Structural and Functional Highlights of Vacuolar Soluble Protein 1 from Pathogen Trypanosoma brucei brucei

Abhishek Jamwal1, Adam R. Round1,2, Ludovic Bannwarth1, Catherine Venien-Bryan1, Hassan Belrhali1,8, Manickam Yogavel1 and Amit Sharma1,2

From the 1Structural and Computational Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India, the 2European Molecular Biology Laboratory, Grenoble Outstation, 38042 Grenoble, France, the 1Unit for Virus Host-Cell Interactions, University Grenoble Alpes-EMBL-CNRS, 38042 Grenoble, France, and the 1IMPMC, UMR 7590, CNRS-UPMC-IRD, 75252 Paris, France

Trypanosoma brucei (T. brucei) is responsible for the fatal human disease called African trypanosomiasis, or sleeping sickness. The causative parasite, Trypanosoma, encodes soluble versions of inorganic pyrophosphatases (PPase), also called vacuolar soluble proteins (VSPs), which are localized to its acidocalcisomes. The latter are acidic membrane-enclosed organelles rich in polyphosphate chains and divalent cations whose significance in these parasites remains unclear. We here report the crystal structure of T. brucei brucei acidocalcisomal PPases in a ternary complex with Mg2+ and imidodiphosphate. The crystal structure reveals a novel structural architecture distinct from known class I PPases in its tetrameric oligomeric state in which a fused EF hand domain arranges around the catalytic PPase domain. This unprecedented assembly evident from TbVSP1 crystal structure is further confirmed by SAXS and TEM data. SAXS data suggest structural flexibility in EF hand domains indicative of conformational plasticity within TbVSP1.

African trypanosomiasis, commonly known as sleeping sickness, affects ~50,000 inhabitants of sub-Saharan Africa yearly (1) with 60 million people at risk of infection (2). Sleeping sickness is caused by two subspecies of T. brucei: T. brucei gambiense and T. brucei rhodesiense. The former alone accounts for ~98% of the cases in humans and livestock (1). T. brucei brucei is another subspecies of T. brucei that is used as an experimental model in laboratory to study sleeping sickness, and this parasite infects animals causing a disease called nagana (3). Left untreated, sleeping sickness is fatal, and there are currently two drugs used to treat the initial phase of the disease: suramin and pentamidine. These are employed in treatment of acidocalcisomiasis caused by T. brucei rhodesiense and T. brucei gambiense infections (4). For the treatment of second or neurological phase, an arsenic-based drug called melarsoprol is used. However, this drug causes severe side effects and can sometimes be lethal (5). A newer and much more expensive drug, eflornithine, is effective only against T. brucei gambiense. (6). A combination of another drug, nifurtimox, with eflornithine has been used for treatment, but unfortunately it is not effective against T. brucei gambiense (6). Therefore, there is a pressing case to find new, safe, inexpensive, and broad spectrum drugs for treating sleeping sickness in humans and livestock.

Soluble inorganic pyrophosphatases (PPase, EC 3.6.1.1)3 is a ubiquitous and essential enzyme that hydrolyzes the PPi generated for treating sleeping sickness in humans and livestock.

Acidocalcisomes are acidic organelles rich in short and long chain polyphosphates complexed with Ca2+ and other divalent cations (16). Trypanosomes carry a subset of soluble PPases called vacuolar soluble proteins (VSPs) that localize to acidocalcisomes and are known for their roles in regulation of acidocalcisome phosphate pool via their PPase and triphosphatase activities (17). There is increasing evidence for the functional importance of VSPs for growth and survival of parasites inside their host (17–19). This has resulted in investigation of VSP as potential inhibitor target and has also highlighted the importance of acidocalcisomal proteins in general (20). A study has shown that small molecule inhibitors of VSP triphosphatase activity can provide protective effects against T. brucei infection in a mouse model (21). However, these inhibitors suffer from poor IC50 values, and further improvement has been suggested for generating more potent compounds. VSPs have mostly been explored from cellular and biochemical perspectives to date, despite the pivotal role of structure-based drug development in modern infectious disease drug discovery (22).

Here, we present the crystal structure of T. brucei brucei VSP1 (TbVSP1) at 2.35 Å resolution in complex with Mg2+ and

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3 The abbreviations used are: PPase, inorganic pyrophosphatase; VSP, vacuolar soluble protein; SAXS, small angle x-ray scattering; TEM, transmission electron microscopy; ITC, isothermal calorimetry; rmsd, root mean square deviation; PDB, Protein Data Bank; polyP, triphosphate.
and IDP. Our analyses reveal an unusual tetrameric quaternary association of TbbVSP1 when compared with other known class I PPases that adopt dimeric or hexameric states. Our TbbVSP1 tetramer structure has been confirmed by SAXS and EM experiments, which indicate flexibility in the EF hands within this protein. This is the first study of structural characterization of an acidocalcisomal protein from protozoan parasites to our knowledge, and provides new structural insights into class I PPases. Our work provides a foundation for further functional dissection and pharmacological exploitation of VSPs.

