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Thiol-induced reduction of antimony(V) into antimony(III): A comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione

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Abstract

Gluthathione (GSH) has been previously shown to promote the reduction of pentavalent antimony (Sb(V)) into the more toxic trivalent antimony (Sb(III)) in the antimonial drug, meglumine antimonate. However, this reaction occurred at acidic pH (pH 5) but not at the pH of the cytosol (pH 7.2) in which GSH is encountered. The aim of the present study was to further characterize the reaction between thiols and antimonial drugs, addressing the following aspects: (i) the reducing activity of cysteine (Cys) and cysteinyl-glycine (Cys-Gly), expected to be the predominant thiols in the acidic compartiments of mammalian cells; (ii) the reducing activity of trypanothione ($T(SH)_2$), the main intracelular thiol in *Leishmania* parasites; (iii) the influence of the state of complexation of Sb(V) on the rate of Sb(V) reduction. We report here that Cys, Cys-Gly and $T(SH)_2$ did promote the reduction of Sb(V) into Sb(III) at 37 °C. Strikingly, the initial rates of reduction of Sb(V) were much greater in the presence of Cys-Gly, Cys and $T(SH)_2$ than in the presence of GSH. These reactions occurred at both pH 5 and pH 7 but were favored at acidic pH. Moreover, our data shows that Sb(V) is reduced more slowly in the form of meglumine antimonate than in its non-complexed form, indicating that the complexation of Sb(V) tends to slow down the rate of its reduction. In conclusion, our data supports the hypothesis that Sb(V) is reduced *in vivo by* $T(SH)_2$ within *Leishmania* parasites and by Cys or Cys-Gly within the acidic compartments of mammalian cells.

Introduction

The pentavalent organoantimonial complexes, meglumine antimonate and sodium stibogluconate, are the first line drugs for the treatment of all forms of leishmaniasis (Marsden 1985; Berman 1988; Berman 1997). While the toxicity of pentavalent antimonials is much less than that of trivalent compounds, it is not negligible. Most side effects appear at the end of courses of therapy, the most common effects being arthralgias, myalgias, anorexia, elevation of hepatocellular enzymes and eletrocardiographic abnormalities (Marsden 1985).

Despite their clinical use for over half a century, the metabolism of pentavalent antimonials in mammals (Gebel 1997) and their mechanisms of action and toxicity (Berman 1997; Frézard *et al.* 2001; Shaked-Mishan *et al.* 2001; Demicheli *et al.* 2002) remain poorly understood. The hypothesis that pentavalent antimony (Sb(V)) acts as a prodrug, that is converted to active and more toxic trivalent antimony (Sb(III)), was first suggested by Goodwin & Page (1943). Later, it was clearly established that a significant fraction of Sb(V) was reduced into Sb(III) following the administration of pentavalent antimonials in humans (Petit de Pena *et al.* 1990; Burguera *et al.* 1993).

Leishmania parasites may also play a role in the metabolism of Sb(V), as hamsters experimentally infected with *Leishmania* and treated with meglumine antimonate showed altered antimony biodistribution and Sb(V)-to-Sb(III) conversion, when compared to healthy treated animals (Lugo *et al.* 1994).

The location where Sb(V) reduction occurs as well as the biomolecule that promotes this conversion have been the subject of two recent investigations. Shaked-Mishan *et al.* (2001) identified an intracellular Sb(V) reducing activity in *Leishmania donovani* amastigotes that was found to be deficient in a stibogluconate-resistant mutant. On the other hand, Frézard *et al.* (2001) demonstrated that reduced glutathione (GSH) promotes the reduction of Sb(V) into Sb(III) in meglumine antimonate, as previously reported for arsenate (Delnomdedieu *et al.* 1994), suggesting that GSH-induced reduction may occur *in vivo*. Nevertheless, this reaction occurred at acidic pH (pH 5) but not at the pH of the cytosol (pH 7.2) in which GSH is encountered. In the proposed model, important aspects still have to be addressed. First, the low rate of reaction between GSH and Sb(V) in the form of meglumine antimonate might be attributed to a slow oxidation-reduction reaction between Sb(V) and GSH and/or to the ability of Sb(V) complexation to N-methylglucamine to protect Sb(V) from reduction. Thus, the extent of influence of Sb(V) complexation state on the rate of reaction should be examined. Secondly, cysteine (Cys) and cysteinyl-glycine (Cys-Gly), but not GSH, are expected to be the predominant thiols within lysosomes (Mego 1985; Gainey *et al.* 1996), the main acidic organelle of the cell. Moreover, the glutathione-spermine conjugate, trypanothione, is the predominant thiol within the parasite (Fairlamb & Cerami 1992). Therefore, the ability of these thiols to promote the reduction of Sb(V) remains to be evaluated.

