Interaction of trivalent antimony with a CCHC zinc finger domain: potential relevance to the mechanism of action of antimonial drugs†

Cynthia Demicheli,*[a] Frédéric Frézard,[b] John B. Mangrum[c] and Nicholas P. Farrell*[c]

Received (in Cambridge, UK) 2nd June 2008, Accepted 10th July 2008
First published as an Advance Article on the web 26th August 2008
DOI: 10.1039/b809186b

Sb^{III} competes with Zn^{II} for its binding to the CCHC zinc finger domain of the NCp7 protein of HIV-1, indicating that zinc finger proteins may be targets for antimony-based drugs and thus responsible for their important pharmacological actions.

In this communication, we report for the first time the interaction of Sb^{III} with a zinc finger peptide model (ZF) and its ability to promote Zn^{II} ejection. These results suggest that zinc finger proteins may be targets for antimony-based drugs and thus responsible for their important pharmacological actions. Sb^{V} needs to be reduced to the trivalent cation before ZF reaction. Mass spectrometric studies further suggest that the incorporation of Sb^{III} may be facilitated by glutathione (GSH), through formation of a novel biomolecule–Sb–GSH conjugate.

trans-Acting factors such as DNA- or RNA-binding proteins have conserved structural domains that mediate and stabilize their binding to nucleic acid molecules. The zinc finger domain is characterized by the coordination of a zinc atom by several amino acid residues, usually cysteines and histidines. These structural elements are associated with protein–nucleic acid and protein–protein interactions as well as extraordinarily diverse functions, including DNA recognition, RNA packaging, protein folding and assembly, lipid binding, transcriptional activation, cell differentiation and growth and regulation of apoptosis.[1]

One subtype of the zinc finger domain is the $CX_2CX_4HX_4C$ (CCHC) structure that occurs twice in the nucleocapsid protein p7 (NCp7) of human immunodeficiency virus type 1 (HIV-1) and is absolutely conserved among all known strains of retroviruses, except human foamy viruses.[2] NCp7 protein contributes to the selection and packaging of the viral genome, as well as exercising additional functions critical to viral replication, through interactions with single-strand nucleic acids and viral proteins.[3]

Several zinc finger proteins sharing the CCHC motif have also been identified in trypanosomatids and have been shown to be involved in different cellular functions. In *Leishmania major*, the protein HEXBP, containing nine CCHC motifs, binds to the hexanucleotide repeat sequence found in the intervening region of the GP63 gene cluster, the most abundant surface glycoprotein of this protozoan, and it is likely to be involved in DNA replication, structure and repair.[4]

Antimony-containing compounds have been used for almost a century in the clinical treatment of the parasitic disease, leishmaniasis. About 60 years ago, trivalent antimonial drugs were substituted by less toxic pentavalent antimonials in the treatment of this disease. More recently, the pentavalent antimony compounds, ammonium-21-tungsto-9-antimoniate and NSC 13778 have been shown to exert anti-HIV activities.[5,6]

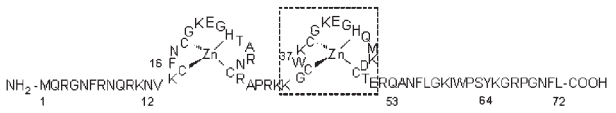
Despite their clinical use for several centuries, the mechanism of action of antimonial drugs is still poorly understood and the final molecular target for Sb^{III} has not yet been identified.[7] The hypothesis that Sb^{V} acts as a prodrug, being converted to active and more toxic Sb^{III}, was first suggested by Goodwin and Page.[8] Later, it was clearly established that a significant fraction of Sb^{V} is reduced to Sb^{III} following the administration of pentavalent antimonials in humans.[9] Whether reduction occurs enzymatically or is simply mediated by plasma or cellular thiols remains to be defined.[10]

We hypothesized that zinc finger domains may be a target for antimonial drugs, based on the following observations. First, Sb^{III} and thiolates are substantially soft electrophilic and nucleophilic species, respectively, with high stability complexes formed between Sb^{III} and the biologically relevant thiols GSH and trypanothione.[11] Moreover, statistically significant correlations were found between the reactivities of nucleophilic cysteine thiolates in NCp7 and the ability of a variety of electrophilic agents to function as soft electrophiles.[12] Secondly, the antimony-containing compound NSC 13778 was found to effectively inhibit the nucleic acid chaperone activity of NCp7 protein, although its detailed mechanism of action may lie in blocking viral entry into CD4 cells.[13] Thirdly, several metals, including Cd^{II}, Co^{II}, Ni^{II} and Pt^{II} have been found to substitute Zn^{II} in CCHC zinc finger domains.[14] As^{III}, Pb^{II}, Cd^{II} and Co^{II} also displace Zn^{II} from CCCC zinc finger domains, such as those encountered in DNA repair proteins such as XPA.[15]

