2 of Cys (δ 2.98). The peaks marked with asterisks (*) are resonances from stibogluconate.

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b2/b210240d/

DOI: 10.1039/b210240d

set of resonances at δ 3.05 and 3.24 could be assigned to the βCH_2 of Cys in T(S–S), whilst the broad resonance at δ 3.34 could be assigned to the bound βCH_2 of Cys in an $Sb^{III}T(S)_2$ complex.[12] As a control, the intensity of the β protons of the Cys residues of $T(SH)_2$ remained almost unchanged in the absence of Sb^V under identical conditions (< 5% after 12 h of incubation at 310 and 298 K, respectively).

We further explored the effects of pH on the kinetics of Sb^V reduction by $T(SH)_2$ at 298 and 310 K. The rate of the decrease in intensity of β protons of Cys (δ 2.98) at pH 7.4 and 310 K was three times slower (k = 1.39 M^{-1} min^{-1}) than at pH 6.4, and was barely detectable at 298 K (Table 1). For comparison, the reduction of Sb^V by glutathione (GSH), the mammalian trypanothione equivalent, under the same experimental conditions (*i.e.* pH 6.4, 310 K and the same thiolate concentration) was found to be at least 200 times slower than for trypanothione ($k < 1.9 \times 10^{-2}$ M^{-1} min^{-1}, Table 1). No reduction of Sb^V by GSH was observed within a week at pH 7.4 and 298 K. This is in agreement with a previous UV–vis study by Frezard *et al.*[4] Cysteine and vitamin C, both important physiological reductants present in humans, were found to be incapable of reducing Sb^V within 3 days at pH 6.4, 310 K (Table 1).

Electronic absorption spectroscopy was also used (with BPR as an indicator) to monitor the kinetics of Sb^{III} formation under similar experimental conditions. An exponential increase of Sb^{III} concentration was observed for the 2:1 $T(SH)_2$:Sb^V mixture at pH 6.4 and 310 K. The rate of Sb^{III} formation was calculated to be k = 4.76 M^{-1} min^{-1} (Table 1), which is in good agreement with ^{1}H NMR data of 4.42 M^{-1} min^{-1}. The combination of ^{1}H NMR and UV–vis spectroscopy allows the reduction of Sb^V to Sb^{III}, and the simultaneous oxidation of $T(SH)_2$ to be readily monitored. We also investigated the rate of reduction of Sb^V by trypanothione in the presence of *Leishmania enriettii* promastigote extracts (20%) and found the rate to be similar to that in aqueous solution (k = 5.62 M^{-1} min^{-1}).†

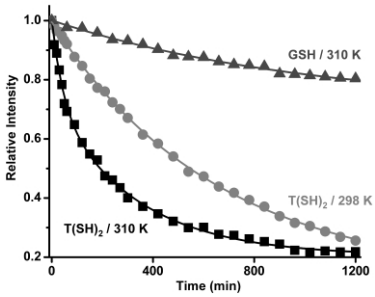


Fig. 2 Plot showing the decrease in intensity of the β-CH_2 of Cys (δ 2.98) of trypanothione and glutathione (*vs.* β-CH_2 of Glu) at pH 6.4, at two different temperatures. The rate of reduction was calculated based on a nonlinear square fit of the data by assuming a second-order reaction. The time for 50% disappearance of $T(SH)_2$ at 310 K (■), 298 K (●) and GSH at 310 K (▲) were 113 min, 402 min and over 3.5 days, respectively.

Table 1 The rate of reduction of Sb^V by $T(SH)_2$ (as half-lives) and selected biological reductants together with their standard reduction potentials

		Reduction rate ($t_{½}$)[a]	
Reductant	$E°$/V	310 K	298 K
GSH	−0.230[8]	> 3.5 days[b]	No reduction[b,d]
Cysteine	−0.355[16]	> 3.5 days[b]	No reduction[b,d]
$T(SH)_2$	−0.242[8]	113 ± 5 min (pH 6.4)[c]	402 ± 5 min (pH 6.4)[c]
		360 min (pH 7.4)[c]	> 20 h (pH 7.4)[e]
Ascorbic acid	−0.058[17]	No reduction[c,d]	No reduction[c,d]

[a] All data were obtained at pH 6.4 unless specified, $t_{½}$ = 1/(k[thiolate]). [b] ^{1}H NMR measurement (pH 6.4 and 7.4) [c] By UV–vis and ^{1}H NMR methods [d] No reduction within 7 days [e] Little reduction within 24 h (< 10% reduction measured after 24 h).