In this paper, we compared Cys, Cys-Gly, $T(SH)_2$ and GSH as reducing agents for Sb(V). Furthermore, the influence of the state of complexation of Sb(V) on the rate of reduction was also investigated, through the evaluation of Sb(V) in the form of meglumine antimonate and antimonate (the non-complexed form of Sb(V)).

Materials and methods

Materials

GSH, oxidized glutathione (GSSG), Cys-Gly and Cys were obtained from Sigma Chemical Co. Potassium hexahydroxoantimonate (antimonate) was obtained from Fluka Chemie GmbH. N-methyl-glucamine, bromopyrogallol red (BPR), potassium antimony tartrate and tris(2-carboxyethyl)phosphine (TCEP) were obtained from Aldrich Chemical Co.. Oxidized trypanothione ($T(S)_2$) was obtained from Bachem. All other reagents were of at least reagent grade. Double-distilled-deionized water was used throughout the experiments.

Preparation of antimonials

Meglumine antimonate was synthesized, as previously described (Demicheli *et al.* 1999), from equimolar amounts of N-methyl-glucamine and pentavalent antimony oxyhydrated. Meglumine antimonate contained approximately 30% of antimony by weight, as determined by atomic absorption spectroscopy.

Reduction of oxidized trypanothione and glutathione

$T(S)_2$ and GSSG were reduced with a 2-fold molar excess of TCEP immediately before use (Burns *et al.* 1991; Mukhopadhyay *et al.* 1996).

Study of the reduction of Sb(V) in the presence of different thiols

Solutions containing 5 mM of $T(SH)_2$ or 10 mM of GSH, Cys-Gly or Cys were prepared in KCl 0.15 M at pH 5 or pH 7. The antimonial compound was then added at a final antimony concentration of 2 mM and the mixture was incubated at 37 °C. All solutions were deoxygenated by bubbling with argon and tubes were flushed with argon before closing, to protect thiols from air oxidation. The amount of Sb(III) was then determined at different times of incubation. The initial rate of reduction was determined in the initial and linear portion of the kinetic of reduction and was given as the molar concentration of antimony reduced per minute.

Determination of Sb(III)

The procedure used to determine Sb(III) was described in details previously (Frézard *et al.* 2001). It is based

on the specific interaction of Sb(III) with the chromogen bromopyrogallol red (BPR). The absorbance of BPR at 560 nm decreases proportionally to the amount of Sb(III) in the analyte solution, as a consequence of the formation of the 1:1 BPR-Sb(III) complex. Briefly, 0.5 ml of analyte solution was prepared from 0.1 ml of 0.1 M phosphate, 0.01 ml of 5% (w/v) tartrate, 0.05 ml of 350 μM BPR solution in 1:1 water/ethanol (v/v) and 0.34 ml of water. The pH was then adjusted to 6.8 using sodium hydroxide. The absorbance was registered at 560 nm before (A_o) and after (A_m) adding 5 μl of the sample to be analyzed, so as to obtain a final antimony concentration of 20 μM. For each experiment, a calibration curve was established using potassium antimony tartrate as the source of Sb(III) and plotting the difference in absorbance (A_o-A_m) as a function of Sb(III) concentration. We checked that neither Sb(V) nor thiols interfered with the colorimetric test. Moreover, we observed that the presence of the thiol in the analyte solution did not interfere with the formation of the BPR-Sb(III) complex.

Results

The fraction of Sb(III) originally present in the antimonial compounds, antimonate and meglumine antimonate, was measured using the chromogen BPR and was found to be less than 0.2% of total antimony, indicating that antimony was initially in the pentavalent state.

Reduction activities of cysteine, cysteinyl-glycine and glutathione

Figure 1 shows the kinetics of reduction of Sb(V) into Sb(III) obtained, upon incubation of antimonate at 37 °C in the presence of 10 mM Cys-Gly, Cys or GSH. Since no significant reduction was observed when antimonate was incubated in the absence of thiols (data not shown), the appearance of Sb(III) could be attributed to the occurrence of an oxidation-reduction reaction between Sb(V) and the thiol compounds. Strikingly, the reduction of Sb(V) was found to be much faster in the presence of Cys-Gly and Cys than in the presence of GSH. Table 1 shows the initial rates of reduction determined from these kinetics. The initial rates obtained at pH 5 in the presence of Cys-Gly and Cys were about 50- and 20-fold greater, respectively, than in the presence of GSH. Reactions were run at two different pH. The pH value of 7 mimics that of the extracellular medium and the cytosol, whereas the pH value of 5 mimics that of the lysosomes. Sb(V) reduction occurred at both pH, however, it was favored at acidic pH, as the initial rates of reduction at pH 5 were about 3 times as high as those at pH 7.