The ZF peptide model used in this study is (34–51)NCp7, a peptide corresponding to the distal (C-terminal) finger motif of NCp7 (outlined sequence in Scheme 1). The choice of this

[a] *Departamento de Química, ICEX, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. E-mail: demichel@dedalus.lcc.ufmg.br; Fax: +55 3134095700; Tel: +55 3134095755*
[b] *Departamento de Fisiologia e Biofísica, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. E-mail: frezard@icb.ufmg.br; Fax: +55 3134092924; Tel: +55 3134092940*
[c] *Department of Chemistry, Virginia Commonwealth University, 1001 W. Main Street, Richmond, VA 23284, USA. E-mail: npfarrell@vcu.edu; Fax: +1 804 828 8599; Tel: +1 804 828 6320*

† Electronic supplementary information (ESI) available: Details on experimental determination of K and K'_{SbF}. See DOI: 10.1039/b809186b



Scheme 1 Sequence of the entire HIV nucleocapsid NCp7 protein.

isolated CCHC motif is justified because: (i) the binding information is essentially encoded by the CCHC motifs themselves; (ii) the peptide is characterized by a single Trp residue that constitutes a sensitive intrinsic fluorescence probe for the binding of Zn^{II}—the high quantum yield of Trp37 depending strongly on Zn^{II} binding and the resulting folded and highly constrained structure of the peptide.[16]

Addition of Sb^{III} (as $Sb(GS)_3$ generated by reduction of 4 mM $KSb(OH)_6$ in the presence of 40 mM GSH with complete reduction confirmed using a specific colorimetric assay) to Zn^{II}-bound peptide resulted in a concentration-dependent decrease of the peptide intrinsic fluorescence.‡ The $Sb(GS)_3$ complex is the expected intracellular chemical form of Sb^{III}.[11a]

Fig. 1 shows the fluorescence titration of the Zn^{II}-associated peptide with Sb^{III} at different pH values, in the absence (a) and presence (b) of the chelator, EDTA. The addition of Sb^{III} led to the extinction of peptide intrinsic fluorescence, indicating the formation of a complex between Sb^{III} and ZF and supporting a change in the original peptide geometry. In the absence of EDTA, the effectiveness of Sb^{III} in quenching peptide fluorescence was strongly dependent on pH, being favored at acidic pH. In the presence of 0.1 mM EDTA, the maximum quenching was achieved at a Sb : peptide ratio of about 1 at both pH 6 and 7 supporting the formation of a 1 : 1 Sb–peptide complex. It is noteworthy that no quenching effect was observed when adding GSH or Sb^{V} in the form of $KSb(OH)_6$ or NSC 13778 (data not shown). Taking into account that the stability constant of the Zn–peptide complex strongly depends on pH and decreases at acidic pH,[16a] our data are consistent with the formation of the Sb^{III}–peptide complex with concomitant Zn^{II} ejection. The existence of a competition between Sb^{III} and Zn^{II} for peptide binding was further supported, as shown in Fig. 1(c), by the observation that addition of $ZnCl_2$ to a preformed 1 : 1 Sb^{III}–peptide complex (prepared by adding to the peptide solution an equimolar amount of $Sb(GS)_3$) promoted the recovery of ZF intrinsic fluorescence. These data also established the reversibility of Sb^{III} binding to the peptide.

The ability of Sb^{III} to form a 1 : 1 Sb–peptide complex and to promote Zn^{II} ejection from its binding site was further confirmed by ESI-MS analysis.§ The ESI(+)-MS spectrum displayed in Fig. 2 shows peak A at m/z 1111.7, peak B at m/z 1172.1 and peak C at m/z 1324.6, corresponding to the 2^+ state of the free peptide, the 1 : 1 Sb^{III}–peptide complex and the 1 : 1 adduct between the peptide and a 1 : 1 Sb–GSH complex, respectively (the total intensity of A,B,C was approximately 10% with major species being free GSH under these conditions). As illustrated in the Fig. 2 insets, the species responsible for peaks B and C were easily characterized as containing Sb by the distinctive isotope pattern of Sb (ratio of ^{121}Sb : ^{123}Sb, 57 : 43). To our knowledge, this is the first demonstration of a conjugate Sb–GSH–biomolecule. The assignment of the peak at m/z 1260.0 is equivocal. Mass

