Structural basis for iron piracy by pathogenic *Neisseria*

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*Neisseria* are obligate human pathogens causing bacterial meningitis, septicaemia and gonorrhoea. *Neisseria* require iron for survival and can extract it directly from human transferrin for transport across the outer membrane. The transport system consists of TbpA, an integral outer membrane protein, and TbpB, a co-receptor attached to the cell surface; both proteins are potentially important vaccine and therapeutic targets. Two key questions driving *Neisseria* research are how human transferrin is specifically targeted, and how the bacteria liberate iron from transferrin at neutral pH. To address these questions, we solved crystal structures of the TbpA–transferrin complex and of the corresponding co-receptor TbpB. We characterized the TbpA–transferrin complex by small-angle X-ray scattering and the TbpA–TbpB–transferrin complex by electron microscopy. Our studies provide a rational basis for the specificity of TbpA for human transferrin, show how TbpA promotes iron release from transferrin, and elucidate how TbpB facilitates this process.

*Neisseria* comprise a large family of Gram-negative bacteria that colonize humans. Two family members, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, are pathogens that invade the urogenital tract and nasopharynx, respectively, causing gonorrhoea, meningitis and other systemic infections. Although vaccines exist for bacterial meningitis and *Neisseria meningitidis* colonize humans. Two family members, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, are pathogens that invade the urogenital tract and nasopharynx, respectively, causing gonorrhoea, meningitis and other systemic infections. Although vaccines exist for bacterial meningitis, they have significant limitations1 and are ineffective against serogroup B *N. meningitidis*. Currently, there are no vaccines to protect against gonococcal infections. The recent emergence of antibiotic-resistant strains2 adds urgency to the need to develop more effective countermeasures for both pathogens.

**Neisseria** require iron for survival and virulence3. Unlike most Gram-negative bacteria, *Neisseria* do not make siderophores but instead extract iron directly from serum transferrin in the human host (TF). The neisserial transport system consists of two large surface proteins: TF-binding protein A (TbpA), a 100-kDa integral outer membrane protein belonging to the TonB-dependent transporters4; and TF-binding protein B (TbpB), an ~80-kDa co-receptor attached to the outer membrane by a lipid anchor (Supplementary Fig. 1). Both proteins are found in all clinical isolates of pathogenic *Neisseria*. TbpA binds apo and iron-containing transferrin with similar affinities, whereas TbpB only associates with iron-bound TF5,6. Although TbpA can extract and import iron without TbpB, the process is considerably more efficient in the presence of the co-receptor7,8. TbpA and TbpB induce bactericidal antibodies in mice against *N. meningitidis*3,10 and *N. gonorrhoeae*11, making both proteins important vaccine targets. To elucidate how TbpA and TbpB function to bind human TF selectively and extract its tightly bound iron (K\text{a} = 10^{23} \text{M}^{-1}) at physiological pH, we combined an approach consisting of X-ray crystallography, small-angle X-ray scattering and electron microscopy to determine a model of the 260-kDa iron import complex from *N. meningitidis* strain K454 (serogroup B). Because *N. gonorrhoeae* strains FA1090 and FA19 express TbpA proteins that are 94% identical to the meningococcal protein, whereas the corresponding TbpB proteins are 61% and 69% identical, respectively, our results are relevant to both pathogens.

**Crystal structure of the TbpA–(apo)hTF complex**

Structural characterization of the neisserial iron import machinery was initiated by crystallizing *N. meningitidis* TbpA with full-length, glycosylated apo human transferrin (hTF) and solving the structure to a resolution of 2.6 Å (Fig. 1, Supplementary Figs 2–4, 17 and Supplementary Table 1). Despite being significantly larger (~20%) than other structurally characterized TonB-dependent transporters4, TbpA retains the classic fold with a 22-strand transmembrane β-barrel encompassing a plug domain (Fig. 1a). Most of the additional mass is found in several extracellular loops which extend up to ~60 Å above the outer membrane. A plug loop implicated in iron uptake12,13 is unusually long and protrudes ~25 Å above the cell surface.