**Experimental Procedures**

Cloning, Overexpression, and Purification of TbbVSP1—The ORF corresponding to full-length TbbVSP1 containing EF hand domain (residues 1–160) and PPase domain (residues 161–414) was optimized for expression in *E. coli* and cloned into pETM11 vector using NcoI and KpnI restriction sites. Briefly, bacterial cells were lysed by sonication in a buffer containing protease inhibitor mixture. Affinity purification was performed on a nickel-nitrilotriacetic acid (His-Trap FF; GE Healthcare)
column using AKTA-FPLC system (GE Healthcare). The His<sub>6</sub> tag of the protein was removed with TEV protease followed by dialysis in low salt buffer (30 mM HEPES, pH 7.2, 25 mM NaCl, and 1 mM DTT), and it was subsequently applied to Q-Sepharose (GE Healthcare) for further purification and removal of TEV protease by anion exchange chromatography. Purest fractions were pooled and saturated with ammonium sulfate to a final concentration of 1 M, and further polishing and purification was achieved by hydrophobic interaction chromatography on a phenyl FF 16/10 column (GE Healthcare). Finally, purest fractions were pooled and concentrated to 10 mg ml<sup>-1</sup> with 10-kDa cutoff centrifugal devices (Millipore) followed by gel permeation chromatography on S200–16/60 column (GE Healthcare) in a buffer containing 30 mM Na-HEPES, pH 7.2, 50 mM NaCl, and 1 mM DTT.

**Analytical Size Exclusion Chromatography—Oligomerization of TbbVSP1** was examined using analytical size exclusion chromatography (Superdex 200 HR 10/300; GE Healthcare) in 30 mM HEPES, pH 7.2, 500 mM NaCl, and 5 mM β-mercaptoethanol. A total of 500 μl of full-length TbbVSP1 at concentration of 4 mg ml<sup>-1</sup> was applied to the column; separately, 100 μl of N-terminally truncated TbbVSP1 at concentration of 0.5 mg/ml was injected into size exclusion chromatography column. The molecular mass standards used for this study (blue dextran (~2000 kDa), ferritin (~400 kDa), aldolase (158 kDa), conalbumin (74 kDa), ovalbumin (66 kDa), carbonic anhydrase (30 kDa), ribonuclease (12 kDa), and apoprotinin (6 kDa) were purchased from GE Healthcare. A standard curve was generated by plotting relative elution volume K<sub>av</sub> against log<sub>10</sub> molecular mass for each standard marker. The curve was fitted to a linear equation, and the apparent molecular masses were deduced from K<sub>av</sub> for respective proteins.

**Crystallization of TbbVSP1—** A single peak corresponding to tetramer in gel permeation chromatography was collected followed by the addition of 3 mM MgCl<sub>2</sub> and 0.5 mM imidodiposphate (Sigma-Aldrich) to protein (concentrated to 30 mg ml<sup>-1</sup>) solution for co-crystallization. Initial crystallization trials were performed at 293 K by the hanging drop vapor diffusion method using commercially available crystallization screens. Single cuboid shaped crystals were obtained within 4 days from MORPHEUS screen (Molecular Dimensions) in a solution containing 10% PEG 8000, 20% ethylene glycol, 0.03 M halide, and 0.1 M bicine/trizma pH 8.3. Further optimization of solution conditions to 9% PEG 8000, 18% ethylene glycol, 0.02 M halide, 0.1 M bicine/trizma, pH 8.0, and 0.1 M spermine tetrahydrochloride produced diffraction quality crystals.

**Data Collection, Structure Solution, and Analysis—** X-ray diffraction experiments were conducted at BM14 European Synchrotron Radiation Facility BioSAXS Beamline BM29 in Grenoble, France (28). Highly purified protein (>95% purity) was used, measurements for 30 μl of protein solution at five different concentrations (8.6, 4.3, 2.1, 1.08, and 0.54 mg ml<sup>-1</sup>) for each sample (and buffer) were defined using the ISPyB BioSAXS interface (29), and the sequence was triggered via BsxCuBE. Ten individual frames were collected for every exposure, each 2 s in duration using the Pilatus 1 M detector (Dectris). Individual frames were processed and merged using PRIMUS (30) and ATSAS package (31), and pair distribution function was computed using GNOM (32). The ab initio models were calculated for each construct using DAMMIF (33) and then averaged, aligned, and compared (which showed minimal variation) using DAMAVER (34). Rigid body modeling of the complex was complicated by the internal cavities that caused artifacts in the calculation of theoretical experimental curves. Therefore, ensemble modeling (35) was used to generate 10,000 models allowing random movements, and the resulting models were using COOT and phenix.refine respectively. An EF hand domain was built manually into electron density maps. Imidodiphosphate and coordinating Mg<sup>2+</sup> ions were added during the manual model building, and ligand density was verified using SA-OMIT maps. The overall stereochemical quality of the built model was assessed using MolProbity (26) and with PDB validation server. All structural figures were generated with PyMOL and Chimera (27). The coordinates for TbbVSP1.PNP.Mg<sup>2+</sup> complex have been deposited with Protein Data Bank with accession code 5C5V.

**Small Angle X-ray Scattering Experiments—** SAXS experiments were conducted at the European Synchrotron Radiation Facility BioSAXS Beamline BM29 in Grenoble, France (28). Highly purified protein (>95% purity) was used, measurements for 30 μl of protein solution at five different concentrations (8.6, 4.3, 2.1, 1.08, and 0.54 mg ml<sup>-1</sup>) for each sample (and buffer) were defined using the ISPyB BioSAXS interface (29), and the sequence was triggered via BsxCuBE. Ten individual frames were collected for every exposure, each 2 s in duration using the Pilatus 1 M detector (Dectris). Individual frames were processed and merged using PRIMUS (30) and ATSAS package (31), and pair distribution function was computed using GNOM (32). The ab initio models were calculated for each construct using DAMMIF (33) and then averaged, aligned, and compared (which showed minimal variation) using DAMAVER (34). Rigid body modeling of the complex was complicated by the internal cavities that caused artifacts in the calculation of theoretical experimental curves. Therefore, ensemble modeling (35) was used to generate 10,000 models allowing random movements, and the resulting models were using COOT and phenix.refine respectively. An EF hand domain was built manually into electron density maps. Imidodiphosphate and coordinating Mg<sup>2+</sup> ions were added during the manual model building, and ligand density was verified using SA-OMIT maps. The overall stereochemical quality of the built model was assessed using MolProbity (26) and with PDB validation server. All structural figures were generated with PyMOL and Chimera (27). The coordinates for TbbVSP1.PNP.Mg<sup>2+</sup> complex have been deposited with Protein Data Bank with accession code 5C5V.

