Crystal Structure of the Tp34 (TP0971) Lipoprotein of *Treponema pallidum*

**IMPLICATIONS OF ITS METAL-BOUND STATE AND AFFINITY FOR HUMAN LACTOFERRIN**

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The Tp34 (TP0971) membrane lipoprotein of *Treponema pallidum*, an obligate human pathogen and the agent of syphilis, was previously reported to have lactoferrin binding properties. Given the non-cultivatable nature of *T. pallidum*, a structure-to-function approach was pursued to clarify further potential relationships between the Tp34 structural and biochemical properties and its propensity to bind human lactoferrin. The crystal structure of a nonacylated, recombinant form of Tp34 (rTp34), solved to a resolution of 1.9 Å, revealed two metal-bound states that may contribute to innate immune responses (11–18). Whereas early experiments revealed only five treponemal lipoproteins (10), more recent analysis of *T. pallidum* genome sequence information has predicted that open reading frames encode proteins with ligand-binding properties, modular components of ATP binding cassette (ABC) transporters, enzymes, receptors, protective immune targets, and proinflammatory agonists that contribute significantly to innate immune responses (11–18). Whereas early experiments revealed only five treponemal lipoproteins (10), more recent analysis of *T. pallidum* genome sequence information has predicted that at least 45 lipoprotein genes (19). Most of these predicted that open reading frames encode proteins with no known homologies to other structurally or functionally characterized bacterial proteins. In addition, the absence of techniques for in vitro cultivation and genetic manipulation of *T. pallidum* has greatly hindered the ability to characterize the functional aspects of *T. pallidum* membrane lipoproteins.

As an alternative approach to investigating the peculiar membrane biology of *T. pallidum*, we have adopted a structure...
to-function approach to formulate new testable hypotheses regarding the potential function(s) of a number of the treponemal lipoproteins. That a structural biology approach for analyzing *T. pallidum* lipoproteins is a fruitful avenue of investigation is exemplified in at least three recent studies on Tp47, Tp32, and PnrA (20–22).

The current structural study focuses on the Tp34 membrane lipoprotein (also known as Tp0971) of *T. pallidum*. Early studies by Stagg et al. (23) denoted a protein, most likely Tp34, as having lactoferrin binding properties. Lactoferrin is a mammalian iron-binding glycoprotein found on mucosal surfaces and within biological fluids, including milk, saliva, and seminal fluid (24–26). Despite the preliminary functional characterization of Tp34, NCBI Clusters of Orthologous Groups database includes 10,000 molecular weight exclusion limit. The concentrated protein was applied to a HiLoad 16/60 Superdex 75 prep grade column and purified on an Äkta fast performance liquid chromatography system (GE Healthcare) using buffer A. Subsequent to elution, peak fractions were analyzed by SDS-PAGE. At this stage, the protein was pure to apparent homogeneity (i.e. >95%). Fractions containing purified rTp34 were pooled and concentrated to 10 mg/ml in buffer A for protein crystallization. Protein concentration was determined spectrophotometrically using an absorption coefficient of 1.491 mg⁻¹ cm⁻¹ at 280 nm (27). To express protein for nuclear magnetic resonance (NMR) characterization, bacteria were grown in M9 minimal medium supplemented with 15NH₄Cl as the sole nitrogen source. The resultant 15N-labeled protein was purified as described above. For the production of a selenomethionine (SeMet) variant of rTp34, the expression vector was transformed into the *E. coli* methionine auxotroph B834 cells (Novagen) and grown in the presence of M9 medium containing 5% LB supplemented with 125 mg/liter each of adenine, uracil, thymine, and guanosine, 2.5 mg/liter thiamine, 4 mg/liter b-2,3-dihydroxybutyrate, 20 mM glucose, 2 mM MgSO₄, 50 mg/liter each of 19 L-amino acids (excluding methionine), and 50 mg/liter L-selenomethionine (Sigma). Labeled protein was prepared as described above with the addition of 15 mM β-mercaptoethanol to the purification buffers.

**NMR Spectroscopic Screening** — Suitability of rTp34 for structural determination was evaluated by NMR screening of 15N-labeled protein (28). All 1H, 13N heteronuclear single quantum coherence (HSQC) spectra of labeled protein were acquired at 25 °C by using a Varian INOVA 500-MHz spectrometer with NMRPipe (29) used for data processing and NMRView (30) for analysis. Data were collected on 500 μM samples in 50 mM K₂PO₄, 50 mM NaCl, pH 7.5.

**Preparation of PnrA, Tp32, Human Milk Lactoferrin (hLf), Bovine Milk Lactoferrin (bLf), and Human Serum Transferrin (hTF)** — The purification procedures for PnrA and Tp32 have been described previously (21, 22). As a final step, both proteins were dialyzed against buffer B (20 mM Hepes, 100 mM NaCl, 2 mM octyl β-glucoside, pH 7.5). Lyophilized hLf, bLf, and hTF were obtained from Sigma and reconstituted in buffer A at protein concentrations of 10 mg/ml. Proteins were then dialyzed overnight into buffer B. AphoLF was prepared in the same buffer after exhaustive dialysis against 0.1 M citric acid (31).

**Crystallization, Data Collection, and Structure Determinations** — Crystals were grown at 20 °C using the vapor diffusion method in hanging drop mode by mixing 4 μl of protein (10 mg/ml in buffer A) with 4 μl of reservoir solution (2.4 M ammonium sulfate, 0.1 M Bicine, pH 9.0) and equilibrating against 500 μl of reservoir solution. Crystals appeared within 24 h and grew to average dimensions of 0.2 × 0.6 × 0.1 mm in about 3 days. The crystals were cryo-protected in 2.8 M ammonium sulfate, 15% (v/v) ethylene glycol, 0.1 M Bicine, pH 9.0, and then flash-cooled in liquid propane. Crystals exhibited the symmetry of space group P2₁2₁2₁, with cell dimensions of *a* = 34 Å, *b* = 66 Å, *c* = 151 Å and contained two molecules per asymmetric unit and 41% solvent. Crystals for the selenomethionine

**Structure of the Tp34 Lipoprotein from *T. pallidum***
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variant of rTp34 (rTp34-SeMet) were produced under the same conditions.

Diffraction data were collected at beamlines 19-ID and 19-BM (SBC-CAT) at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL) and processed with HKL2000 (32). Crystals diffracted to a minimum Bragg spacing of about 1.63 Å.

Phases for rTp34-SeMet were obtained from a single anomalous diffraction experiment using x-rays with an energy near the selenium K absorption edge. Using data to 2.5 Å, 6 of 12 possible selenium sites were located and refined with the program CNS (33), resulting in a figure of merit of 0.35. Phases were extended to 1.7 Å and improved by density modification (CNS), resulting in a final overall figure of merit of 0.92.

The resulting electron density map was of sufficient quality to automatically construct an initial model using the program ARP/wARP (34). Manual rebuilding was carried out with the program Coot (35). Refinement was accomplished using the program REFMAC5 (36) from the CCP4 package (37).