Human TF is a bilobal glycoprotein (~80 kDa) with a single ferric (Fe\textsuperscript{3+}) ion tightly bound within a cleft in each lobe (Fig. 1a and Supplementary Fig. 8). Each lobe of TF consists of two subdomains which form the cleft: N1, N2, C1 and C2. In the absence of iron, each lobe adopts an open conformation (Protein Data Bank (PDB) accession code 2HAV)14. To obtain the best model of neisserial iron import, we solved the structure of diferric hTF at 2.1 Å resolution (Fig. 2d and Supplementary Fig. 8). In our diferric structure, each lobe is found in a fully closed conformation, nearly identical to the diferric structures for both porcine (PDB code 1H76) and rabbit (PDB code 1NF) TF.

When TbpA binds hTF, it sequesters ~2,500 Å\textsuperscript{2} of buried surface, with 81 TbpA residues and 67 hTF residues participating in the interaction (Fig. 1a, c, e and Supplementary Table 2). TbpA binds exclusively to the C lobe of hTF, where electrostatic complementarity exists between the extracellular surface of TbpA (electropositive) and the C1 subdomain of hTF (electronegative) (Fig. 1b, d). Two notable features of the interface include: (1) the unusually long TbpA plug loop (residues 121–139) interacts directly with the C1 subdomain

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(Figs 1a, 2a, c and Supplementary Fig. 5), and (2) an α-helix in TbpA extracellular loop 3 (residues 351–361, the L3 ‘helix finger’) is inserted directly into the cleft of the C lobe between the C1 and C2 subdomains (Figs 1a, 2a, b and Supplementary Fig. 5). The interaction between TbpA and hTF was found to be relatively tolerant to point mutations in TbpA, as might be expected given the large binding interface (Supplementary Fig. 6).

The mechanism of species specificity of neisserial TbpA for hTF is unknown. In in vitro assays, gonococcal and meningococcal TbpA proteins have been shown to bind human TF, but not TF from cow, horse, rabbit, mouse, rat, sheep, duck or pig. Infection with N. meningitidis displayed a higher mortality rate when the iron source was Fe₃hTF rather than bovine TF. From the TbpA–hTF crystal structure, seven sites spanning both the C1 and C2 subdomains of hTF participate in binding TbpA (Fig. 1e and Supplementary Fig. 7), with each site containing one or more residues unique to human TF.

Because TbpA shows limited sequence variation (Supplementary Fig. 5 and Supplementary Table 4), is present in all clinical isolates, and nearly all the interactions with hTF are mediated by extracellular loops of TbpA, we attempted to disrupt the TbpA–hTF interface to see if this would be a viable therapeutic strategy. Peptides from four loops

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**Figure 1** | Crystal structure of the TbpA–(apo)hTF complex. **a**, The TbpA β-barrel is lime, the plug is red, the helix finger is magenta. For hTF, the C lobe is gold and the N lobe is salmon. A ferric ion has been modelled into the C lobe as a red sphere. **b**, Electrostatic potential of TbpA viewed from the extracellular surface with hTF shown in gold ribbon. **c**, Residues of TbpA that bind hTF: gold, hydrophobic interactions; green, hydrogen bonds; red, salt bridges (residues labelled). **d**, Electrostatic potential of hTF viewed from the extracellular surface with TbpA shown in green ribbon. **e**, Surface representation of hTF showing regions that bind TbpA.

**Figure 2** | TbpA distorts the iron coordination site in the hTF C lobe by inserting a helix from extracellular loop three. **a**, TbpA (green) inserts a helix finger from extracellular loop 3 (magenta) into the cleft of the hTF C lobe (gold). **b**, The helix finger interacts with the hTF C-lobe residues through main-chain and side-chain interactions. **c**, The long TbpA plug loop (pink) interacts with residues from the C1 subdomain. **d**, Comparison of C-lobe conformations for holo (green), apo (grey, PDB code 2HAU), and TbpA-bound TF (gold). **e**, Superposition of residues coordinating iron in diferric hTF (grey) with the same residues in hTF when bound to TbpA (gold). Distances for the residues coordinating iron are shown for the TbpA-bound state.
This page discusses the structure of the TbpA–TbpB–holohTF complex and how TbpB facilitates iron extraction and uptake. It mentions the utility of competition ELISA to determine the relative binding affinities of iron to the complex. It also describes the use of SAXS structures to understand the interactions of the complex and the role of TbpA and TbpB in iron binding and release.