**Table 1**

|                                                                 | TbbVSP1-Mg-PNP |
|-----------------------------------------------------------------|----------------|
| **Data collection**                                             |                |
| Space group                                                     | P4,2,2         |
| α, β, γ (°)                                                     | 104.2, 104.2, 215.5 |
| R<sub>free</sub>/R<sub>free</sub> (%)                          | 0.07 (0.69)    |
| I/σ(I)                                                         | 32.8 (2.71)    |
| Completeness                                                    | 98.1 (97.2)    |
| Redundancy                                                      | 10.9 (11.0)    |
| **Refinement**                                                 |                |
| Resolution                                                      | 39.8–2.35      |
| No. of Reflections                                              | 49,471 (3366)  |
| R<sub>free</sub>/R<sub>free</sub> (%)                          | 19.9/23.8      |
| **No. of atoms**                                               |                |
| Protein                                                         | 5580           |
| Ligand/Ions                                                     | 20/15          |
| Water                                                           | 200            |
| **B-factor (Å²)**                                               |                |
| Protein                                                         | 64             |
| Ligand/Ions                                                     | 56/59          |
| Water                                                           | 59             |
| **rmsd**                                                        |                |
| Bond length (Å)                                                 | 0.0073         |
| Bond angle (°)                                                  | 1.061          |

**TABLE 1**

**Data collection and refinement statistics**

The values in parentheses are for the highest resolution shell.
screened using Pepsi-SAXS. The resulting representative model and ab initio models for each construct selected by DAMAVER were overlaid using PyMOL. The fits to the experimental data of the models and the theoretical scattering of the structures were prepared with SAXSVIEW.

Electron Microscopy—Small aliquots (3.5 μl) of diluted protein (0.025 mg ml\(^{-1}\)) were deposited on carbon-coated electron microscope grids and stained with 2% uranyl acetate. Specimens were observed using a JEOL JEM2100 LaB6 at a nominal magnification of \(\times39600\) with a pixel size of 3.5 Å at the given magnification. Pictures were recorded using a GATAN 2K × 2K cameras. A total of 5268 particles were selected, normalized using EMAN software (36), and classified using SPIDER (37) and K-means classification. The average classes obtained were compared with the two-dimensional projections of three-dimensional crystal structure of TbbVSP1 filtered at 30 Å obtained using the PJ 3Q function in SPIDER.

Isothermal Titration Calorimetry—All ITC experiments were performed on a GE-Micro Cal ITC 200, and Origin was used for data analysis. Direct titration of EF hand domain of TbbVSP1 with Ca\(^{2+}\) was done in 50 mM Na-HEPES, pH 7.2, and 50 mM NaCl. Before titration, the protein was made divalent cation free and decalcified using sequential treatment of first 10 mM EDTA followed by dialysis against decreasing concentrations of EGTA (20 to 0.5 mM). Finally, the protein was dialyzed against experimental buffer (30 mM Na-HEPES, pH 7.2, and 100 mM NaCl) prepared in deionized water. Ca\(^{2+}\) solutions were prepared by diluting 1 M CaCl\(_2\) stock solution with dialysate/
experimental buffer. A typical titration was carried out over 40 injections with initial injection of 0.4 μl followed by 39 injections of 1 μl of 0.2 mM Ca^{2+} into protein solution (50 μM) with 150-s intervals between injections and 750 rpm stirring speed. Independent titrations were performed at 25 and 30 °C. All titrations were done twice, and the values are reported in Table 3.

Results

Domain Analysis and Characterization of TbbVSP1—The T. brucei brucei genome contains two isoforms of VSPs present on chromosome XI: TbbVSP1 (TriTrypDB ID Tb927.11.7060, characterized in this study) and TbbVSP2 (TriTrypDB ID Tb927.11.7080). Both forms are nearly identical and diverge only in three amino acids (F32V, S32L, and L33H). Sequence and domain analysis shows that TbbVSP1 belongs to class I PPases with best identity with animal/fungal PPases in the C-terminal region, but it also contains an EF hand domain in the N-terminal region (Fig. 1a). In particular, this EF hand domain is present in all VSPs of kinetoplastida parasites (Fig. 1a). TbbVSP1 ORF encodes a protein of 414 amino acids with relative molecular mass of 47.2 kDa. Nevertheless, native size analysis by gel permeation chromatography indicated that both full-length and N-terminal truncated TbbVSP1 (residues 160–414; PPase domain) predominantly elute as tetramers with molecular masses of 183 and 138 kDa respectively, suggesting that PPase domain is sufficient for tetramer formation in solution (Fig. 1b). This is in contrast to animal/fungal PPases that associate as dimers (38). Because of the putative EF hand
domain in the N-terminal region of the protein, we tested effect of divalent cations on protein oligomerization state. Gel permeation chromatography data indicated that addition of Ca\(^{2+}\) and Mg\(^{2+}\) had no influence on oligomeric stoichiometry of TbbVSP1 (Fig. 1b, top panel). Thus, collectively these data suggested that TbbVSP1 possesses an unusual and specific structural organization, which is different from conventional class I PPases.