The rTp34-SeMet model was used as a starting model for the refinement of native rTp34. The electron density revealed strong, spherical features near His-124 in each monomer, indicative of metal ions. These features were not present in rTp34-SeMet. An anomalous difference Fourier electron-density map showed peaks of about 6.4 and 4.7 σ that coincided with the features. Subsequently, a crystal of rTp34 was incubated with 10 mM zinc acetate (rTp34-Zn), and diffraction data were collected using x-rays with an energy near the zinc K absorption edge. The anomalous difference Fourier electron-density map revealed very strong peaks of about 32 and 33 σ at the previously observed positions. Zinc ions were, therefore, included in the model. An additional six zinc ions were located, but they had rather high B values and are, thus, minor sites. Although the nature of the metal ions in native rTp34 was not unambiguously clear, the peaks were interpreted as zinc ions at this stage. These metal-binding sites were not fully occupied as indicated by the comparatively weak electron density and high B factors. Furthermore, a second conformation appeared for some of the zinc-ligand residues, whereas these residues adopted only a single conformation in rTp34-Zn, where the main zinc sites were presumably fully occupied. As a control, a dataset was collected from a crystal of rTp34 that was grown in the presence of 1 mM EDTA (rTp34-EDTA). Density observed at the zinc sites was weak and was interpreted as water molecules.

Final models had an $R_{work}$ and $R_{free}$ of 18.4 and 21.5% for rTp34-SeMet, 18.8 and 23.5% for native rTp34, 19.0 and 23.3% for rTp34-Zn, and 18.9 and 22.0% for rTp34-EDTA, respectively. About 31 N-terminal residues could not be located in all cases.

Figure Generation—Figures that feature structural details of rTp34 were generated using PyMOL. They were rendered using POVRay.

Isothermal Titration Calorimetry—The binding of rTp34 or other T. pallidum lipoproteins to various target proteins was analyzed by injecting a solution of each lipoprotein into a solution of target protein and monitoring the resultant heat changes upon binding using a VP-ITC titration microcalorimeter (MicroCal, Inc.). All proteins were exhaustively exchanged into buffer B before the titration experiment. Protein concentrations were obtained by measuring the absorbance of protein-containing solutions spectrophotometrically followed by the calculation of the protein concentration using Beer’s Law ($A = ecl$, where $A$ is absorbance, $e$ is the molar extinction coefficient, $c$ is the protein concentration, and $l$ is the path length). In the case of rTp34, hLF, and bLF, the extinction coefficients were experimentally determined following the method of Pace et al. (27). For other proteins $ε$ was calculated by the ProtParam tool available at the ExPASy Proteomics Server. The titration was carried out using 31 serial injections of 8 µl of lipoprotein at concentrations of 500–600 µM into a stirred reaction cell (1.4 ml) containing solutions of the target protein at ~30 µM. To study the potential binding of hTF to rTp34, a solution of hTF (126 µM) was injected into a solution of rTp34 (13.6 µM) as described above. The heats of interaction resulting from the titration were monitored by the VP-ITC instrument until the target protein was saturated with the lipoprotein. Integration of the resultant thermogram yielded the binding isotherm, which was analyzed using the Microcal-ORIGIN software package to yield the association constants and the thermodynamic parameters associated with binding. Titrations using rTp34 as the injectant and hLF as the target protein were carried out in triplicate; others were performed only once.

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed in a Beckman XL-1 analytical ultracentrifuge. For rTp34 sedimentation, samples of 390 µl of reference buffer C (20 mM Tris-Cl, 20 mM NaCl, pH 7.5) or rTp34 (19 µM) diluted in reference buffer were loaded into a dual-sector charcoal-filled epon centerpiece. In experiments that contained divalent metal ions, they were included at a concentration of 3 mM unless otherwise noted. The samples were centrifuged at 50,000 rpm in an An60-Ti rotor, and sedimentation was monitored using either laser interferometry or absorbance spectrophotometry at a wavelength of 280 nm. Data were analyzed using the program SEDFIT, which generates a continuous $c(s)$ distribution for the sedimeting species (38). For experiments designed to monitor the binding of rTp34 to another protein, the same buffer was used. The volumes, rotor, centrifugation speed, and instrumentation were as described above. Three simultaneous sedimentation experiments were performed in these cases, with the solution contents being (i) rTp34 (15 µM) alone, (ii) target protein (5 µM) alone, and (iii) a mixture of rTp34 (7.5 µM) and the target protein (2.5 µM). For such experiments, two signals from the proteins were concurrently used to follow their sedimentation. For some experiments, the two signals were absorbance at 280 nm and refractive index detection by laser interferometry; for others, the signals were absorbances at 280 and at 250 nm. Such data were analyzed according to the multisignal $c(s)$ methodology of Balbo et al. (39), as implemented in the program SEDPHAT. Briefly, the sedimentation profiles obtained from experiments i and ii were used to calculate the unknown extinction coefficient of the protein at 250 nm or the unknown interferometric extinction coefficient. This is possible because the extinction coefficient of the proteins at 280 nm is known. Once the extinction coefficient at both signals is known, a characteristic signal
increment $e_k$ of each protein component $k$ at each signal $\lambda$ can be defined that allows the spectral decomposition of the c($s$) distribution into two $c_k(s)$ distributions, one for each of the protein components. Integration of a $c_k(s)$ peak yields the concentration of a given component in that peak. The concentrations of co-sedimenting proteins may, thus, be derived by analyzing the two $c_k(s)$ distributions; comparison of the two concentrations yields the stoichiometry of the proteins in that peak. The program SEDNTERP was used to estimate the partial specific volume of the proteins as well as the density and viscosity of the buffer solutions. All sedimentation coefficients quoted in this study have been corrected to values that would be observed under standard conditions (i.e. $s_{20,w}$).

Cultivation and Isolation of Treponemes—$T. pallidum$ subspecies $pallidum$ (Nichols strain) was maintained and passaged by intratesticular inoculation of adult male New Zealand White rabbits as previously described (40). Spirochetes were harvested in $T. pallidum$ medium (41) and flushed with a reduced oxygen atmosphere of 3% O$_2$, 5% CO$_2$, and the balance of nitrogen. The medium consisted of Earle’s balanced salt solution, minimum essential medium (MEM) (Hyclone) amino acids, MEM nonessential amino acids, MEM vitamin, 2 mM L-glutamine, 550 $\mu$M D-mannitol, 320 $\mu$M L-histidine, 24 mM sodium bicarbonate, 25 mM MOPS, 900 $\mu$M sodium pyruvate, 14 mM D- (+)glucose, 650 $\mu$M dithiothreitol, and 20% (v/v) fetal bovine serum (Mediatech, Inc.) with a final pH of 7.5. Rabbit testicular debris was removed from treponemal suspensions by two successive rounds of slow-speed centrifugation (200 $\times$ g for 8 min). Spirochetes remaining suspended were enumerated by darkfield microscopy and diluted to a concentration of $10^8$ ml$^{-1}$ in $T. pallidum$ medium.