**X-ray and SAXS structures of TbpB and TbpB–holohTF**

Although only TbpA can acquire iron from hTF, the reaction is enhanced by expression of the co-receptor TbpB, which preferentially binds holo-hTF. To understand how TbpB facilitates iron extraction and uptake, we solved the structure of *N. meningitidis* TbpB (Fig. 3a, Supplementary Figs 12, 18 and Supplementary Table 1). TbpB consists of two lobes that are structurally similar, sharing an eight-strand β-barrel subdomain flanked by a four-strand ‘handle’ domain.

TbpB proteins from different isolates vary substantially in size and sequence (Supplementary Fig. 11), but the overall fold is conserved. Our structure aligns closely with three TbpB structures from porcine pathogens and shows that sequence and conformational variations are found primarily in the N lobe (Fig. 3a and Supplementary Fig. 12b). Specifically, residues affecting TF binding lie on the distal surface of the N lobe and confer much of the TF species specificity (Fig. 3a). We made point mutants at four sites on this surface that reduced or abolished binding to hTF (Fig. 3a and Supplementary Fig. 12). An analysis of our mutants, and those reported previously, demonstrates that the major site of interaction lies in the N lobe.

To clarify the interactions of purified TbpA and TbpB with hTF, an enzyme-linked immunoassay (ELISA) was used to probe binding to Fe₃-hTF, monoferric hTF with iron only in the N lobe (Fe₃N), or in the C lobe (Fe₃C), and apo-hTF (incapable of binding iron open conformation). Notably, interaction with TbpA causes the C lobe to adopt a conformation midway between open and closed, with a 24° rotation required to align C1 and C2 subdomains with dixeric hTF (Fig. 2d and Supplementary Fig. 10). The TbpA L3 helix finger is inserted into the cleft, where it interacts with D634 from the C1 subdomain and several residues from the C2 subdomain (Fig. 2a, b). The long TbpA plug loop also interacts with the surface of the C1 subdomain of hTF (Fig. 2c). These interactions induce a partial opening of the cleft in the hTF C lobe, thereby destabilizing the iron coordination site to facilitate the release of iron from the C lobe to TbpA. Figure 2e shows the residues coordinating iron in the hTF C-lobe structure and the significant increase in these distances when hTF binds TbpA. Such increases are clearly incompatible with tight binding of iron in the C lobe.
in either lobe). Consistent with earlier studies using apo- or holo-
hTF, TbpA binds all four hTFs with equal affinity regardless of the 
iron status of either lobe (Fig. 3b). In contrast, TbpB has a strong 
preference for hTF constructs with iron bound in the C lobe, regardless 
of the coordination state of the N lobe. These experiments clearly show 
that, at least in vitro, hTF interacts with TbpA and TbpB solely through 
the C lobe and is not affected by the presence or absence of iron in the N 
lobe. Our results indicate that Neisseria cannot use the entire serum TF 
iron supply and that the primary function of TbpB is to select and 
concentrate on the cell surface only those forms of TF that are able to 
provide iron to the bacterium.

Because TbpB primarily binds the C lobe of hTF through its N lobe, 
we performed steered molecular docking for the TbpB–hTF complex 
based on previous docking studies for the porcine complex and on 
our mutagenesis results. We collected small-angle X-ray scattering 
(SAXS) data on the TbpB–(holo)hTF complex (Supplementary Figs 12 
and 13) and used GASBOR to construct the SAXS envelope (Fig. 3c 
and Supplementary Fig. 13). The resulting molecular envelope 
describes the spatial arrangement of TbpB and hTF, and was used to 
fit the TbpB–hTF complex structure. Binding TbpB to hTF buries 
1,300 Å² of surface area and, notably, uses a region of the hTF C lobe 
distinct from the site where TbpA binds.

Structure of the triple complex by single–particle EM

On the basis of the TbpA–(apo)hTF crystal structure and the SAXS 
solution structure of the TbpB–(holo)hTF complex, we formed an 
in silico model for the TbpA–TbpB–(holo)hTF triple complex by 
superposing the two complexes along the C1 subdomain of hTF 
(Fig. 4a). To test this model, we assembled the triple complex from 
its components (Fig. 4b) and visualized the resulting particles by 
negative staining electron microscopy (EM) (Fig. 4c and Supplementary 
Fig. 14). A set of 4,240 particles was subjected to a reference-free 
classification to identify subsets of like images, representing molecules 
viewed in the same orientation; the members of each class were then 
averaged to suppress noise. Several of the class averages show a central 
density, ~45 Å across, to which two small globular densities are 
appended at points about 120° apart around its periphery (for 
example, Fig. 4c (asterisk) and Supplementary Fig. 14). A plausible 
interpretation is that the central density corresponds to the β-barrel 
domain of TbpA and the two appended densities to TbpB and hTF 
(Supplementary Fig. 14), in agreement with our model for the triple 
complex.