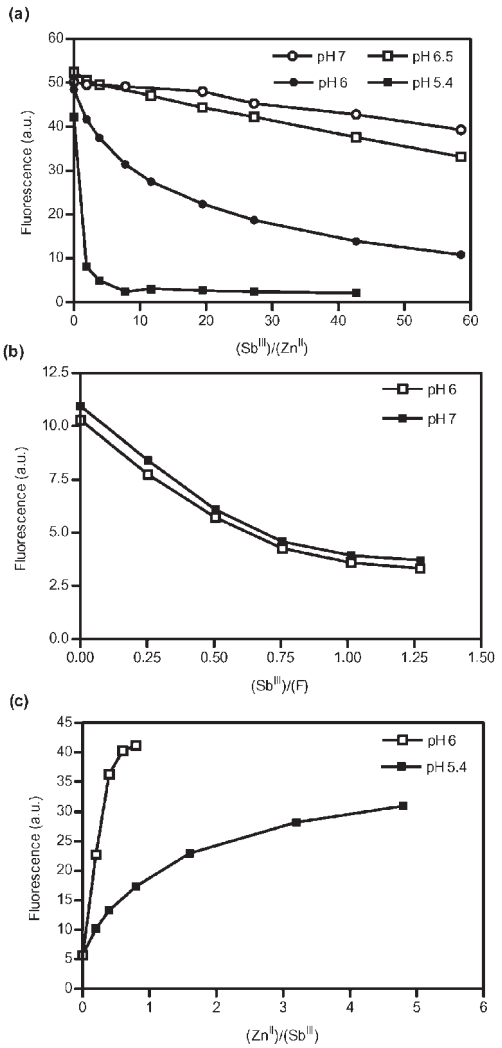


Fig. 1 Binding of Sb^{III} to (34–51)NCp7 peptide (F) and competition with Zn ion at different pHs and 298 K, as measured using peptide intrinsic fluorescence. Fluorescence titration of 3.3 μM ZF with Sb^{III} in 20 mM phosphate buffer: (a) in the absence or (b) presence of 0.1 mM EDTA; (c) titration of Sb^{III}-bound peptide with $ZnCl_2$ in phosphate buffer. Fluorescence intensities were measured with excitation and emission at 280 nm and 360 nm, respectively, and were corrected for dilution effects.

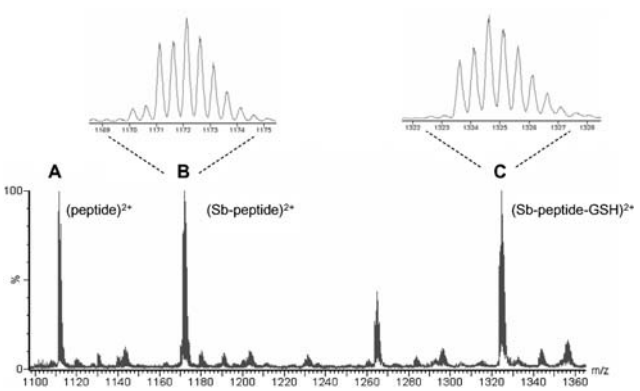


Fig. 2 ESI-MS spectrum in the positive mode of (34–51)NCp7 peptide in the presence of equimolar amounts of $Sb^{III}(GS)_3$ and $Zn^{II}(acetate)_2$.

measurement suggests a 1 : 1 free peptide : GSH species but isotopic distribution shows the presence of Sb; peptide cleavage may have occurred.

To determine the Sb^{III} binding constant to the peptide, preformed ZF peptide was titrated with the trivalent antimonial compound, potassium antimony tartrate, at pH 6, 298 K and $I = 0.1$ M (see ESI†).

The following reaction and equilibrium constant were considered:

$$SbT^- + ZnF^- + 2H^+ \leftrightarrow SbF + H_2T^{2-} + Zn^{II} \quad (1)$$

$K = [SbF][H_2T^{2-}][Zn^{II}]/[SbT^-][ZnF^-][H^+]^2$, where H_2T^{2-}, Zn^{II}, SbT^-, SbF and ZnF^- represent tartrate with deprotonated carboxylic groups, the free Zn^{II} cation, the 1 : 1 Sb^{III}–tartrate, the 1 : 1 Sb^{III}–peptide and the 1 : 1 Zn^{II}–peptide complex, respectively.