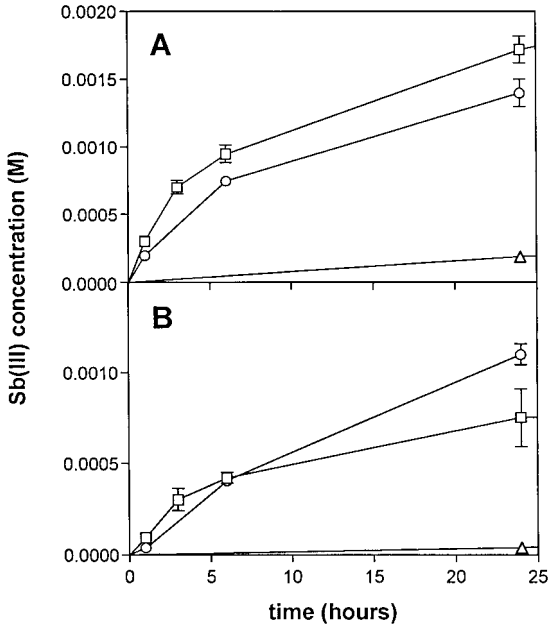

Fig. 1. Kinetics of reduction of Sb(V) into Sb(III) at 37 °C in antimonate (potassium hexahydroxoantimonate), in the presence of Cys-Gly (□), Cys (◯) or GSH (△), at pH 5 (A) or pH 7 (B). Antimonate was initially present at a final concentration of 2 mM. Thiol concentration = 10 mM. The results are expressed as means ± standard deviations (error bars) ($n = 3$).

Table 1 displays also the initial rates of Sb(V) reduction determined in the presence of the three thiols, when Sb(V) was presented as meglumine antimonate. The same tendencies were observed as with antimonate. First, reactions were faster at acidic pH when compared to neutral pH. Secondly, the rates of reduction observed with the different thiols could be ordered according to the following sequence: Cys-Gly > Cys >> GSH. On the other hand, the reactions were found to be much slower with meglumine antimonate than with antimonate. For instance, the initial rates determined at pH 5 were about 8-fold lower with meglumine antimonate than with antimonate. Since antimonate is a non-complexed form of Sb(V), our data indicates that the complexation of Sb(V) slows down the rate of its reduction by the thiol compound.

Table 1. Initial rates of reduction (μM min^{-1}) of 2 mM Sb(V) at 37 °C in the presence of 10 mM Cys-Gly, Cys or GSH. Data are given as means ± standard deviations ($n = 3$).

Thiol	Antimonate		Meglumine antimonate	
	pH 5	pH 7	pH 5	pH 7
Cys-Gly	5.1 ± 0.4	1.6 ± 0.3	0.60 ± 0.16	0.17 ± 0.07
Cys	3.3 ± 0.3	1.1 ± 0.1	0.44 ± 0.03	0.13 ± 0.01
GSH	0.19 ± 0.01	0.026 ± 0.007	0.042 ± 0.001	0.013 ± 0.001

Table 2. Initial rates of reduction (μM min^{-1}) of Sb(V) at 37 °C in the presence of $T(SH)_2$, GSH, Cys and TCEP. Data are given as means ± standard deviations ($n = 4$).

Reactants	Antimonate		Meglumine antimonate	
	pH 5	pH 7	pH 5	pH 7
2 mM Sb(V) + 5 mM $T(S)_2$ 10 mM TCEP	13 ± 1	2.1 ± 0.2	2.2 ± 0.2	0.50 ± 0.16
2 mM Sb(V) + 10 mM GSSG 10 mM TCEP	2.2 ± 0.2	0.11 ± 0.03	0.54 ± 0.02	0.10 ± 0.08
2 mM Sb(V) + 10 mM Cys 5 mM TCEP	22 ± 1	2.9 ± 1.3	3.9 ± 0.5	0.43 ± 0.03
2 mM Sb(V) + 5 mM TCEP	0.37 ± 0.21	–[a]	0.061 ± 0.005	–[a]

[a]No significant reduction.