Iron extraction and import

The X-ray, SAXS and EM structures support a consistent arrangement 
for the TbpA–TbpB–(holo)hTF complex. Although TbpA and TbpB 
each bind hTF tightly through the C lobe, they have unique, non-
overlapping binding sites (Fig. 5a). A consequence of the assembly of 
the triple complex is the formation of an enclosed chamber (volume of 
~1,000 Å³) at the union of the three protein components, which sits 
directly above the plug domain of TbpA (Fig. 3b). This chamber may 
serve two important roles for iron acquisition by the bacteria: (1) 
prevent diffusion of iron released from hTF; and (2) guide the iron 
towards the β-barrel domain of TbpA for subsequent transport.

Figure 5 | Mechanism for iron import. a, Binding surfaces of TbpA (green) 
and TbpB (cyan) mapped onto the hTF C lobe. b, Enclosed chamber formed by 
TbpA–TbpB–(holo)hTF (left, magenta sphere). A cutaway view (right) from 
inside the chamber illustrates the proximity of the iron (red). c, Model for iron 
release. Conserved K359 in the L3 helix finger is positioned to interact with 
residues that regulate iron release in eukaryotic iron uptake. d, Import of iron 
through TbpA. 1, an electrostatic surface depicts cavities between the TbpA 
barrel and plug domain; 2, plug domain constrictions close the tunnel; 3, 
molecular dynamics simulations show removal of constrictions upon 
interaction with TonB.
A plausible mechanism for iron extraction from hTF is shown in Fig. 5c. Insertion of the TbpA L3 helix finger into the cleft between the C1 and C2 subdomains of hTF positions a conserved lysine (TbpA K359; Supplementary Fig. 4) near the hTF triad of charged residues (hTF K534, R632 and D634) that has been implicated in iron release from the C lobe26. TbpA K359 is perfectly situated to interact with D634, which would disrupt the charge neutralization it normally provides to the two basic triad residues K534 and R632. This potential charge repulsion between the hTF C1 and C2 subdomains could induce cleft opening (as observed in the TbpA–(apo)hTF crystal structures), resulting in distortion of the C-lobe iron-binding site and subsequent iron release. Notably, a recent study indicates that an hTF D634A mutant has a rate of iron release that is 80-fold faster at pH 5.6 than the control under the same conditions26.

To investigate iron transport across the outer membrane, steered molecular dynamics was used to simulate interactions between TbpA and TonB (Supplementary Fig. 15). In the ground state structure, a large, highly negative transmembrane cavity is located between the barrel wall and the plug domain, but access is restricted on the extracellular side by residues 91–96 (restriction loop) and on the periplasmic side by residues 65–71 (helical gate) from the plug domain (Fig. 5d). When force (designed to mimic interaction with TonB) is applied to the plug domain, it sequentially unfolds beginning with removal of the helical gate followed by the restriction loop, producing an unobstructed pathway from the extracellular space to the periplasm (Supplementary Movie 1). This pathway is lined by the EIEYE motif of the plug domain18, which contains multiple oxygen donor groups that could transiently bind iron as it is transported through TbpA.

Concluding remarks

Humans and bacteria have each developed unique strategies to acquire iron from serum transferrin27,28 (Supplementary Fig. 16). Our TbpA–TbpB–hTF X-ray, SAXS and EM structures indicate a mechanism for bacterial uptake of iron with the following characteristics: (1) a large TbpA–hTF binding interface with many human-TF-specific interactions; (2) iron removal from the hTF C lobe by insertion of a helical element from TbpA into the iron-binding cleft; and (3) iron transport across the outer membrane after TonB-dependent conformational changes in the TbpA plug domain. This system allows efficient extraction of iron despite the extremely high affinity of hTF bound iron at neutral pH. The TbpB co-receptor, which is tethered to the cell surface by a long, unstructured polypeptide chain, is able to attract and preferentially bind hTF with iron in the C lobe, thereby increasing the efficiency of the system. Crucial to the mechanism, TbpA and TbpB associate with different regions of the hTF C lobe, creating an enclosed chamber above the plug domain to ensure that iron is efficiently sequestered and directionally transported through the TbpA barrel (Supplementary Movie 2). Finally, as TbpA and TbpB are surface-exposed, antigenic and required for neisserial infections29, our structures provide the necessary information for structure-based vaccine and drug design30.