Using the fluorescence data obtained from the titration experiment, a value of $K = 22\,820 \pm 1890$ was determined.†

The Sb^{III} binding constant to the peptide, $K'_{SbF} = [SbF]/[SbO^+][H_3F]$ (where SbO^+ and H_3F represent the reactive antimony species and apopeptide), was determined by (i) taking into account the known values for the equilibrium constants, K'_{ZnF}[16b] and K_{SbT},[17] defined as follows:

$$K'_{ZnF} = [ZnF^-]/[Zn^{II}][H_3F] = 10^{7.5}\ M^{-1}$$

$$K_{SbT} = [SbT^-]/[SbO^+][H_2T^{2-}] = 1.64 \times 10^8\ M^{-1}$$

and (ii) using the following equation:

$$K = K'_{SbF}/K_{SbT}K'_{ZnF}[H^+]^2$$

K'_{SbF} was determined at pH 6 and 298 K as $= 1.18 \times 10^8\ M^{-1}$.

We conclude that Sb^{III} competes with Zn^{II} for its binding to the CCHC zinc finger domain, this competition being favored for Sb^{III} at pH lower than 7. Such pH dependence may contribute to the specificity of Sb^{III} action toward HIV-infected cells and *Leishmania* parasites. Indeed, HIV infection of $CD4^+$ T-lymphoblastoid cells was found to reduce intracellular pH, from approximately 7.2 to below 6.7.[18] On the other hand, a value of 6.4 was previously reported for the intracellular pH of *Leishmania* amastigotes.[19] It is therefore suggested that zinc finger proteins exhibiting the CCHC domain may be targets for antimony-based drugs and thus contribute to their important pharmacological actions. Sb^V needs to be reduced to the trivalent cation before ZF reaction. Further, the results suggest a functional role for glutathione conjugates beyond simply chemical reduction. While GSH has long been recognized as a chemical reductant for biologically active Sb and As species,[10a] to our knowledge no functional role in biomolecule binding has been heretofore observed.

This research was supported by US NSF (CHE0616768) to NPF and postdoctoral fellowships from the Brazilian agencies, CNPq (to C.D.) and CAPES (to F.F.).

Notes and references

‡ Preparation of ZF and Sb^{III}: the free peptide KGCWKCGKEGHQMKDCTE was obtained from Gen Script Corporation (Piscataway, NJ, USA) at a purity higher than 98%. The Zn^{II}-associated peptide was obtained as described previously,[14b] by incubating 1 mM peptide with $Zn(acetate)_2$ at 1 : 1.2 molar ratio for 2 h at pH 6 and 310 K under nitrogen atmosphere. Sb^{III} was obtained, as described previously,[10] through reduction of 4 mM $KSb(OH)_6$ in the presence of 40 mM GSH for 2 h at 310 K under nitrogen atmosphere, followed by pH adjustment to 6. Complete reduction was confirmed through photometric determination of Sb^{III}, utilizing its specific interaction with the bromopyrogallol red chromogen.[10]

§ ESI-MS analysis: the sample was prepared by adding to an aqueous solution of ZF (1 mM) an equimolar amount of the $Sb(GS)_3$ (obtained as above). Positive electrospray ionization (ESI) was performed on a Waters/Micromass QTOF-2 mass spectrometer and was introduced into the inlet at 4.0 uL min^{-1} in water. ESI capillary voltage was held constant at 2.85 kV and a cone voltage of 38 V was used. The ESI source was maintained at 105 °C and N_2 desolvation gas at 100 °C was used throughout the experiment. All spectra were internally calibrated using polyalanine over the entire mass range. Data were processed using Masslynx 4.0 software.