Indirect Immunofluorescence Detection of Antigens in $T. pallidum$—The agarose gel microdroplet method (42) was utilized to assess whether lipoproteins were exposed on the surface of $T. pallidum$. $T. pallidum$ was encapsulated within agarose gel microdroplets as previously described (42). The encapsulation method stabilizes the fragile outer membrane of $T. pallidum$ during antibody exposure and washing treatments. Mouse monoclonal anti-Tp34 (clone 11E3) (43) and mouse monoclonal anti-Tp34 (44) were diluted 1:20 and added directly to 1 ml of aliquots of microdroplets with or without 0.15% (v/v) Triton X-100. The microdroplets were incubated overnight at 4 °C. The microdroplets were collected by centrifugation at (500 $\times$ g, 5 min) and washed 5 times with fresh $T. pallidum$ medium. Two $\mu$g of goat anti-mouse Alexa Fluor 488 (Molecular Probes) were added to the microdroplets for 2 h before being re-washed 5 times and viewed on glass slides with a Olympus BH2 microscope (darkfield lens and BP490 (green) filter (Olympus America Inc.)). Images were obtained using a SPOT Pursuit camera (Diagnostic Instruments, Inc.). Slides were prepared from each of 3 independent microdroplet preparations, and ~100 bacteria were counted per slide. Any spirochete with a fluorescent signal, which was often punctuated, was considered positive and compared with the total number of spirochetes observed via darkfield microscopy.

SDS-PAGE and Immunoblotting—Samples for protein analysis were separated on 12.5% polyacrylamide resolving gels. Proteins were transferred electrophoretically to a 0.45-$\mu$m-pore-size nitrocellulose filter (Schleicher & Schuell) for immunoblotsting. Immunoblots were incubated with a 1:100 dilution of mouse monoclonal antibody 4A4. This was followed by incubations with a dilution of 1:10,000 of goat anti-mouse immunoglobulin G (heavy and light chain-specific) peroxidase conjugates (Jackson ImmunoResearch). Immunoblots were colorimetrically developed with 4-chloro-1-naphthol as the substrate.

RESULTS AND DISCUSSION

Production of rTp34 and Screening by $^{15}$N HSQC NMR Spectroscopy—Recombinant Tp34 was created as a nonacylated fusion protein with an N-terminal His$_6$ tag followed by a tobacco etch virus protease cleavage site. The expression of this fusion protein in E. coli resulted in the production of non-lipidated, soluble protein. Typical yields were 20–25 mg of protein per liter of culture (>95% pure protein). Unless otherwise noted, hereafter “rTp34” shall refer to the recombinant Tp34 that has had its N-terminal His tag removed by the action of tobacco etch virus protease. To assess the suitability of rTp34 for structure determination, $^{15}$N-labeled rTp34 was subjected to $^{1}H$, $^{15}$N HSQC NMR spectroscopy. HSQC spectra of $^{15}$N-labeled rTp34 revealed the appropriate number of peaks according to the protein sequence with good peak dispersion and roughly equal intensity (data not shown). These results indicated that rTp34 was well folded, conformationally homogeneous, and, thus, likely suitable for crystallization.

The Crystal Structure of rTp34—The crystal structure of rTp34 was determined using single-wavelength anomalous diffraction of a crystal containing selenomethionyl-substituted protein (Table 1). The native structure (Fig. 1) was refined using data to a resolution of 1.9 Å. Residues 2–27 of rTp34 were not visible in electron-density maps. The asymmetric unit of rTp34 crystals contained two rTp34 monomers. Monomeric rTp34 contains mainly two antiparallel $\beta$-sheets that are packed on one another (Fig. 1A). The structure and topology of these $\beta$-sheets is similar to the classical immunoglobulin fold (Ig-fold (45)). Accordingly, the topological nomenclature of Ig-folds has been adopted for rTp34, with the $\beta$-strands consecutively lettered A, B, C, C’, D, E, F, and G; the $\beta$-sheets shall hereafter be referred to as the “front” and “back” $\beta$-sheets according to their orientation in Fig. 1A. The fold of rTp34 is in the “hybrid” subcategory of Ig-folds, inasmuch as strands C’ and D appear to form a single, interrupted $\beta$-strand, but C’ participates in the front $\beta$-sheet, whereas D is part of the back $\beta$-sheet.

The structure of rTp34 deviates from the classical Ig-fold in several places. Near to the protein’s N terminus, there is an extra, interrupted $\beta$-strand that participates in the back $\beta$-sheet; the $\beta$-strand portions of this feature are designated $A_1$’ and $A_2$’ (Fig. 1A). Some long regions of peptide between the $\beta$-strands of the Ig-fold harbor secondary structural elements. Such features are designated by lowercase letters, indicating the $\beta$-strands of the Ig-fold that occur N-terminal and C-terminal to the element. For example, a portion of the polypeptide chain between $\beta$-strands B and C harbors a 3$_{10}$ helix called bc. Other deviations from the Ig-fold include long insertions that contain $\beta$-strands ab and fg. Although these strands appear to be isolated in the monomeric structure of rTp34 (Fig. 1A), they par-
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A dimer of rTp34 (Fig. 1B) is generated by a 2-fold rotation axis relating the two monomers present in the asymmetric unit of the rTp34 crystals. The total surface area buried at the dimer interface is \(3300 \text{Å}^2\), with about 18% of the total surface area of a monomer contributing to the dimer interface. The two monomers are structurally very similar; a superposition of the two results in a root mean square deviation of 0.4 Å between the 157 equivalent Cα atoms. In the dimer, the aforementioned β-strands ab and fg extend the back β-sheet by two strands; interdigitated between β-strands D and ab of one monomer is β-strand fg of the neighboring molecule. The antiparallel hydrogen-bonding pattern of the back β-sheet is broken by the parallel interaction between β-strands D and fg.

In electron-density maps derived from a native structure of rTp34 determined at 1.9 Å (Table 1), density (not shown) is present near to the side chains of residues His-70, Glu-72, Met-117, His-124, and His-155 (the \(\lambda\) denotes that this residue is from the neighboring molecule in the dimer; see Fig. 1B). The identity of the atom in this density was assigned as the divalent metal cation Zn\(^{2+}\), based on several lines of evidence. First, all atoms near to the cation are chemically compatible ligands of Zn\(^{2+}\), and the ligation geometry is commensurate with the fact that Zn\(^{2+}\) may have a coordination number of four, five, or six. In addition, a crystal of rTp34 was soaked with 10 mM zinc acetate. Diffraction data were collected from this crystal at an x-ray wavelength near to the K absorption edge for zinc; any zinc atoms present in the structure would, therefore, scatter such x-rays anomalously. A 33-σ peak is present at the putative metal ion-binding site in an anomalous difference Fourier map calculated from these data, demonstrating bound Zn\(^{2+}\) (Fig. 2A). These data were also used to refine a structure of rTp34 with Zn\(^{2+}\) included in the model (Fig. 2A; rTp34-Zn in Table 1); the atom at the metal ion-binding site refined well as a Zn\(^{2+}\), with B factors that were comparable with those of the surrounding atoms. As a final piece of evidence, diffraction data were collected from a crystal of rTp34 that was grown in the presence of the divalent metal ion chelator EDTA. Because these crystals are sufficiently isomorphous to the native crystal, a 33-σ peak present at the putative metal ion-binding site (Fig. 2A), indicating that density present there in the native structure is not present when free divalent metal ions are sequestered from solution by EDTA. The structure derived from these data (rTp34-EDTA in Table 1), refined using data to a resolution of 1.6 Å, exhibits almost no electron density at the metal ion-binding site, and the liganding side chains in general move away from the site (not shown). All of these crystallographic data indicate that there is a Zn\(^{2+}\)-binding site present at the dimeric interface of rTp34 and support the assignment of the aforementioned electron density in the native rTp34 structure as Zn\(^{2+}\). Because two monomers contribute to this zinc-binding site, it is a “protein interface zinc site,” as characterized by Auld (46).