METHODS SUMMARY

The TbpA–(apo)hTF complex was crystallized from TbpA expressed in Escherichia coli and apo-hTF purchased from Sigma-Aldrich. For the TbpA–(apo)hTF C-lobe structure, hTF incorporating a protease cleavage site between N and C lobes was expressed in BHK cells and purified as described32. Full-length N-His-tagged hTFs including holoh, authentic apo, and both monoferric forms were expressed in BHK cells and purified as described32. The dimeric hTF structure was solved using protein purchased from Sigma-Aldrich. The hTF C-lobe structure was solved using hTF C lobe from a construct containing a TEV protease site between the N and C lobes, expressed in BHK cells, and purified31. hTF was expressed in E. coli, X-ray data were collected at GM/CA and SER-CAT beamlines of the Advanced Photon Source synchrotron. SAXS data was collected on beamline BL4-2 at the Stanford Synchrotron Radiation Lightsource. EM data were collected on a CM120-LaB6 electron microscope (FEI), operating at 120 kV. Molecular dynamics simulations were performed using the program NAMD31.
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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** N.N., N.C.E., M.O. and S.K.B. expressed, purified and crystallized TbpA, TbpB and various hTFs. N.N. solved all crystal structures and the SAXS structure and analysed all data. A.B.M. and A.N.S. designed and purified apo-hTF, holo-hTF, hTF–FeFe and hTF–FeFe for binding experiments with TbpA and TbpB; they also expressed and purified hTF C-lobe for the corresponding structure (PDB code 3SKP). P.A. and O.Z. expressed and purified hTF C-lobe for the TbpA–(apo)hTF C-lobe structure (PDB code 3V89). N.M. and A.C.S. designed, conducted and analysed EM experiments. E.T. and J.G. designed, conducted and analysed molecular dynamics simulations. E.B. participated in the data collection and analysis of the SAXS data. R.W.E., A.R.G. and S.K.B. conceived and designed the original project. N.N. and S.K.B. wrote the manuscript.

**Author Information** Coordinates and structure factors for TbpA–(apo)hTF, TbpA–(apo)hTF C-lobe, diferric hTF, apo-hTF C-lobe and TbpB are deposited in the Protein Data Bank under accession codes 3V8X, 3V89, 3V83, 3SKP and 3V8U, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.K.B. (skbuchan@helix.nih.gov).
**METHODS**

Cloning, expression and purification of TbpA. The *N. meningitidis* TbpA sequence from strain K454 (B15:P1.7,16) was subcloned into pcET2b (Novagen) containing an N-terminal 10×-His tag. TbpA mutants were created using site-directed mutagenesis using QuikChange (Stratagene). For structural studies, mutation of M889 to Tyr improved expression levels. TbpA was expressed in BL21(DE3) cells at 20 °C without induction in terrific (TB) and carbenicillin. Expression for the mutants followed the same protocol.

For purification, cells were re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM MgCl2, 10 μg ml−1 DNase, 100 μg ml−1 AEBFS) and lysed by two passages through an Emulsiflex C3 (Avantium) homogenizer at 4 °C. The lysate was centrifuged at 12,000g for 10 min to remove unlysed cells and the supernatant was incubated with 2% Triton X-100 for 30 min at room temperature. The mixture was centrifuged at 160,000g for 90 min at 4 °C. The membrane pellets were re-suspended in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 20 mM imidazole and solubilized by constant stirring using 5% Elugent for 16 h at 4 °C. Solubilized membranes were centrifuged at 265,000g for 60 min at 4 °C and the supernatant filtered before crystallization. Sparse matrix screening was performed using a 3% final concentration, incubated on ice for 30 min and then the protein sample was filtered before crystallization. Several crystallization conditions were observed; however, none was red in colour as might be expected for iron-bound crystals and most contained citrate, which is a known iron chelator. Data were collected and analysed as for TbpB. The space group was R32 with 1 mol of hTF C lobe per ASU with final cell parameters *a* = 95.847, *b* = 95.847, *c* = 204.140, *α* = 90.00°, *β* = 90.00°, *γ* = 90.00°. No TbpB N lobe was present in the crystals.