Crystal Structure of TbbVSP1—The TbbVSP1 crystallized in tetragonal space group (P4\(_{2}2\), and its structure was solved using molecular replacement method with yeast PPase as template (PDB code 1M38). Electron density for most of the residues was generally good; however, residues 1–71 were not traceable in the model because of poor or no electron density. The final refinement and statistical parameters are stereochemically sound (Table 1). The crystal structure of TbbVSP1 reveals bidomain architecture (Fig. 1c). A DALI search against PDB using the complete structure of TbbVSP1 failed to identify any structure with significant similarities over entire polypeptide, suggesting a unique overall architecture. However, searches using individual domains revealed closest structural homolog of the N-terminal domain (residues 72–142) as EF hand calcium binding protein (PDB code 2BE4; Z = 8.2), and for C-terminal domain (residues 167–414) the yeast PPase (PDB code 1E6A; Z = 33.3). Therefore, hereon we refer to the N- and C-terminal domains as EF hand and PPase domains.

FIGURE 4. SAXS analysis of TbbVSP1 solution structure indicates flexibility. a, Guinier plot from raw data recorded at a range of protein concentrations. b, Individual domains of homodimer subunits in the asymmetric unit are superimposed. EF hand domains show more conformational variation relative to the PPase domains. c, SAXS curve showing logarithm of scattered intensity plotted as a function of momentum transfer, \(s = 4\pi \sin(\theta) / \lambda\), where \(\theta\) is the scattering angle, and \(\lambda\) is the x-ray wavelength. d, two views of TbbVSP1 model (PPase domains (blue sticks) and EF hand domain (orange sticks)) in solution by SAXS overlaid on ab initio low resolution envelope colored in green. e, left panel shows temperature factor variation in TbbVSP1 crystal structure colored from low to high values (40–105 Å\(^2\)) where arrows indicate the bridge helix. The right panel shows sequence conservation in the bridge helix region where residue positions with highest variability are boxed.
respectively. The EF hand domain has a compact globular fold and consists of four $\alpha$-helices, h1–h4 (residues 75–87, 98–108, 118–125, and 133–142) (Fig. 1c). The PPase domain consists of a five-stranded $\beta$ barrel made from strands $\beta$4 and $\beta$7–$\beta$10 in the core region, and in addition a $\beta$ sheet is formed away from the core by strands $\beta$1–$\beta$3. The $\beta$-barrel is flanked by two long $\alpha$-helices, h8 and h11, three short $3_{10}$ helices (residues 341–343 (h6), 370–372 (h9), and 380–382 (h10)) and helix h7, which is a combination of a $3_{10}$ helix and $\alpha$ helix. The h7 lies at the base of the $\beta$-barrel. The EF hand and PPase domain are bridged via a 24-residue interdomain region (residue 145–165). The interdomain region is composed of a helix (“bridge helix,” residues 145–159) present at the base of the EF hand domain followed by a small unstructured region (residues 160–165) that leads into PPase domain (Fig. 1c). Overall folds of two TbbVSP1 protomers in the asymmetric unit are similar. However, an approximately 12° orientation shift is observed at bridge helix (Fig. 1c, bottom panel), which suggests flexibility in this region.

**Quaternary Structure of TbbVSP1**—The protomers in asymmetric unit make contact via their PPase domain and extend EF hand domain in opposite directions, thus presenting an inward facing concave surface (Fig. 2a). The tetramer can be generated by application of 2-fold crystallographic operator resulting in a dimer of dimers assembly (Fig. 2b). The assembly contains two dimers, AB and A’B’, where each uses its concave surface to intimately lock with another to form a tetramer of $84 \times 72 \times 120 \text{Å}$ dimensions. The central core of the tetramer contains PPase domains, whereas EF hand domains jut out in the oligomeric assembly (Fig. 2c). This assembly is stabilized by four unique intersubunit interfaces (interface I–IV). Interface I (1840 Å$^2$) and II (700 Å$^2$) are hydrophilic, whereas interface III (825 Å$^2$) and IV (300 Å$^2$) are hydrophobic in nature (Fig. 3a). As
indicated by buried surface areas, interface I is much more extensive than other interfaces and involves interaction between residues from PPase domain and interdomain region of monomers A-A’ or B-B’ (Fig. 3b). Interface II is majorly formed by contacts between EF hand and PPase domains of two protomers (Fig. 3c). Crystal structure showed that His$^{92}$ of EF hand domain forms a salt bridge with Asp$^{396}$ present on h11 of PPase domain, and this could be an important contributing factor to stability of this interface. In addition, the bridge helix also makes contribution to interface II stability via Asp$^{158}$, which makes a salt bridge with Arg$^{410}$ of h11 (Fig. 3c). Interface III involves interaction between monomer A-B or A’-B’, which is braced by hydrophobic stacking interactions between Trp$^{233}$ and Trp$^{353}$ (Fig. 3d) and further stabilized by hydrogen bonding and N-H...π between Arg$^{235}$ and His$^{265}$ residues. Further, the aromatic ring stacking interactions between Phe$^{243}$ and Phe$^{353}$ are observed at interface IV (Fig. 3e). Phe$^{243}$ and Phe$^{353}$ are highly conserved among VSPs, suggesting that interface IV is another specific feature of Tb$_b$VSP1. Thus, these interactions demonstrate extensive connections between the monomers of the assembly. In line with above, the theoretical free energy value estimated for the assembly by PISA ($\Delta G_{\text{diss}} = 26.5$ kcal mol$^{-1}$) identified the tetramer as stable. This oligomer is further confirmed by SAXS and EM (see below) data.