The B factors of the Zn\(^{2+}\) ions in the native structure are higher than those of the surrounding atoms, indicating that the cations may have a lower occupancy. Another factor that indi-

### Table 1

Data collection and refinement statistics

<table>
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<tr>
<th></th>
<th>rTp34-SeMet(^a)</th>
<th>rTp34</th>
<th>rTp34-Zn</th>
<th>rTp34-EDTA</th>
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<td>P2,2,2</td>
<td>P2,2,2</td>
<td>P2,2,2</td>
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<td>34.47</td>
<td>34.45</td>
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<td>b (Å)</td>
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<td>65.88</td>
<td>65.92</td>
<td>66.03</td>
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<td>150.61</td>
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<td>27.54-1.86 (1.89-1.86)</td>
<td>33.60-1.87 (1.90-1.87)</td>
<td>26.03-1.63 (1.66-1.63)</td>
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<td>Completeness</td>
<td>99.2 (88.6)</td>
<td>96.0 (74.5)</td>
<td>99.7 (98.2)</td>
<td>95.3 (94.1)</td>
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<td>29,534 (1,449)</td>
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<td>4.1 (3.7)</td>
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<td>5.6 (73.1)</td>
<td>5.0 (50.8)</td>
<td>10.6 (74.5)</td>
<td>3.8 (48.7)</td>
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<td>Rwork (%)</td>
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<td>24.5 (2.0)</td>
<td>19.5 (1.8)</td>
<td>32.0 (2.2)</td>
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<td>23.9</td>
<td>28.9</td>
<td>19.4</td>
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**Phases determination**

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<th>rTp34-SeMet(^a)</th>
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<th>rTp34-Zn</th>
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<td>Anomalous scatterer</td>
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<td>Figure of merit</td>
<td>Selenium (6 of 12 possible sites)</td>
<td>0.35-2.5 Å (0.92 after density modification to 1.7 Å)</td>
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**Phases determination**

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<th>rTp34-Zn</th>
<th>rTp34-EDTA</th>
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<td>No. of reflections R(<em>\text{work}^b)/R(</em>\text{free}^b)</td>
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<td>26,782 (1,418)</td>
<td>26,688 (1,413)</td>
<td>40,384/1,508</td>
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<td>18.4/21.5</td>
<td>18.8/23.5</td>
<td>19.0/23.3</td>
<td>18.9/22.0</td>
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<td>Average B-factor (Å(^2))</td>
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<td>30.5</td>
<td>23.7</td>
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<td>Water molecules</td>
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<td>Root mean square deviations</td>
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<td>Correlation coefficient (F_0^\text{free} - F_0^\text{experimental})</td>
<td>0.962</td>
<td>0.964</td>
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<tr>
<td>Ramachandran plot (% of atoms in most favored/allowed/disallowed)</td>
<td>97.8/1.9/0.3</td>
<td>97.8/1.6/0.6</td>
<td>98.1/1.9/0.0</td>
<td>97.7/1.9/0.3</td>
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</table>

\(^a\) Bijvoet pairs were kept separate for data processing.

\(^b\) \(R_{\text{merge}} = 100 \sum_{h,i} ||I_h|| - (I_h + I_i)/\sum_{h,i}||I_h||\), where the outer sum \(h\) is over the unique reflections, and the inner sum \(i\) is over the set of independent observations of each unique reflection.

\(^c\) As defined by the validation suite MolProbity (88).
icates low occupancy of the Zn$^{2+}$ in the native structure is that the side chain of His-124 adopts multiple conformations (not shown), but in the Zn$^{2+}$-soaked structure, the side chain is locked into a single, Zn$^{2+}$-proximal conformation (Fig. 2A). The native crystals of rTp34 were not grown in the presence of added metal ions; any such ions present in this structure must have co-purified with rTp34 (from *E. coli*) or been scavenged from the crystallization medium. This fact may account for the apparently low occupancy of the metal ions in the rTp34 native structure. It also indicates a high affinity for metal ions by the observed interfacial metal-binding sites.

The inner-sphere coordination environment of the Zn$^{2+}$/H11001 bound at the dimeric interface of the rTp34-Zn structure is shown in detail in Fig. 2B. The cation has one oxygen ligand, one sulfur ligand, and three nitrogen ligands. They are arranged around the Zn$^{2+}$ with a distorted square pyramidal geometry. All of the oxygen-Zn$^{2+}$ and nitrogen-Zn$^{2+}$ distances conform to established norms for inner-sphere ligandation (2.0–2.1 Å). The distance between the δ-sulfur of Met-117 and the Zn$^{2+}$ is 3.1 Å, a distance that is significantly longer than the sum of the ionic radii of sulfur and zinc(II) ion (~2.4 Å). However, ligand-zinc distances are known to grow larger as the coordination number increases (47), and distances on this order have been observed between the methionine δ-sulfur and divalent cations whose radii are similar to that of Zn$^{2+}$ (48).

Intriguingly, there is no crystal structure currently in a metalloprotein data base (49) that features a δ-sulfur of methionine coordinated to Zn$^{2+}$. Therefore, the interfacial Zn$^{2+}$-binding site of rTp34 may represent a novel Zn$^{2+}$-binding site. Alternatively, Zn$^{2+}$ could be adventitiously binding to a site that would bind to a different metal ion in vivo. For example, both copper and iron ions can be coordinated by the δ-sulfur of methionine when bound...
species exhibited a sedimentation coefficient of 2.1 S, the other, 3.0 S (Fig. 3A). The slower sedimenting species accounted for about 90% of the signal in the experiment. A molecular mass calculated for the 2.1 S species (25,893 Da) corresponded to that of an rTp34 monomer (23,390 Da; protein with a His6 tag was used). The molecular mass calculated for the 3.0 S species (48,354 Da) was roughly twice that of monomeric rTp34. It is, therefore, likely that a monomer-dimer equilibrium is present in solutions of rTp34. The koff of the dimer dissociation must be slow on the time scale of the SV experiment; otherwise, two distinct peaks would not have been observed in the c(s) analysis (51). Similar experiments at higher protein concentrations were conducted, but the ratio of monomer to dimer did not change significantly (Fig. 3A). These data support the hypothesis that, under the solution conditions used, even the highest protein concentration (50 μM) is well below the Kd of the monomer-monomer interaction.