**Structure determination.** For TbpA–hTF, we were unable to collect useful heavy atom derivatives for experimental phasing, and selenomethionine-substituted TbpA proteins yielded insufficient for crystallization. We eventually used molecular replacement in Phaser-CCP435 to solve the TbpA–hTF complex structure. Here, we first searched for each of the two domains (N lobe and C lobe) of hTF using the deposited coordinates (PDB code 2H4V), which had good solutions with Z-scores above 8. However, although the electron density for the hTF molecule was reasonable, the electron density for TbpA was poor and could not be used for model building. Our attempts at using known TonB-dependent transporter structures as search models (barrel and plug, together and individually) were unsuccessful (low Z-scores and LLG scores). We then aligned the TbpA sequence to our structure-based sequence alignment reported in our recent review47 and found that TbpA contained many conserved regions characteristic of TonB-dependent transporters. Using the alignment between TbpA and its closest relative, FhuA (10% identity, ClustalW), and trimming the extracellular loops, 50 models within a root mean squared deviation of 5 Å were produced using Modeller (Accelrys). Each of these models was then used for molecular replacement within Phaser-CCP435, with two of them producing Z-scores above 8. The solution with the highest LLG (containing both hTF and the TbpA model) was refined in PHENIX48 producing an initial *R*/*R*free value of 0.43/0.48. Further model building was performed using COOT49 and subsequent refinement done in PHENIX50 and BUSTER-TNT.51 During the final stages of refinement, extra density was observed which was mapped to residues N413 and N611 of hTF, both of which are reported as possible N-linked glycosylation sites. Therefore, N-linked glycans were built for these two residues.

The final structure was solved to 2.60 Å with *R*/*R*free values of 0.22/0.28. The TbpA–hTF C lobe structure was solved by molecular replacement using the coordinates from the TbpA–hTF (full-length) structure reported here. Two search models were formed, one for TbpA and one for hTF C lobe. PHASER-CCP45 was used for molecular replacement and subsequent refinement performed using PHENIX50. The structure was solved to 3.1 Å resolution with final *R*/*R*free values of 0.24/0.29. The TbpB structure was solved by molecular replacement using PDB code 3HOL. An initial model was created using the Swiss Model server52 that was subsequently divided into four different search domains. PHASER-CCP45 was used for molecular replacement and subsequent refinement performed using PHENIX50. The structure was solved to 2.40 Å resolution with final *R*/*R*free values of 0.25/0.30. The differr hTF crystal structure was solved by molecular replacement using Phaser-CCP45. Search models for the N lobe and C lobe were created separately with the program Chainsaw (CCP4) using the existing differr porcine TF coordinates (PDB code 1H76). Six copies of each lobe (six molecules of hTF total) were found in the ASU and the iron sites were easily observed in the difference density. These iron sites were further verified in an anomalous difference electron density map. Refinement was performed using PHENIX50 and the structure was solved to 2.1 Å resolution with final *R*/*R*free values of 0.19/0.23.

The non-glycosylated hTF–C lobe structure was solved by molecular replacement using PDB code 2HAU. An initial search model was formed by truncating the N-lobe domain. PHASER-CCP45 was used for molecular replacement and subsequent refinement performed using PHENIX50. The structure was solved to 1.7 Å resolution with final *R*/*R*free values of 0.17/0.19. For all structures, figures were made with PyMOL (Schrödinger) or Chimera53 and annotated and finalized with Adobe Illustrator.
Dot blots. Whole cells (2 μl, 0.01 g ml⁻¹) and cell lysates (unmodified for TbpB samples, or incubated for 3 h with 2 mM EDTA and 1% DDM at room temperature for TbpA samples) were spotted onto nitrocellulose membrane and allowed to dry at room temperature. The membranes were then blocked with PBS 2% BSA for 15 min, washed and probed with HRP-conjugated hTF (1:1,000) (Jackson ImmunoResearch) for 15 min. The membrane was then washed and imaged using the colorimetric substrate 3,3',5,5'-diaminobenzidine (Sigma) where the appearance of a red dot indicated specific binding of the hTF–HRP conjugate.