**SAXS Reveals Conformational Flexibility of Tb$_b$VSP1**—To study solution structure of Tb$_b$VSP1, we conducted SAXS studies with highly pure full-length protein. The Guinier plot of Tb$_b$VSP1 exhibits good linearity (Fig. 4a), suggesting the protein solution was free of large aggregates or higher molecular mass contaminants. The molecular masses estimated from experimental volume and ab initio envelopes are 150–200 and 203 kDa respectively, which correlate well with values expected for a tetrameric species. However, the experimental volume estimated from ab initio modeling (302 ± 50 nm$^3$) does not correlate with volume from tetramer crystal structure (248 nm$^3$). This increase in volume is likely to be a result of flexibility...
in Tb₂VSP1 structure in solution state. We further noticed difference in $D_{\text{max}}$ between SAXS (157 ± 3 Å) and the crystal structure (120 Å), suggesting again that the crystal structure described a compact conformation for this protein. To investigate the SAXS curves at molecular level, we probed the tetrameric crystal structure model, but it did not fit with experimental SAXS curves (the associated $\chi^2$ were >10). Consistent with this observation, the overlay of tetrameric crystal structure...
form produced poor fits with the SAXS envelope. We further noticed that PPase domains docked nicely into the core region of the envelope but the peripheral EF hand domains fit poorly and in fact protruded outside the envelope. Considering the increase in size observed by SAXS, we attributed this disagreement to absence of flexibility in our atomic model based on crystal structure data.

In line with the above, alignment of homodimer subunits in the asymmetric unit identified a significant difference in the positioning of EF hand domains (rmsd 0.75 Å for 70 Cα atoms) and bridge helices (rmsd 1.3 Å for 18 Cα atoms; Figs. 4b and 1c, bottom panel). However, PPase domains did not display significant deviation (rmsd 0.12 Å for 247 Cα atoms; Fig. 4b). These observations collectively suggest inherent flexibility in EF hand domain and bridge helix with respect to the PPase domain backbone. Therefore, to assess the likelihood that crystal packing may stabilize a more flexible structure, we deployed an ensemble modeling (EOM) approach to predict the likely flexible movements and multiple conformations in solution by allowing hinge motions in EF hand domain. We also tested the bridge helix as a potential source of hinge motion, which could act as pivot between EF hand domain and PPase domain. Indeed, we found that the hinge motion produces better fit to the SAXS data (χ² = 2.22) (Fig. 4c). Overlay of model into ab initio envelope indicates that EF hand domains bend toward each other about the PPase domains, which hold together the tetramer core preventing it dissociating into lower oligomeric state (Fig. 4d). Although a single atomic model suffices to explain the experimental SAXS curve, this atomic state may represent a true dominant conformation in solution or an average of an ensemble spanning a subset of conformational spaces. Either of the above interpretation indicates structural malleability of TbVSP1 in solution in the absence of external forces such as those exerted by crystal packing.

Finally, crystal structure derived B-factors display a gradient along the body of bridge helix ranging from low (proximal to PPase domains) to high (near the base of EF hand domain, consistent with the three-dimensional crystal structure presented here (Fig. 5, d and e).

Electrostatic Potential—The molecular surface of TbVSP1 tetramer displays a bipolar distribution of electrostatic potentials. A highly negatively charged pocket concentrated on the distal face of the tetramer whereas positively charged pockets are formed near the tetramer inner core, toeing the tetramer midline (Fig. 6a). The negatively charged pocket consists of clusters of multiple aspartate residues (Asp291, Asp296, Asp323, and Asp328) and one glutamate residue (Glu329). This charged congregation in appropriate proximity suggests a metal binding site. Structural superposition with other known PPases shows high degree of structural conservation in the arrangement and position of these residues, all of which have been shown in previous studies to coordinate metal ions (38). In contrast, the positive patch results from tetramerization and causes arrangement of basic residues from discontinuous regions of the PPase domain (Fig. 6b). The underlying subset of residues of this pocket are Lys215, Arg223, Glu309, Lys314, Arg342, Lys345, and Lys388 of PPase domain that are present in both faces of the tetramer (Fig. 6b). Lys215, Arg223, Glu309, and Arg342 are buried, whereas Lys314, Lys345, and Lys388 are accessible. All these residues show high conservation in VSP homologs from trypanosome family (Fig. 6c).

PPase Activity and Pyrophosphate Binding Site—Studies on T. brucei gambiense have shown that VSP1 (TbVSP1) is an active PPase (17). Also, the TbVSP1 PPase domain showed high structural similarity to yeast PPase. Therefore, we examined PPase activity of TbVSP1 using malachite green assay developed for phosphate estimation in solution (39). At a protein tetramer concentration of 0.15 nm, the PPi hydrolysis rate had a turnover number (kcat) of 294 s⁻¹ at 37 °C (Fig. 7a, left panel). We found that the protein actively hydrolyzes PPi in presence of magnesium in an alkaline pH range (7.8-8.5) with kcat/Km of 1.475 × 10⁷ M⁻¹ s⁻¹, suggesting that activity is physiological. The main roles attributed to EF hand domain are the regulation of cellular activity and/or buffering/transporting calcium (40). To investigate whether EF hand domain has any role in PPi hydrolysis, we also used N-terminal EF hand domain truncated enzyme (Δ166 TbVSP1) that contains the PPase activity of TbVSP1 using malachite green assay developed for phosphate estimation in solution (39). In this case, the resulting kinetic constants for PPi hydrolysis were similar to full-length protein, suggesting that EF hand was not essential for PPi hydrolysis (Fig. 7b, right panel). It has been suggested that substitution of Mg⁺² by transition metal ions results in efficient hydrolysis of triplyphosphate (polyP₃) by class I PPases (41); indeed, we did observe this effect for TbVSP1 (Table 2). Again, this activity is independent of the EF hand domain. In contrast to PPi hydrolysis, the optimum pH for poly P₃ hydrolysis shifted to a neutral pH of 7.1, when Co⁺² and Mn⁺² were co-factors (Fig. 7b).