The c(s) protocol used fits a single frictional coefficient ratio (ffo) for the entire range of sedimentation values considered; most compact, globular proteins have a value of about 1.2 for ffo. In these experiments, ffo was about 1.5, which indicates that rTp34 has a moderately elongated structure in solution. Such a ratio comports with the known disorder and presumed flexibility of the N terminus of rTp34. Large ffo values have been observed in proteins with disordered and apparently flexible N termini (e.g. see Ref. 52).

The presence of Zn2+ at the dimeric interface of rTp34 (Figs. 1B and 2) prompted the examination of the solution behavior of rTp34 in the presence of divalent metal ions. SV experiments demonstrated that the dimeric form of rTp34 is indeed stabilized when divalent cations are included in solution (Fig. 3B). All divalent metal ions tested, i.e. Mg2+, Ca2+, Co2+, Ni2+, Mn2+, Cu2+ and Zn2+, displayed an ability to stabilize the dimeric form of the protein relative to the monomeric form at a concentration of 3 mM (300 μM for Zn2+, pH 7.5, and Cu2+, pH 5.5, because higher concentrations caused the precipitation of rTp34; see Table 2). To examine the ability of iron to dimerize rTp34, an SV experiment was conducted in the presence of

<table>
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<tr>
<th>Table 2</th>
<th>Stabilization of the rTp34 dimer by divalent metal ions</th>
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<tbody>
<tr>
<td>Metal ion</td>
<td>Monomers</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>s20,w (%)</td>
</tr>
<tr>
<td>None</td>
<td>2.0</td>
</tr>
<tr>
<td>Na+</td>
<td>2.0</td>
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<tr>
<td>Mg2+</td>
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<tr>
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<tr>
<td>Zn2+</td>
<td>2.4</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>2.0</td>
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<tr>
<td>pH 5.5</td>
<td>None</td>
</tr>
<tr>
<td>Mg2+</td>
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<tr>
<td>Mn2+</td>
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<tr>
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<tr>
<td>Zn2+</td>
<td>2.2</td>
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<tr>
<td>Cu2+</td>
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a 100 mM NaCl instead of 20 mM NaCl used in all other experiments.

b 300 μM; 3 mM caused rTp34 to precipitate.

to proteins (48). In addition, Zn2+ is known to occupy iron-binding sites in some cases (50). The rTp34 dimer completely surrounds the bound zinc ions; they have no exposure to the solvent.

Solution Properties of rTp34—The large dimeric interface present in the crystal structure of rTp34 suggested that the protein may be a dimer in solution. Analytical ultracentrifugation sedimentation velocity (SV) analysis was employed to determine the oligomerization state of the protein in solution. Under similar solution conditions to those used for purification (20 mM Tris, 20 mM NaCl, pH 7.5), rTp34 displayed two sedimenting species when analyzed using the c(s) method (38). One
ferric citrate (i.e. chelated Fe\(^{3+}\)). Inclusion of ferric citrate caused 9% of the monomers in solution to form dimers (Table 2). SV experiments in the presence of unchelated Fe\(^{2+}\) and Fe\(^{3+}\) were attempted at pH 5.5 because of the low solubility of those cations at pH 7.5. SV in the presence of Fe\(^{2+}\) exhibited artifacts that were possibly because of oxidation of the cation during the course of the sedimentation. Inclusion of Fe\(^{3+}\) in the buffer caused rTp34 to aggregate (not shown), whereas Zn\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\) encouraged dimerization at pH 5.5 (Table 2). It is noteworthy that lowering the pH to 5.5 from 7.5 had a deleterious effect on the ability of most metal ions to induce dimerization of rTp34 (Table 2). The lone exception is Cu\(^{2+}\); even at only 300 \(\mu\)M concentration, this metal ion induced the complete dimerization of rTp34 (Table 2).

If the metal ions bind at the interfacial site observed in the crystal structure, this site must be promiscuous with respect to its metal ion binding preferences; although Cu\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) appear to be more efficient at stabilizing the dimer, no strong metal ion preference is observed at pH 7.5. The promiscuity is surprising for the “hard” metal ions (Mg\(^{2+}\) and Ca\(^{2+}\)) given their normally strong preference for oxygen ligands and the preferred octahedral coordination environment for Mg\(^{2+}\). Such insensitivity to metal ion identity may be a result of site malleability; several of the ligating residues exhibit significant movements depending on the occupancy of the metal ion-binding site. This flexibility could assist in binding metal ions with differing preferred coordination numbers.

The inclusion of more sodium ions (100 mM) caused about 60% of the protein to form dimers (Fig. 3B; Table 2). It is not known whether this latter effect is due to Na\(^{+}\) binding at the metal ion-binding site or to a more generalized effect whereby mutual repulsion of the rTp34 monomers is overcome by the increased ionic strength of the solution. Given that the normal concentration of sodium and chloride ions in human plasma is about 100 mM, it is plausible that Tp34, a lipoprotein believed to be periplasmic in T. pallidum (see below), is mostly dimeric in vivo. The highly flexible N-terminal portion of the protein would allow the dimerization to occur even when the protein is tethered to the inner membrane of T. pallidum by its acyl moiety, which occurs at Cys-1 in the native protein. Thus, rTp34 appears to be a metal ion-stabilized dimer in vitro and probably in vivo as well.

It is important to note that the SV assay does not directly measure metal ion binding; it simply measures the efficacy of metal ions in inducing the dimerization of rTp34. However, two pieces of evidence indicate that the observed dimerization phenomenon is a direct result of the cations binding at the known metal ion site in rTp34. First, the metal ion-binding site occurs at the dimeric interface of rTp34, with residues from both monomers participating in metal ligation (Figs. 1B and 2). Second, as noted above, lowering the pH has a negative effect on the efficiency with which metal ions induce dimerization (Table 2). For example, in the presence of Co\(^{2+}\), c(s) distributions show a significant peak for dimeric rTp34 at pH 7.5 but almost none at pH 5.5. This indicates that an ionizable group must contribute to the metal ion-binding site. The pK\(_a\) of His is about 6.5, and 3 His residues participate in the interdimeric metal ion-binding site of rTp34 (Fig. 2). To approximate the pK\(_a\) values of these histidines in the context of rTp34, the structure of monomeric, metal-free rTp34 was subjected to electrostatic calculations (89). These calculations were performed on both monomers of the rTp34-EDTA structure independently, and the two pK\(_a\) values thus obtained for each His were averaged. The results demonstrate that the pK\(_a\) values of His-70 (7.3) and His-155 (7.7) are probably titratable in the pH range used for the SV experiments (5.5–7.5). The tendency for less metal-induced dimerization at pH 5.5, thus, buttresses the notion that the observed metal ion-binding site is responsible for the metal ion-induced dimerization of rTp34.