The results from ELISA mutants were compared to wild-type TbpA to determine their effect on hTF binding.

ELISA. Whole cells (100 μl at 10 mg ml⁻¹ or 1 mg ml⁻¹ in PBS) of wild-type TbpAa, empty vector control (pET20b) and TbpA mutants were added to a NUNC polystyrene 96-well plate (Fisher Scientific) and incubated at 37°C overnight. Wells were washed 2× with PBS and then blocked with PBS 2% BSA for 30 min and probed with hTF–HRP (1:1,000) for 15 min. Wells were washed 2× in PBST, 2× in PBS, and then developed using 100 μl 3,3',5,5'-tetramethylbenzidine substrate (TMB, Sigma) for 5 min and terminated using Stop solution (Sigma). Absorbances of each well were determined using a BioRad iMark plate reader at 450 nm and data normalized and compared to wild-type TbpAa. Each experiment was performed in triplicate and data reported with standard errors.

Antibody blocking assays. Using the TbpA–hTF crystal structure reported here, we designed four different peptides based on four loops from TbpAa (loops 3, 7, 11 and plug loop) to be used as antigens for polyclonal antibody development (Precision Antibody). A fifth polyclonal antibody was developed using purified full-length TbpAa (1× PBS 7.4, 0.1% DDM). An ELISA was designed to probe whether or not these antibodies could block hTF binding. Here, TbpA–His (20 ng) was incubated for 20 min in a final volume of 100 μl either alone or in the presence of each antibody (1:20) individually in PBS containing 0.05% Cymal-6 (Anatrace). In addition, we tested the antibodies that targeted TbpAa loops in combinations to determine if an additive effect could be observed. Each sample was then transferred to a 96-well Ni-NTA Agarose HisSorb plate (Qiagen) and incubated for 30 min and washed 2× with PBST + 0.05% Cymal-6. Assays were performed as described in the previous section. In a second set of ELISAs, TbpAa–His was first bound to the Ni-NTA Agarose HisSorb plate before incubation with antibodies. Results were analysed and initial graphs made using Microsoft Excel. The graphs were then imported, annotated and finalized with Adobe Illustrator.

Protease accessibility of TbpAa and TbpA mutants. To confirm that TbpAa and the TbpA mutants were being properly presented at the surface of the bacteria, we treated whole cells with trypsin (5 μg ml⁻¹) for 1 min and then filtered using a 0.2 μm spin filter. Data were collected at concentrations of 1, 2.5 and 5 mg ml⁻¹ at Stanford Synchrotron Radiation Lightsource beamline BL4-2. Data reduction and analysis were performed using the beamline software SAStool. The program AutoGNOM of the ATSAS suite was used to generate P(r) curves and to the determine maximum dimension (Dmax) and radius of gyration (Rg) from the scattering intensity curve (I(q)) versus q in an automatic, unbiased manner, and rounds of manual fitting in GONOM were used to verify these values. Ab initio molecular envelopes were computed by the programs GASBOR. Ten iterations of GASBOR were averaged using DAMAVER. Docking of the TbpB and dimeric hTF crystal structures into the molecular envelope was performed manually, guided by both previous docking studies and mutagenesis results. Figures were made with PyMOL and annotated and finalized with Adobe Illustrator.

Modelling the TbpA–TbpB–hTF triple complex. The in silico TbpA–TbpB–hTF triple complex was assembled based on our crystal structures (TbpA–apo-hTF, diferric hTF, TbpB) and SAXS analysis (TbpB–(holoh)hTF) reported here. The crystal structure (TbpA–hTF) was aligned with our TbpB–hTF model using the Cl subdomain of hTF as a reference, yielding a triple complex containing a 1:1:1 ratio of TbpAa, TbpB and hTF. Figures were made with PyMOL and/or Maya (Autodesk) and annotated and finalized with Adobe Illustrator.