### Table 2

| Cofactor | Concentration (mM) | pH | Km (μM) | kcat (s⁻¹) | kcat/Km (μM⁻¹ s⁻¹) |
|---------|--------------------|----|---------|------------|---------------------|
| Zn⁺²    | 0.5                | 6.2| 44.8 ± 6.7 | 15.8 ± 3.7 | 3.69 × 10⁴         |
| Co⁺³    | 1.0                | 7.1| 96.8 ± 11.2| 7.3 ± 1.2  | 0.75 × 10⁵         |
| Mn⁺²    | 1.0                | 7.1| 142.3 ± 24.7| 1.3 ± 0.9  | 0.91 × 10⁴         |

**Table 2**: Kinetic parameters of polyP₃ hydrolysis in the presence of different transition metal co-factors.
FIGURE 8. Calcium binding by EF hand domain and interhelical angles. a, representative ITC titrations. The upper panel shows heat flows observed during the experiment; the lower panel shows integrated heats of each Ca$^{2+}$ injection; lines show fit of data to the binding model describing ligand interaction with a single binding site. Panel i, buffer Ca$^{2+}$ titrations showing heat of dilutions. Panels ii and iii, EF hand domain Ca$^{2+}$ titration in 50 mM Na-HEPES and 100 mM NaCl, pH 7.2, at 25 °C (panel ii) and 30 °C (panel iii). b, structural superposition of EF hand domain and calmodulin showing differences in interhelical packing between helices h1 and h2 (left panel) and helices h3 and h4 (right). Left panel shows helix h1 of Tb$_b$VSP1 (yellow) and calcium bound calmodulin (orange) are bent by 18° and 42°, respectively, relative to closed state (pink). Whereas, the right panel shows helices h3 are bent by 14° and 40° relative to closed state. PDB codes of calcium bound and unbound forms calmodulin are mentioned in parentheses. Interhelical angles (bottom) are shown in a tabular form.

| Name/ PDB ID | h1-h2 | h3-h4 |
|--------------|-------|-------|
| 1EXR         | 87°   | 94°   |
| 1CFD         | 139°  | 142°  |
| Tb$_b$VSP1   | 123°  | 130°  |
activity was discernable at acidic pH of 6.2 in presence of Zn\(^{2+}\) (Fig. 7b), and this property of Tb\(_b\)VSP1 is in line with previous findings on VSP1 from *Trypanosoma brucei gambiense* and *Leishmania amazonensis* (17–18). The \(K_\text{cat}\) values suggest that overall efficiency of polyP\(_3\) hydrolysis by Tb\(_b\)VSP1 in presence of transition metal follows the order Zn\(^{2+}\) > Co\(^{2+}\) > Mn\(^{2+}\) (Table 2). Taking these results together, our data indicate that recombinant Tb\(_b\)VSP1 hydrolyzes inorganic phosphates over a wide pH range of 6.2–8.5.

We were successful in co-crystallizing Tb\(_b\)VSP1 with magnesium and IDP (a slow turnover analog of PPi). Omit maps revealed electron density for IDP and Mg\(^{2+}\) ions at the active site of PPase domain (Fig. 7d). The complex comprises of four Mg\(^{2+}\) ions (M1, M2, M3, and M4) and one IDP molecule, which contain two phosphate groups P1 and P2 (Fig. 7e). The active site consists of residues from loops and \(\beta\)-strands that are highly conserved in PPases. In the complex, phosphate group P1 forms hydrogen bonding and ionic interactions with Arg\(^{259}\) (bidentate interaction), Tyr\(^{368}\) and Lys\(^{369}\), whereas P2 forms hydrogen bonding interactions with Lys\(^{237}\) and Tyr\(^{270}\) of the active site (Fig. 7e). M1 and M2 are coordinated exclusively by protein side chain carboxylates: M1 with Asp\(^{291}\), Asp\(^{296}\), and Asp\(^{326}\) and M2 with Asp\(^{290}\) (Fig. 7e). M3 and M4 are coordinated with phosphate groups of IDP and side chain carboxylates: M3 with Glu\(^{339}\) and M4 with Asp\(^{323}\) and Asp\(^{328}\) (Fig. 7e). Coordination geometry for all Mg\(^{2+}\) ions in the active is nearly octahedral, which we validated using the Check My Metal tool (42).