**Lactoferrin Binding**—Previous reports (23, 53) had indicated that Tp34 associates with hLF, a protein that serves as a ferric ion carrier within mammalian mucosal tissues. Isothermal titration calorimetry (ITC) and analytical ultracentrifugation were used to verify the previous reports of Tp34 binding to hLF. The ITC experiments showed that rTp34 specifically and avidly binds to iron-free hLF (apo-hLF; Fig. 4A). The thermogram obtained from this experiment is complex; it clearly displays two different modes of binding. The first mode is endothermic and starts to saturate before the second, which is exothermic. Control experiments established that the heat of dilution of Tp34 is minimal. Satisfactory fits to the isotherm could be obtained using a two-independent-site model (Fig. 4A) or a sequential binding model (not shown). Fitting the data to the former model, the dissociation constant obtained for the endothermic binding site is 66 ± 7 nM; for the second, exothermic site, it is 540 ± 10 nM. For both sites, the stoichiometry is 1:1; therefore, the overall stoichiometry of binding is two rTp34 proteins per apo-hLF molecule. Significantly, the structure of hLF is bilobed, with both lobes exhibiting very similar structures (54). Further experiments are warranted to examine the facile interpretation that one rTp34 monomer binds one lobe of hLF, resulting in the observed 2:1 stoichiometry. Titrations carried out using iron-loaded hLF (Fe-hLF) were qualitatively similar to those using apo-hLF (not shown). Notably, the buffer in which these experiments were conducted contained 100 mM NaCl, which enables Tp34 dimerization (Fig. 3B). The potential, thus, exists for multiple heat-producing reactions to occur upon injection of concentrated solutions of rTp34 into apo-hLF, e.g. dimer dissociation, monomer association, dimer-apo-hLF association, and monomer-apo-hLF association. Given the complexity of this system, proposing a definitive model for the rTp34-hLF interaction must await further biochemical and structural characterization of these proteins.

The rTp34-hLF interaction was also studied using SV. Alone, apo-hLF exhibited a sedimentation coefficient of 5.3 S, which is in agreement with its status as a monomer in solution (Fig. 5A). Upon the addition of a 3-fold molar excess of rTp34, this peak in the c(s) profile is fully shifted to 6.3 S. The calculated molecular mass of the 6.3 S peak (133,000 Da) is consistent with 2 molecules of rTp34 (23,000 Da) binding to one molecules of apo-hLF (76,000 Da). By using multi-signal c(s) analysis (39), the approximate concentrations of the individual components making up the 6.3 S peak can be calculated (Fig. 5B). The calculated concentration of rTp34 in the 6.3 S peak is 5.7 \(\mu\)M, whereas it is 2.7 \(\mu\)M for apo-hLF. The calculated molar ratio of rTp34:apo-hLF is, therefore, 2:1.1. Hence, two independent bio-

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**Structure of the Tp34 Lipoprotein from T. pallidum**

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Given that the rTp34 dimer is stabilized by divalent metal ions, experiments were conducted to study the rTp34-apo-hLF interaction with such cations present in solution. Unfortunately, at the high protein concentrations necessary for ITC, both proteins were subject to precipitation and other aberrant behaviors in the presence of Zn\(^{2+}\). Nonetheless, SV could be employed at lower protein concentrations and with 300 \(\mu\)M Zn\(^{2+}\). The results (supplemental Fig. 1) demonstrated that rTp34 binds to apo-hLF with a stoichiometry of \(~2:1\), even in the presence of the divalent metal ion, implying that dimeric rTp34 binds to apohLF. Further experiments are planned to explore the dimeric rTp34:apoHLF association.

Interestingly, an earlier report demonstrated that bLF bound to rTp34 (23). We also found that apo-bLF bound to rTp34, but the stoichiometry was 1:1, and only the endothermic phase of the binding was observed with a dissociation constant of 630 nM (Fig. 4B). Because bLF and hLF are structurally similar (55), it is not surprising that one of the binding sites is preserved between the two proteins. The 1:1 stoichiometry of the rTp34-bLF interaction could illustrate a lack of conservation of the second exothermic binding site.

Several control experiments were performed to confirm the specificity of the observed rTp34-hLF interaction. We first examined whether the interaction between Fe-hLF and rTp34. Similar results to those presented above were obtained (not shown).

Although no kinetic experiments were performed on the rTp34-hLF interaction, two lines of evidence lead to the conclusion that \(k_{\text{off}}\) for the interaction is slow. First, the SV studies demonstrate that the rTp34-hLF complex sediments as a single peak (Fig. 5A). As mentioned above, the calculated mass of the species in that peak comports with the mass of one hLF and two rTp34 molecules. Simulations have demonstrated that this type of \(c_{\text{s}}\) distribution is consistent with \(k_{\text{off}}\) of \(10^{-4}\) to \(10^{-5}\) s\(^{-1}\) (51). Also, rTp34-hLF complex that had been subjected to ITC was subsequently subjected to size-exclusion chromatography. The two proteins co-eluted (not shown), with no evidence of a shoulder in the complex UV elution profile. This latter experiment also indicates a slow \(k_{\text{off}}\) for the rTp34-hLF interaction.

hTF, a serum iron-transport protein with structural homology to hLF (56), could bind to rTp34. In neither ITC (Fig. 4C) nor analytical ultracentrifugation experiments (not shown) could an interaction between hTF and rTp34 be demonstrated. The lack of an rTp34:hTF interaction is in accord with earlier results demonstrating that hTF does not interact with proteins from \(T.\ pallidum\) (23, 53). Other \(T. pallidum\) lipoproteins (PnrA and rTp32) failed to bind to hLF specifically in the ITC assay (Fig. 4C). From these control experiments, we conclude that the avid binding of rTp34 to hLF is specific.

The simplest deduction from the combined data is that rTp34 is a treponemal receptor for hLF. As such, it may be responsible for iron uptake by \(T. pallidum\). Other pathogenic bacterial species are known to utilize hLF to acquire iron from their mammalian hosts. Specifically, under conditions of iron starvation, certain \(Neisseria\) express outer-membrane hLF receptors (LbpAs) that specifically bind Fe-hLF (57). Once the
LbpAs bind to Fe-hLF, accessory proteins are recruited to the complex, and ferric iron is stripped from Fe-hLF. The cations are then transported into the periplasm. The process is dependent on energy transduction via a TonB system present in the bacterial inner membrane. There are several facts that belie the operation of a TonB system present in the Neisseria-like iron import system in *T. pallidum*. LbpA, an integral protein of the outer membrane, has no detectable sequence homology to Tp34, a putative periplasmic lipoprotein (see below). Also, there is no evidence that the *T. pallidum* genome encodes the requisite accessory proteins akin to those used for iron transport into the periplasm of *Neisseria*. If *T. pallidum* uses rTp34 as a receptor for Fe-hLF with the purpose of acquiring iron, it must carry this out via an uncharacterized mechanism.