Reduction activities of trypanothione and glutathione, pre-reduced with TCEP

Considering that trypanothione is the main thiol in *Leishmania* (Fairlamb & Cerami 1992) and that *Leishmania* amastigotes can promote the reduction of Sb(V) into Sb(III) (Shaked-Mishan *et al.* 2001), $T(SH)_2$ is a likely candidate as a reducing agent for Sb(V). Therefore, the ability of $T(SH)_2$ to promote the reduction of Sb(V) was evaluated and compared to that of GSH. As trypanothione is sold commercially as the disulphide, it had to be reduced. Reduction was carried out just before use by incubating $T(S)_2$ with a 2-fold molar excess of TCEP (Mukhopadhyay *et al.* 1996). Taking into account that only half of TCEP is expected to be oxidized in this first step (Burns *et al.* 1991), the ability of TCEP to promote the reduction of Sb(V) also had to be evaluated.

The kinetics of reduction of Sb(V) at 37 °C in the presence of TCEP (5 mM) and freshly reduced $T(SH)_2$ (5 mM) and GSH (10 mM) are shown in Figures 2 and 3 for antimonate and meglumine antimonate, respectively. TCEP promoted the reduction of Sb(V) at pH 5, but not at pH 7. Nevertheless, the extent of Sb(V) reduction by TCEP at pH 5 was much lower than in the presence of GSH and $T(SH)_2$. Importantly, the initial rates presented in Table 2 establish that the reaction in the presence $T(SH)_2$ is about 4-fold faster at pH 5 and more than 7-fold faster at pH 7 than in the presence of GSH. In the presence of $T(SH)_2$, like with the other thiols, the reaction was found to depend on the pH and on the state of complexation of Sb(V). The initial rates of reduction were more than 4-fold higher at pH 5 than at pH 7. Moreover, at pH 5, Sb(V) was reduced at a 6-fold higher rate in the form of antimonate than in the form of meglumine antimonate.

It is noteworthy that GSH-mediated reduction at pH 5 was faster in the presence of TCEP (Table 2) than in its absence (Table 1). This difference may be attributed to the fraction of TCEP remaining in the freshly reduced glutathione that is expected to

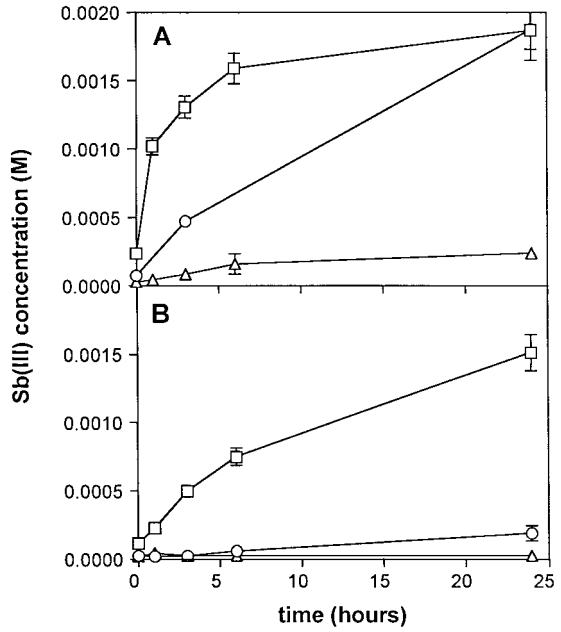

Fig. 2. Kinetics of reduction of Sb(V) into Sb(III) at 37 °C in antimonate (potassium hexahydroxoantimonate), in the presence of 5 mM $T(SH)_2$ (□), 10 mM GSH (◯) or 5 mM TCEP (△), at pH 5 (A) or pH 7 (B). Antimonate was initially present at a final concentration of 2 mM. The results are expressed as means ± standard deviations (error bars) ($n = 4$).