**Ca\(^{2+}\) Binding Affinity of EF Hand Domain**—We investigated whether EF hand domain is a functional Ca\(^{2+}\) binder. We deployed ITC to determine Ca\(^{2+}\) binding and measure binding affinities along with thermodynamic parameters. Because PPase domains have divalent cation binding capabilities, we generated a protein encompassing 1–160 residues that contains the EF hand domain. Initial titration of Ca\(^{2+}\) in HEPES buffer against 50 \(\mu\)M protein showed a sharp transition in ITC curves, indicating high affinity interaction. The results presented in Fig. 8a show good fit to a simple one-site model, describing specific binding driven by both favorable enthalpy and entropy changes. As in Table 3, binding affinity of EF hand domain is in nanomolar range (\(K_\text{d} = \sim 65 \text{ nm at } 25 \degree \text{C}\), and this affinity decreases slightly upon increase in temperature, which is again accompanied by favorable changes in heat and entropy. Note that the structure of EF hand domain presented in this work is in Ca\(^{2+}\)-free form. Comparison of EF hand domain with an archetypal Ca\(^{2+}\) binding protein calmodulin (PDB ID 1EXR; Ca\(^{2+}\)-bound form and 1CFD in unbound form) revealed that the putative Ca\(^{2+}\) binding loop which connects the helices h1 and h2 in EF hand domain adopts a conformation similar to Ca\(^{2+}\)-bound form of calmodulin (Fig. 8b, left panel). Further, measurement of interhelical angles and angular displacement of helices shows that the EF hand domain is in a partially open state, deviating from geometry proposed for a closed state (Ref. 43 and Fig. 8b, bottom panel). Finally, the loop connecting helices h3 and h4 shows poor overlap with either Ca\(^{2+}\)-bound or unbound forms (Fig. 8b, right panel).

**Comparison Tb\(_b\)VSP1 Structure with Other PPases**—A sequence-based phylogenetic tree construction supports the notion that Tb\(_b\)VSP1 is a class I PPase because it clusters with animal/fungal class I PPases. However, the branch length of animal/fungal PPase clade tends to be longer than acidocalcisominal VSPs, suggesting a more rapid rate of evolution (Fig. 9a). A survey of crystal structures of class I PPases in the PDB reveals that Tb\(_b\)VSP1 architecture presented in this study repre- sents an atypical class I PPase, not only in terms of its domains but also its oligomer state (Fig. 9a). The PPase domain structure of Tb\(_b\)VSP1 is similar to yeast PPase (rmsd 0.6 Å for 202 C\(_\alpha\) atoms). A notable difference is the presence of a longer loop in Tb\(_b\)VSP1, which results from insertion of 11 amino acids (resi- dues 208–218). This loop contributes to tetramer stability by burying 400 Å\(^2\) of accessible area at interface l. Another notable difference is an auxiliary space proximal to PP binding, which is absent in yeast PPase. We find that this structural difference translates to residue Gly\(^{374}\) of Tb\(_b\)VSP1 PPase domain. The corresponding structurally equivalent residue in yeast PPase is Lys\(^{198}\), which points toward the active site and thus occludes the space formation near the PPi binding site. Again, Gly\(^{374}\) is highly conserved among kinetoplastid VSPs. However, the most striking difference is the absence of C-terminal extension from Tb\(_b\)VSP1 PPase domain (Fig. 9c). This C-terminal extension is also absent from bacterial and plant PPases (Fig. 9c). Superposition of yeast PPase dimer as a whole to dimer component of Tb\(_b\)VSP1 (PPase domains only) gives a very high rsmsd value of \(\sim 4\) Å, indicating that spatial arrangement of monomers forming the primary dimer interface in Tb\(_b\)VSP1 is different from yeast PPase (Fig. 9d). This difference can also be judged by examining the orientation of catalytic center in each monomer of yeast and Tb\(_b\)VSP1; in the former, they are arranged on opposite faces, whereas in Tb\(_b\)VSP1, they consort in a sideways manner (Fig. 9d). Overlay of aforementioned dimers also reveals a notable difference in their packing angles, which entails a rotational shift of 20° in Tb\(_b\)VSP1 monomer with respect to yeast PPase monomer; this rotational shift is accompanied by translation of 101 Å. The dimer interface of yeast PPase (total buried area, \(\sim 1920 \text{ Å}^2\)) is also more substantial than Tb\(_b\)VSP1 (\(\sim 1650 \text{ Å}^2\)). This may be explained by the fact that C-terminal extension of yeast PPase contributes significantly (\(\sim 43\%\) total buried surface) to dimerization interface of yeast PPase. Further, theoretical free energy of dissociation from PISA indicates that C-terminal is important for stability of the yeast PPase dimer. Therefore, C-terminal extensions of yeast/animal PPases might act as clamps holding monomers, and their absence in Tb\(_b\)VSP1 might increase the fluidity of dimer interface region, resulting in different spatial arrangement.

**Discussion**

Tb\(_b\)VSP1 has a bidomain architecture consisting of the EF hand domain and the PPase domain. Although most known class I PPases are single domain proteins that form hexameric/
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FIGURE 9. Comparison of crystal structures/biological assemblies and phylogeny. a, maximum likelihood tree based on protein sequences of soluble PPase domains. Bootstrap values from 500 iterations are shown in red font, and branch lengths are shown in black font. The 0.2 bar represents amino acid substitution per site. Putative cytosolic PPases of kinetoplastid are used as the out group. b, biological assemblies of TbVSP1 and PPases from E. coli and S. cerevisiae. c, schematic shows the C-terminal extension present in animal/fungal PPases. Residues that contribute to dimer interface of yeast PPase are conserved and highlighted in purple/magenta in the sequence alignment. d, ribbon cartoon of PPase domain of yeast and TbVSP1 showing different spatial arrangement of monomers, which is also indicated by topology of active site in each dimer. The C-terminal extension of ScPPase colored in magenta.
dimeric assemblies, TbbVSP1 is distinct in being tetrameric. Aside from TbbVSP1 crystal structure, SAXS and EM confirm the unprecedented tetrameric assembly of TbbVSP1. Although crystal structure analysis indicates a static tetrameric arrangement, our SAXS data hint at a rather dynamic assembly in solution. Together, the SAXS modeling and crystal structure suggest that the flexibility manifests itself via hinge motion in EF hand domains, although the functional implication of flexibility remains unclear. This is also a nice example where “in solution” and “in crystallo” structural techniques together are more informative than either alone.