**Structure of the Tp34 Lipoprotein from T. pallidum** - That rTp34 contained metal ions and bound the iron-sequestering protein hLF in receptor-like fashion(s) implied that native Tp34 might be exposed on the outer membrane of *T. pallidum*, where it would be accessible to such nutrients within human body fluids. However, despite decades of intensive efforts and some claims of success, not a single protein has yet been shown definitively to be located on the outer leaflet of the *T. pallidum* outer membrane (7, 8). A previous study by Cox et al. (42) using polyclonal antibodies provided initial evidence that Tp34 was not located on the surface of the *T. pallidum* outer membrane. In that study (42), an agarose gel microdroplet method, the current reference method for assessing mem-

**FIGURE 5. SV studies of the rTp34-hLF interaction.** A, the c(s) profiles of two separate experiments are shown. In one (black line), only hLF was sedimented, and it sedimented as a monomer. The second experiment (gray line) was performed with a 3-fold excess of rTp34. The hLF peak was completely shifted by about 1 S. B, shown are the spectrally decomposed c(s) distributions that correspond to the experiment represented by the gray line in A. The black line denotes the signal present from hLF. The gray line denotes the signal for rTp34. The rTp34 protein is present in the 6.2 S peak at twice the concentration of hLF.
brane surface topology in the pathogenic spirochetes *T. pallidum* and *Borrelia burgdorferi* (61) was utilized. Encapsulation of spirochetes in agarose gel microdroplets protects the fragile *T. pallidum* outer membrane from physical disruption (during reactivity with antibodies) by providing a supporting matrix but one that is sufficiently porous to allow reagent (e.g., antibody probes, secondary antibody conjugates) access and removal (42, 61). The location of proteins beneath the surface is inferred by observing immunofluorescence of the bacteria upon treatment of encapsulated *T. pallidum* with low levels of Triton X-100 (0.15%) (which partially disrupts outer membrane integrity) in the presence of the primary antibody probes (42, 61). In our study the anti-Tp34 monoclonal antibody (4A4) was used to further investigate Tp34 membrane topology using the gel microdroplet technique. Of note, in immunoblots, monoclonal antibody 4A4 reacted with rTp34 as well as with a broader-migrating 34-kDa protein band in *T. pallidum* whole cell lysates (Fig. 8A); this broadly migrating pattern for Tp34 has been well documented (44, 62). For these studies, we also employed an antibody directed against Tp47, a subsurface penicillin binding lipoprotein of *T. pallidum* (63), as a known marker for subsurface localization. When monoclonal antibody 4A4 or anti-Tp47 antibodies were used to probe intact *T. pallidum* encapsulated within agarose gel microdroplets (in the absence of detergent), spirochetes were not labeled (Fig. 8B). In contrast, when spirochetes were treated with a low concentration of Triton X-100, they fluoresced when probed with the same antibody preparations (Fig. 8B). Quantitatively, less than 6% of spirochetes fluoresced when exposed to anti-Tp34 or anti-Tp47 antibodies, whereas in the presence of Triton X-100, immunolabeling of treponemes was observed at 92 and 93% when reacted with anti-Tp47 or anti-Tp34 antibodies, respectively (Fig. 8C). These results as well as those previously reported by Cox et al. (42) suggest strongly that Tp34 is not surface-exposed in *T. pallidum*.

**Implications**—As a non-cultivable bacterium, *T. pallidum* remains intractable to many conventional approaches for pathogen characterization, such as site-directed mutagenesis or gene disruption experiments. A structural biology approach to investigating the structure and function of various *T. pallidum* membrane lipoproteins, thus, has emerged as a fruitful avenue of syphilis research (20–22). Although the lipoproteins of *T. pallidum* and other bacteria are considered to be integral membrane proteins, the hydrophobic properties of the lipoproteins are engendered principally by their three covalently attached long chain fatty acids that serve as membrane anchors. When these acyl chains are absent (as was performed herein for rTp34), the polypeptides tend to be water-soluble, consistent with their presumed native locations in either the periplasm or extracellular environments; this feature also allows the proteins (deacylated) to be amenable to protein crystallization.
**Structure of the Tp34 Lipoprotein from T. pallidum**

Tp34 was selected for a structural determination based on an early report that it bound the iron-sequestering protein lactoferrin (23, 53). Thus, the only preconceived notion before solving the structure of Tp34 was that it might be involved in protein-protein interactions. That dimeric rTp34 contained two interfacial metal-binding sites likely occupied by Zn$^{2+}$ was unexpected. Apparently, the metal-binding sites can accommodate a wide array of ions, but the two ions that most efficiently induce the dimerization of rTp34 are Zn$^{2+}$ and Cu$^{2+}$ (Table 2), of which zinc would appear to be the most biologically relevant (64).

The demonstration that rTp34 binds to Zn$^{2+}$ (Fig. 2) adds to the increasing body of evidence suggesting that zinc is an important metabolic cofactor for *T. pallidum*. Indeed, *T. pallidum* encodes a large number of predicted metalloproteins that typically bind Zn$^{2+}$ (65, 66). In this regard there has been some speculation that *T. pallidum* might have evolved enzymatic machinery that preferentially employs zinc (rather than iron) in its major metalloproteins. In support of this view, from a biological perspective, Zn$^{2+}$ is the second (next to iron) most abundant trace metal in the human body (64). Zinc also may be more easily scavenged than iron (which is very tightly bound to its iron-sequestering proteins) from human tissues and body fluids. Regarding zinc uptake by *T. pallidum*, convincing evidence has been presented that TroA, a periplasmic protein tethered to the cytoplasmic membrane via an uncleaved leader peptide, is a zinc-containing ABC-type transporter (66–68). Heretofore, TroA, thus, was assumed to be the major, if not sole, receptor for the transport of zinc across the cytoplasmic membrane of *T. pallidum* (66). However, given our findings, Tp34 may also play a role in zinc acquisition by *T. pallidum*.

Although Cu$^{2+}$ binding to rTp34 has not yet been directly observed, the high efficiency with which that metal induces the dimerization of rTp34 (Table 2) suggests that it binds to the interfacial metal ion site. The suitability of the rTp34 metal ion site (Fig. 2) as a copper binding motif is established by the fact that His nitrogens, Glu carboxylate oxygens, and δ sulfurs of Met are known ligands for copper ions (48). The physiological relevance of the putative Cu$^{2+}$ binding to rTp34 is unknown; Cu$^{2+}$ is thought to be almost wholly sequestered in vivo by metal-binding proteins (69), and no other treponemal proteins are known to require copper. However, examination of the genomes of organisms that carry *tp34*-like genes shows a striking correlation; in all cases but one (*Chromobacter violaceum*), an open reading frame within two genes of the *tp34*-like gene is a putative Fe$^{2+}$/Pb$^{2+}$ permease belonging the FTR1 family. This finding is significant because in yeast, FTR1 works in concert with a copper-containing protein (FET3) to import iron (70). There is, thus, the intriguing possibility that Tp34 acts either as a treponemal FTR1 accessory protein or as a copper-sequestering protein that supplies the cation to another proteogel microdroplets were exposed to either a mouse monoclonal antibodies anti-Tp34 (4A4) or anti-Tp47 (11E3) in the presence or absence of 0.15% (v/v) Triton X-100 (TX). After secondary antibody exposure (anti-mouse Alexa Fluor 488), spirochetes were examined by either darkfield (DF) or fluorescent microscopy. C, the number of fluorescent treponemes were quantified from at least three independent microdroplet preparations and plotted. The error bars represent S.D.