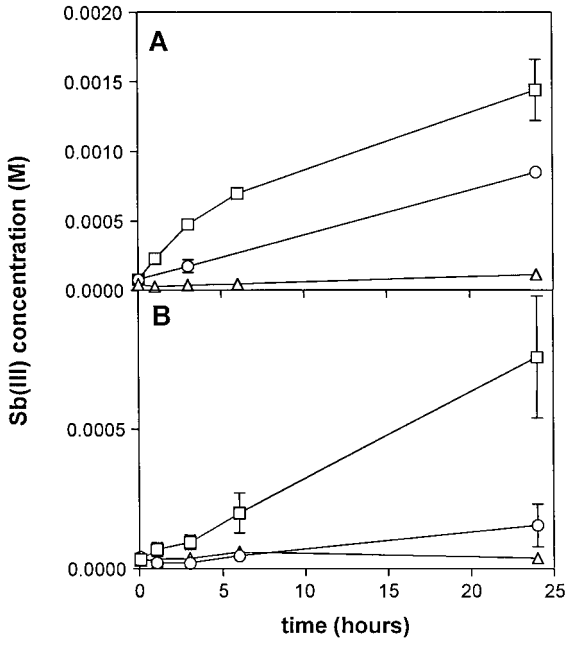

Fig. 3. Kinetics of reduction of Sb(V) into Sb(III) at 37 °C in meglumine antimonate, in the presence of 5 mM $T(SH)_2$ (□), 10 mM GSH (◯) or 5 mM TCEP (△), at pH 5 (A) or pH 7 (B). Meglumine antimonate was initially present at a final concentration of 2 mM. The results are expressed as means ± standard deviations (error bars) ($n = 4$).

re-reduce GSSG produced by the oxidation-reduction reaction. Our interpretation is further supported by the observation that Cys reduced Sb(V) more rapidly in the presence of TCEP (Table 2) than in its absence (Table 1).

Discussion

The present data extends our previous study about the influence of thiols on the reduction of Sb(V) into Sb(III) (Frézard *et al.* 2001). We reported previously that GSH promotes the reduction of Sb(V) into Sb(III) in meglumine antimonate and proposed the following oxidation-reduction reaction:

$$SbO_3^- + 5GSH + H^+ \leftrightarrow Sb(GS)_3 + GSSG + 3H_2O, \quad (1)$$

where SbO_3^- is the main soluble form of Sb(V), GSSG is the oxidized form of glutathione and $Sb(GS)_3$ is the complex between Sb(III) and GSH (Sun *et al.* 2000).

The present study establishes that $T(SH)_2$, Cys and Cys-Gly also promote the reduction of Sb(V) into Sb(III) in conditions close to physiological ones. More importantly, the rate of reduction was much higher in the presence of $T(SH)_2$, Cys or Cys-Gly than in the presence of GSH. Several different factors may account for this difference, such as steric factors and the presence of a charged amino group at the cysteine Cα position in Cys and Cys-Gly (electrostatic factor). In the case of $T(SH)_2$, the dithiol character of the molecule may contribute to its elevated reducing activity. The following oxidation-reduction reaction can also be proposed:

$$SbO_3^- + 2T(SH)_2H^+ + H^+ \leftrightarrow SbT(S)_2^+ + T(S)_2H^+ + 3H_2O, \quad (2)$$

where $SbT(S)_2$ is the expected Sb(III)-trypanothione complex in solution, as previously evidenced using MALDI mass spectrometry (Mukhopadhyay *et al.* 1996). Obviously, further studies are needed in order to clarify the mechanism(s) responsible for the higher rate of reduction in the presence of these thiols.

The state of complexation of Sb(V) was found to influence the rate of its reduction by thiols. The presentation of Sb(V) as an organoantimonial complex slowed down the rate of its reduction, suggesting that either Sb(V) is reduced at a slower rate in its complexed state or antimony complexes first have to dissociate to allow for Sb(V) reduction. As an implication of this data, the rate of reduction should also depend on the type of ligand involved in the antimony complex.

Reactions occurred at both pH 5 and pH 7, but were favored at acidic pH. Such a pH-dependence was expected, since protons participate as reactants in reactions (1) and (2). From the pharmacological point of view, this observation is also important as *Leishmania* parasites reside in the secondary lysosomes of macrophages, whose pH is about 5 (Alexander & Russel 1992), and Sb(V) has to cross this acidic compartment to reach the parasite.

The present study represents an important step toward the understanding of the metabolism of antimonials in mammals and their mechanisms of action and toxicity. It led us to the identification of three potential reducing biomolecules for Sb(V). Moreover, it suggests two potential locations where this conversion may occur: the acidic compartments of mammalian cells, including the phagolysosome of macrophages in which *Leishmania* resides, and the cytosol of the parasite. In this context, $T(SH)_2$ would exhibit two antagonist actions with respect to pentavalent antimonials. According to our results, $T(SH)_2$ may increase the activity of these drugs by promoting the reduction of Sb(V). On the other hand, $T(SH)_2$ is expected to promote a detoxification for the parasite by forming a complex with Sb(III), subsequently extruded by an ATP-coupled efflux pump (Mukhopadhyay *et al.* 1996). Importantly, our data also supports the concept that thiol-induced reduction can take place in vivo without the need for enzymatic catalysis. Future studies should be aimed at the investigation of Sb(V) reducing activity in mammalian cells as well as at the demonstration of the effective role of thiols in this conversion.

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