1 (*a*) O. Leon and M. Roth, *Biol. Res.*, 2000, **33**, 21; (*b*) C. O. Pabo and R. T. Sauer, *Annu. Rev. Biochem.*, 1992, **61**, 1053.
2 (*a*) J. M. Berg, *Science*, 1986, **232**, 485; (*b*) T. South, P. Blake, R. Sowder, L. Arthur, L. Henderson and M. Summers, *Biochemistry*, 1990, **29**, 7786.
3 (*a*) J. Darlix, M. Lapada-Tapolsky, H. de Rocquigny and B. Roques, *J. Mol. Biol.*, 1995, **254**, 523; (*b*) S. Ramboarina, N. Romellet, M. C. Fournié-Zalusky and B. P. Roques, *Biochemistry*, 1999, **38**, 12196.
4 J. R. Webb and W. R. McMaster, *J. Biol. Chem.*, 1993, **268**, 13994.
5 (*a*) J. Ficher, L. Richard and R. Weiss, *J. Am. Chem. Soc.*, 1976, **98**, 3050; (*b*) M. Souyri-Caporale, M. G. Tovey, K. Ono, C. Jasmin and J. C. Chermann, *J. Gen. Virol.*, 1984, **65**, 831; (*c*) C. Jasmin, J. C. Chermann, M. Raynaud, G. Werner, N. Raybaud, F. Sinoussi and C. B. Loustau, *Prog. Chemother.*, 1975, **2**, 956; (*d*) R. H. Kimberlin and C. A. Walker, *Arch. Virol.*, 1983, **78**, 9.
6 Y. Quan-en, A. G. Stephen, J. W. Adelsberger, P. E. Roberts, W. Zhu, M. J. Currens, Y. Feng, B. J. Crise, R. J. Gorelick, A. R. Rein, R. J. Fisher, R. H. Shoemaker and S. Sei, *J. Virol.*, 2005, **79**, 6122.
7 S. Yan, J. Jin and H. Sun, in *Metallotherapeutic Drugs and Metal-Based Diagnostic Agents: The Use of Metals in Medicine*, ed. M. Gielen and E. R. Tiekink, John Wiley & Sons, 2005, pp. 441–461.
8 L. C. Goodwin and J. E. Page, *Biochem. J.*, 1943, **22**, 236.
9 (*a*) J. L. Burguera, M. Burguera, Y. Petit de Pena, A. Lugo and N. Anez, *Trace Elem. Med.*, 1993, **10**, 66; (*b*) N. Miekeley, S. R. Mortari and A. O. Schubach, *Anal. Bioanal. Chem.*, 2002, **372**, 495.
10 (*a*) F. Frézard, C. Demicheli, C. S. Ferreira and M. A. P. Costa, *Antimicrob. Agents Chemother.*, 2001, **45**, 913; (*b*) C. S. Ferreira, P. S. Martins, C. Demicheli, C. Brochu, M. Ouellette and F. Frézard, *BioMetals*, 2003, **16**, 441; (*c*) S. Yan, I. L. Wong, L. M. Chow and H. Sun, *Chem. Commun.*, 2003, **21**, 266; (*d*) C. S. Ferreira, A. M. C. Pimenta, C. Demicheli and F. Frézard, *BioMetals*, 2006, **19**, 573.
11 (*a*) H. Sun, S. C. Yan and W. S. Cheng, *Eur. J. Biochem.*, 2000, **267**, 5450; (*b*) S. Yan, F. Li, K. Ding and H. Sun, *JBIC, J. Biol. Inorg. Chem.*, 2003, **8**, 689.
12 A. T. Maynard, M. Huang, W. G. Rice and D. G. Covell, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 11578.
13 M. Cruceanu, A. G. Stephen, P. J. Beuning, R. J. Gorelick, R. J. Fisher and M. C. Williams, *Anal. Biochem.*, 2006, **358**, 159.
14 (*a*) A. Witkiewicz-Kucharczyk and W. Bal, *Toxicol. Lett.*, 2006, **162**, 29; (*b*) A. Anzellotti, Q. Liu, M. J. Bloemink, J. N. Scarsdale and N. Farrell, *Chem. Biol.*, 2006, **13**, 539.
15 (*a*) T. Schwerdtle, I. Walter and A. Hartwig, *DNA Repair*, 2003, **2**, 1449; (*b*) H. A. Godwin, J. C. Payne and M. A. ter Horst, *J. Am. Chem. Soc.*, 1999, **121**, 6850; (*c*) E. Kopera, T. Schwerdtle, A. Hartwig and W. Bal, *Chem. Res. Toxicol.*, 2004, **17**, 1452.
16 (*a*) Y. Mély, H. De Rocquigny, N. Morellet, B. P. Roques and D. Gérard, *Biochemistry*, 1996, **35**, 5175; (*b*) E. Bombarda, N. Morellet, H. Cherradi, B. Spiess, S. Bouaziz, E. Grel, B. P. Roques and Y. Mély, *J. Mol. Biol.*, 2001, **310**, 659.
17 K. C. Samantora, D. V. Raman Rao and S. Pani, *J. Indian Chem. Soc.*, 1955, **32**, 197.
18 A. Makutonina, T. G. Voss, D. R. Plymale, C. D. Fermin, C. H. Norris, S. Vigh and R. F. Garry, *J. Virol.*, 1996, **70**, 7049.
19 N. Marchesini and R. Docampo, *Mol. Biochem. Parasitol.*, 2002, **119**, 225.