Although structurally intriguing, biochemically the TbbVSP1 is similar to TbgVSP1. Both substrate specificity profile and active site structure strongly indicate that PPi is a physiological substrate for TbgVSP1; however, PPase activity is optimal at alkaline pH range only, indicating that PPi, may not hydrolyzed constitutively by TbbVSP1 in acidocalcisomes. However, depending on identity of the co-factor, catalytic capabilities of TbbVSP1 expand beyond PPi, resulting in hydrolysis of polyP3 over a range of pH, both acidic and neutral. This suggests a regulatory role for TbbVSP1 in acidocalcisome, which is rich in polyphosphates. In this context, Lemercier et al. (17) have previously suggested that to prevent polyP3 accumulation in the cell compartment where polyP hydrolysis is occurring, polyP3 is further hydrolyzed by VSP1 in the presence of Zn$^{2+}$ at an acidic pH. That followed by an increase in pH and release of Ca$^{2+}$, H$^+$ ions by VSP1 and exopolyphosphatase could drive the reaction forward producing P$_i$ and PPi$_i$ within acidocalcisomes (17). Furthermore, acidocalcisomes seem active in several biological processes after alkalization, which also involves hydrolysis of polyphosphates to short chain phosphates and pyrophosphates (44, 45). This may well provide the basis for biological activity of TbbVSP1.

Our ITC data show that the EF hand domain of TbbVSP1 is a functional and specific Ca$^{2+}$ ion binder. Surprisingly, TbbVSP1 structure reveals a conformation similar to an open or Ca$^{2+}$-bound state, despite absence of metal bound in the putative binding site (Fig. 8b). Inspection of the TbbVSP1 tetramer structure reveals that conformation of calcium binding loops is stabilized by electrostatic and hydrogen bonding interactions with a symmetry-related molecule (Interface II; Fig. 3c). Thus, crystal forces might partially mimic a Ca$^{2+}$ atom, stabilizing an apparent “open” conformation. However, this remains to be verified using crystals in which TbbVSP1 is packed in alternate forms.

We also observed that Ca$^{2+}$ is able to inhibit both full-length TbbVSP1 and Δ166 TbbVSP1 alike (IC$_{50}$ ~ 50 μM in presence 3 mM Mg$^{2+}$) (Fig. 7c), suggesting that EF hand binding to Ca$^{2+}$ does not play a role in inhibition of PPi hydrolysis; this was also observed previously for TbgVSP1 (17). Instead, the Ca$^{2+}$-PP$_i$ complex acts a competitive inhibitor of Mg$^{2+}$-PP$_i$ complex, inhibiting PPase activity by a mechanism suggested previously (46). Furthermore, EF hand domain deletion also has no effect on either PP$_i$ or polyP$_i$ hydrolysis by TbbVSP1. In this context, the crystal structure of TbbVSP1 presented in this study indicates that relative positions of EF hand domain in TbbVSP1 monomer, dimeric, or tetrameric states disallow role for EF hand in direct interactions with substrate or co-factor metal binding site (in the PPase domain). This hence explains the inertness of the EF hand domain toward TbbVSP1 enzyme activity, and this explanation may also hold true for TbgVSP1, although spatial arrangement of EF hand and PPase domains in this assembly will vary, because the protein is reportedly hexameric (17).

A comparison between class I eukaryotic (animal/fungal) PPase family and TbbVSP1 suggests an intriguing evolutionary variety from dimeric to tetrameric states. On the basis of structural and phylogenetic data, a possible scenario emerges in which C-terminal extension has been acquired by animal/fungal PPases, independently after divergence from a protein that formed the last common ancestor between animal/fungal and VSPs. From atomic resolution crystal structures of yeast PPase and TbbVSP1, it is apparent that the C-terminal extension holds PPase domains like a clamp, thus resulting in a stable dimeric state. From the structural data so far, it seems that prokaryotic/archaeal and plant class I PPases also lack this typical C-terminal extension and interestingly show higher or non dimeric oligomerization state (trimers and hexamers). It might also be intriguing to suggest that the absence of such an extension in TbbVSP1 leads to a different packing arrangement of PPase domains in primary dimers, which is amenable to tetramer formation. Indeed, studies in past have shown that loss or addition of certain sequences or structural elements can result in spatial reorientation of subunits that can induce different oligomeric states (47, 48).

In conclusion, our study presents an unprecedented structural assembly of the EF hand and the PPase domain within one TbbVSP1. This work provides a structural foundation both for mechanistic exploration of TbbVSP1 and for scoring feasibility of small molecule inhibitor development in future.

Author Contributions—A. S. and A. J. conceived the study. A. J. performed purification of the enzyme, biochemical assays, ITC, determined and analyzed the x-ray structure. M. Y. and H. B. collected x-ray data. A. R. R. performed and analyzed SAXS experiments. L. B. and C. V.-B. conducted TEM studies. The manuscript was written primarily by A. S. and A. J., but all authors contributed.

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