Electron microscopic analysis. The triple complex (TbpA–TbpB–(holoh)hTF) was prepared from separately purified components by first forming a complex between TbpB and (holoh)hTF, which was purified by size-exclusion chromatography in 1× PBS. Cymal-6 was added to a final concentration of 0.05% and purified TbpAa (1X PBS, 0.05% Cymal-6) was added to the mixture up to an excess of the TbpB–(holoh)hTF complex. The triple complex (which retains a 1:1 stoichiometry) was isolated by size exclusion chromatography in buffer A (1× PBS, 0.05% Cymal-6) and used immediately for EM experiments. The complex was diluted with buffer A to an optimal concentration for EM (determined empirically to be ~1 μg ml⁻¹). Drops (4 μl each) were applied to carbon-coated, glow-discharged EM grids (EMS). After 1 min, the grid was blotted, washed twice with buffer A, once with distilled water, and then stained with 2% uranyl acetate. Grids were observed with a CM120 LaB₆ electron microscope (FEI), operating at 120 kV. Micrographs were recorded on SO163 film (Kodak) at a nominal magnification of 45,000, and digitized on a Nikon Coolscan 9000 at a rate corresponding to 1.55 Å per pixel. The large majority of particles distributed evenly on the grid and were essentially uniform in size (~90–110 Å in diameter), indicative of a homogeneous population.

The particles were variable in substructure, suggesting that the molecules deposit on the grid in a variety of orientations. Accordingly, a data set of 4,240 particles was subjected to a ‘reference free’ classification, using SPIDER⁴⁴, EMAN³⁸ and Boof⁴⁰. Images were picked using a 256 × 256 pixel box, and binned four times (to 6.2 Å per pixel) to increase the signal-to-noise ratio and the speed of calculation. Initial reference-free classification and averaging were performed using EMAN; further classification was done in SPIDER, using principal component analysis (PCA) with three cycles of iteration. We chose to obtain 56 final class averages, based on a cluster distribution obtained from PCA.

The coordinates of the modelled triple complex were converted to a density map and sharpened with Coot. The sharpening radius for TbpAa was 14 Å, always optimal for EM (determined empirically to be ~1 μg ml⁻¹). Drops (4 μl each) were applied to carbon-coated, glow-discharged EM grids (EMS). After 1 min, the grid was blotted, washed twice with buffer A, once with distilled water, and then stained with 2% uranyl acetate. Grids were observed with a CM120 LaB₆ electron microscope (FEI), operating at 120 kV. Micrographs were recorded on SO163 film (Kodak) at a nominal magnification of 45,000, and digitized on a Nikon Coolscan 9000 at a rate corresponding to 1.55 Å per pixel. The large majority of particles distributed evenly on the grid and were essentially uniform in size (~90–110 Å in diameter), indicative of a homogeneous population.

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Molecular dynamics simulations. For simulations of TbpAa bound to apo-hTF, a membrane-water system containing the protein complex was first built using VMD⁹. The complex was placed in a DMPE bilayer as used previously, with the barrel of TbpAa aligned with the membrane’s hydrophobic core, and then fully solvated. Disulphide bonds for three pairs in TbpAa and 19 pairs in hTF were added based on S–S proximity. Ca²⁺ and Cl⁻ ions were added to a concentration of 100 mM, resulting in an initial size of 264,000 atoms. The system was equilibrated in stages for 13.5 ns, including 10 ns of fully unrestrained dynamics. The simulations were run using NAMD 2.7⁵ and in the NPT ensemble at a temperature of 310K and a pressure of 1 atm; after the first 3.5 ns of equilibration, the area of the membrane was fixed. Other simulation parameters were set identically to those used previously. For steered molecular dynamics (SMD) simulations, the Cx atom of the TbpB N-terminal plug domain residue was pulled in the direction, away from the membrane, at a constant velocity of 5 Å ns⁻¹ (refs 33, 49). To counterbalance the pulling force, six residues at the extracellular periphery of the barrel domain were restrained in the z direction. An adaptive procedure was used to limit the maximum required system size during SMD simulations. When the extension of the unfolded region of the plug domain brought it near to the periodic boundary, the simulation was stopped, the unfolded region of the plug
domain distant from the barrel and membrane was cleaved, and the simulation
restarted after a short equilibration of the water with the new N-terminal residue
being pulled. With this procedure, used three times, approximately 150 Å
of pulling was accomplished while keeping the system sizes below 300,000 atoms.

**Sequence analysis and alignments.** Sequence analysis and alignments were per-
formed and analysed with the programs STRAP and JalView. Figures were
annotated and finalized with Adobe Illustrator.

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