Based on these data, we propose that the reaction between Sb^V and $T(SH)_2$ involves two (discrete) steps: the reduction of Sb^V to Sb^{III} by trypanothione, followed by the binding of the Sb^{III} to a second trypanothione molecule.[12] A similar mechanism was suggested 70 years ago for arsenic,[13] although arsenate reductase was shown to be more relevant later.[2] In each step, one mole equivalent of $T(SH)_2$ is consumed, hence two mole equivalents of $T(SH)_2$ are thus required for the complete reduction/complexation of one mole Sb^V.

In conclusion, $T(SH)_2$ can readily reduce antimony(v) (as sodium stibogluconate) to Sb^{III} under both mildly acidic (pH 6.4) and neutral conditions (pH 7.4) at 310 K, in contrast to the tripeptide glutathione, the mammalian analogue of trypanothione.[4] Indeed, pharmacokinetic studies have demonstrated that only a few percent of Sb^V was transformed to Sb^{III} in human subjects.[14] Despite $T(SH)_2$ being present in both life-cycle stages of the parasites, the lower intracellular pH,[15] and higher temperature of amastigotes (pH 6.4, 310 K) compared with promastigotes (pH *ca.* 6.8, 298 K) suggest the rapid reduction of Sb^V by the unique $T(SH)_2$ is favored in amastigotes compared to promastigotes.[15] These data are in good agreement with recent measurements of Sb^{III} and Sb^V (levels) in *Leishmania donovani* based on hydride generation-inductively coupled plasma-mass spectrometry (HG-ICP-MS).[1] Up to 30% of the Sb^V was reduced to Sb^{III} in amastigotes (310 K) and no reduction of Sb^V to Sb^{III} was observed in promastigotes (299 K). Therefore, the unique low molecular thiol-containing molecule $T(SH)_2$ may play an important role in this reduction process and consequently in the activation of the drug. Further biological experiments are warranted to confirm this hypothesis.

This work was supported by the Research Grant Council of Hong Kong (HKU7110/02P), Area of Excellence Scheme of University Grants Committee, University of Hong Kong (UGC), Hung Hing Ying Physical Sciences Research Fund.

Notes and references

1 P. Shaked-Mishan, N. Ulrich, M. Ephros and D. Zilberstein, *J. Biol. Chem.*, 2001, **276**, 3971.
2 (*a*) I. Zegers, J. C. Martins, R. Willem, L. Wyns and J. Messens, *Nat. Struct. Biol.*, 2001, **8**, 843; (*b*) P. Martin, S. DeMel, J. Shi, T. Gladysheva, D. L. Gatti, B. P. Rosen and B. F. P. Edwards, *Structure*, 2001, **9**, 1071; (*c*) M. J. Tamás and R. Wysocki, *Curr. Genet.*, 2001, **40**, 2; (*d*) B. P. Rosen, *Trends Microbiol.*, 1999, **7**, 207.
3 J. Messens, J. C. Martins, E. Brosens, K. V. Belle, D. M. Jacobs, R. Willem and L. Wyns, *J. Biol. Inorg. Chem.*, 2002, **7**, 146.
4 F. Frezard, C. Demicheli, C. S. Ferreira and M. A. P. Costa, *Antimicrob. Agents Chemother.*, 2001, **45,** , 913.
5 R. Mukhopadhyay, J. Shi and B. P. Rosen, *J. Biol. Chem.*, 2000, **275**, 21149.
6 A. H. Fairlamb, P. Blackburn, P. Ulrich, B. T. Chait and A. Cerami, *Science*, 1985, **227**, 1485.
7 A. H. Fairlamb, *Progress in Polyamine Research: Novel Biochemical, Pharmacological, and Clinical Aspects.* 1988, pp. 667–674.
8 A. H. Fairlamb and A. Cerami, *Ann. Rev. Microbiol.*, 1992, **46**, 695–729.
9 C. Dumas, M. Ouellette, J. Tovar, M. L. Cunningham, A. H. Fairlamb, S. Tamar, M. Olivier and B. Papadopoulou, *EMBO J.*, 1997, **16**, 2590.
10 J. Tovar, M. L. Cunningham, A. C. Smith, S. L. Croft and A. H. Fairlamb, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 5311.
11 J. A. Burns, J. C. Butler, J. Moran and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 2648.
12 S. Yan, K. Y. Ding, L. Zhang and H. Sun, *Angew. Chem., Int. Ed.*, 2000, **39**, 4260.
13 A. Cohen, H. King and W. I. Strangeways, *J. Chem. Soc.*, 1932, 2505.
14 T. Gebel, *Chem-Biol. Interact.*, 1997, **107**, 131.
15 N. Marchesini and R. Docampo, *Mol. Biochem. Parasitol.*, 2002, **119**, 225.
16 R. A. Albert, *Arch. Biochem. Biophys.*, 2001, **398**, 94.
17 M. B. Davids, J. Austin and D. A. Partridge, *Vitamin C: Its Chemistry and Biochemistry*, Royal Society of Chemistry, 1991, pp.115–146.