**FIGURE 8.** Membrane topology assessment of Tp34 in *T. pallidum*. A, immunoblot (using monoclonal antibody 4A4) of either 1 μg of rTp34 or 2.5 × 10$^7$ *T. pallidum* whole cells. B, *T. pallidum* bacteria encapsulated in agarose gel microdroplets were exposed to either a mouse monoclonal antibodys anti-Tp34 (4A4) or anti-Tp47 (11E3) in the presence or absence of 0.15% (v/v) Triton X-100 (TX). After secondary antibody exposure (anti-mouse Alexa Fluor 488), spirochetes were examined by either darkfield (DF) or fluorescent microscopy. C, the number of fluorescent treponemes were quantified from at least three independent microdroplet preparations and plotted. The error bars represent S.D.
tein. The similarity of the metal-binding site in rTp34 to a treponemal iron-binding site in the superoxide reductase (Tp0823) of *T. pallidum* (71), however, suggests that Tp34 could be involved in binding and transporting iron.

The proteins of the Tp34-containing Hogenport family 261060 are encoded by the genomes of 23 species of bacteria; in 9 of these organisms, the *tp34*-like genes are clustered with an ABC-type permease and ATPase. We classify the proteins from these nine organisms (*Bifidobacterium longum*, *Erwinia carotovora*, *Pasteurella multocida*, *Treponema denticola*, *Wolinella succinogenes*, *Y. pestis* KIM 5, *Y. pestis* CO-92, *Y. pestis* 91001, and *Yersinia pseudotuberculosis*) as belonging to subfamily 261060ABC. The genome of *T. pallidum* does not encode a member of 261060ABC, although that of the related spirochete *T. denticola*, does. ABC-type operons usually encode a protein (a periplasmic LBP) that binds the targeted ligand (72, 73). However, in the genomes of the members of subfamily 261060ABC, such a protein is not encoded near *tp34*-like genes. It can be speculated that Tp34 assumes the role of the binding protein for an ABC-type metal-ion-transport system. Although Tp34 is not structurally homologous to LBPs, the two proteins apparently employ similar strategies for binding ligands. LBPs utilize a “clamshell” motion to enclose their ligands, effectively surrounding the ligand with protein. The Tp34 dimer is stabilized upon metal ion binding, and this event also shields the metal from solvent. That the genome of *T. pallidum* does not include either an ABC permease or an ATPase in proximity to the *tp34* gene does not preclude, however, Tp34 from acting as a receptor for such a metal ion-transport system; the permease and ATPase may be encoded at distant genomic loci. In fact, in the case of the methionine transporter (Tp32) in *T. pallidum*, its hypothetical ATPase (tp0120) and permease (tp0119) are together but distant from *tp32* (tp0821) (21, 74). Whether such non-operonic ABC transport systems are yet another unusual feature of *T. pallidum* remains to be more fully explored via additional functional studies on other treponemal ABC transport systems in this organism.

Although iron generally is an essential element for microorganisms, it is not readily available in most biological systems. This poses a formidable metabolic obstacle when a virulent bacterium, like *T. pallidum*, invades the human host. In humans, the amount of free iron is extremely low (∼10^{-18} M) and usually insufficient to sustain bacterial growth (75). The majority of iron is found intracellularly as myoglobin, ferritin, or hemoglobin, and smaller quantities of extracellular iron are complexed with transferrin (TF) or lactoferrin (LF) (76–78). TF is present at very high levels (25–44 μM) in serum but is only a minor component (0.2–1.3 μM) of mucosal secretions. On the other hand, LF is present at very low levels in serum (3.8–8.7 nm) but is in relatively high concentrations (6–13 μM) (79) in the mucosa, sites where *T. pallidum* typically establishes its initial infection. Thus, in order for iron to be accessible to the bacterium, it must be extracted from the human host carrier molecules by specialized uptake mechanisms (77, 80, 81). The major role for iron is its involvement in enzymatic redox reactions, but iron in proteins can also play a structural role. *T. pallidum* lacks major iron-containing proteins typically found in other bacterial pathogens, including the oxidative defense enzymes superoxide dismutase, catalase, and peroxidase (65). However, the spirochete is believed to contain superoxide reductase (Tp0823) and rubredoxin (Tp0991), both of which are likely to participate in oxygen detoxification (50, 82–84). This is consistent with the prevailing view that *T. pallidum* is microaerophilic (1). Genome analysis has further revealed that there likely are at least nine *T. pallidum* proteins (Tp0053, Tp0080, Tp0735, Tp0823, Tp0842, Tp0939, Tp0991, Tp1008, and Tp1038) that require iron as a cofactor (50, 65, 71, 85). Thus, it is plausible that *T. pallidum* requires iron.

The potential role of Tp34 as a hLF receptor is intriguing in the light of the belief that *T. pallidum* lacks an outer membrane receptor for iron acquisition and probably does not produce siderophores to trap extracellular iron. Nonetheless, it is tempting to speculate that Tp34 is involved in iron acquisition via its propensity to bind lactoferrin. This, however, poses an interesting paradox. In early studies it was reported that a molecule likely to be Tp34 was capable of binding mammalian iron transport proteins (23). However, those findings were difficult to reconcile, given the fact that there was no evidence that Tp34 was on the surface of *T. pallidum* (where Tp34 could interact with lactoferrin). Our structural studies herein as well as the fact that *T. pallidum* likely utilizes iron now prompt a reevaluation of the earlier work of Staggs et al. (23). First, Tp34 contains a dimeric Ig-fold that is stabilized by divalent metal ions. The ability of the Ig-fold to serve as a receptor for a proteinaceous ligand is well known (e.g. see Refs. 59 and 86). Second, we have garnered additional biochemical evidence via ITC and SV that Tp34 indeed binds avidly to human lactoferrin. Third, although agarose gel microdroplet encapsulation and immunofluorescence microscopy indicated that Tp34 is not on the outer surface of *T. pallidum*, Hazlett et al. (87) recently provided compelling evidence that the outer membrane of *T. pallidum* can be transiently perturbed by TP0453, a membrane lipoprotein that has several membrane-interacting amphipathic α-helices. In this scenario TP0453 is believed to be tethered via its acyl chains to the inner leaflet of the treponemal outer membrane whereby transient interactions of the α-helices of the polypeptide with the inner leaflet of the outer membrane then perturbs the lipid bilayer. This transient disruption of outer membrane integrity, however, is not thought to be sufficient to allow large proteins, such as antibodies, to traverse the outer membrane. On the other hand, such a mechanism could be more forgiving within the human host, where extracellular matrix or other host proteins within blood or interstitium might contribute to outer membrane integrity, much the way that soft agarose can stabilize the *T. pallidum* outer membrane in gel microdroplet assays (42). If so, then other larger nutrients, including lactoferrin, may be able to enter the periplasm of the organism, whereby binding to cognate receptors could occur. It is also important to note that mucosal antibody, namely secretory IgA (∼150–300 kDa) is 2–4-fold the molecular mass of hLF (∼80 kDa). It is, thus, possible that membrane perturbation by Tp0453 could allow moderately sized proteins (like hLF) to traverse the outer membrane, whereas large, IgA-sized molecules are barred.
passage. All of these provocative possibilities warrant further investigation.

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REFERENCES

Structure of the Tp34 Lipoprotein from T. pallidum


