Meningococcal Transferrin-Binding Proteins A and B Show Cooperation in Their Binding Kinetics for Human Transferrin

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Neisseria meningitidis, a causative agent of bacterial meningitis and septicaemia, obtains transferrin-bound iron by expressing two outer membrane-located transferrin-binding proteins, TbpA and TbpB. A novel system was developed to investigate the interaction between Tbps and human transferrin. Copurified TbpA-TbpB, recombined TbpA-TbpB, and individual TbpA and TbpB were reconstituted into liposomes and fused onto an HPA chip (BLAcore). All preparations formed stable monolayers, which, with the exception of TbpB, could be regenerated by removing bound transferrin. The ligand binding properties of these monolayers were characterized with surface plasmon resonance and shown to be specific for human transferrin. Kinetic data for diferric human transferrin binding showed that recombined TbpA-TbpB had $K_a$ and $K_d$ values similar to those of copurified TbpA-TbpB. Individual TbpA and TbpB also displayed $K_a$ values similar to those of copurified TbpA-TbpB, but their $K_d$ values were one order of magnitude higher. Chemical cross-linking studies revealed that TbpA and TbpB, in the absence of human transferrin, formed large complexes with TbpA as the predominant species. Upon human transferrin binding, a complex was formed with a molecular mass corresponding to that of a TbpA-human transferrin heterodimer as well as a higher-molecular-mass complex of this heterodimer cross-linked to TbpA. This indicates that TbpA and TbpB form a functional meningococcal receptor complex in which there is cooperativity in the human transferrin binding kinetics. However, iron loss from the diferric human transferrin-TbpA-TbpB complex was not greater than that from human transferrin alone, suggesting that additional meningococcal transport components are involved in the process of iron removal.

Infections with the bacterial pathogen Neisseria meningitidis manifest themselves as bacterial septicemia and cerebrospinal meningitis, causing a global estimate of 135,000 fatalities every year; 10 to 15% of survivors also experience neurological complications and other disabilities which can have a seriously negative effect on quality of life (52). While newly developed conjugate vaccines have been effective against serogroup C infections, at present there is no universal vaccine against se- rogrou B meningococci. Only by characterizing the proteins essential for pathogenesis can effective vaccine strategies be developed, which is essential for the control of such a devastating disease.

A problem facing all bacterial pathogens is the bioavailability of iron within the host. Neisseria meningitidis overcomes this barrier to proliferation by acquiring its iron directly from the human iron-binding protein transferrin (80 kDa). It is able to do this by expressing two transferrin-binding proteins, TbpA and TbpB (28), both of which are potential candidates for inclusion in a meningococcal vaccine (35, 51). TbpA, the larger of the two proteins, has a molecular mass of 98 kDa and is an integral outer membrane protein (24) which is highly conserved between strains (38). TbpB is the smaller of the two proteins and has a high degree of heterogeneity between different strains, with molecular masses of 65 to 85 kDa (21, 29, 47). TbpB is attached to the outer membrane via an N-linked terminal lipid anchor.

TbpA is a member of the family of TonB-dependent outer membrane proteins that includes siderophore receptors (48) and has been demonstrated to interact with TonB (32). This has led to the suggestion that TbpA uses the same mechanism for iron uptake as the siderophore transporters (34, 41). After crossing the outer membrane, iron is then bound by ferric binding protein A (FbpA), a 37-kDa periplasmic iron transporter protein (12). FbpA is the periplasmic component of an ATP-binding cassette transport system (10) and is thought to interact with an FbpB-FbpC permease complex in the inner membrane, which itself is responsible for transporting iron into the cytoplasm (28).

Studies with isogenic mutants of N. meningitidis and Neisse- ria gonorrhoeae lacking TbpA or TbpB have shown that both proteins are required for optimal iron uptake (3, 15, 17, 31, 40). In addition, TbpB purification from mutants lacking TbpA required the presence of exogenous TbpA (31). These results suggest that TbpA and TbpB probably interact to form a functional cell surface receptor complex for human transferrin.

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Purified TbpA and TbpB have been shown to form complexes in solution (9), and Tbps from other pathogens are also known to interact with one another (22). Further evidence for complex formation by TbpA and TbpB is provided by the fact that both can be copurified with a human transferrin affinity protocol (25). However, both TbpA and TbpB are capable of independently and specifically binding human transferrin, as shown by whole-cell studies (15, 27, 40, 49) and with isolated TbpA and TbpB proteins (8). Their structural differences suggest that they have separate roles in human transferrin binding and iron uptake (16).

TbpB has been shown to be able to discriminate between iron-loaded and iron-free human transferrin, whereas TbpA does not (7, 15, 43, 46). The facts that TbpB is able to detect the iron-loaded status of human transferrin and is more surface exposed than TbpA (15, 18) provide evidence that TbpB probably acts as the initial human transferrin binding site, bringing the ligand into contact with TbpA. The primary Tbp binding region on human transferrin has been localized to the N-lobe with fragments of human transferrin (2) and chimeric transferrins constructed from human and bovine transferrin (44). A more detailed study with these chimeric transferrins and the technique of surface plasmon resonance revealed that there was a secondary, strain-specific TbpB binding site in the C-lobe of human transferrin (8). This suggests that multiple domains on human transferrin are likely to be involved in the interaction with Tbps.

It has been proposed that the antagonism between the TbpA and TbpB binding sites possibly produces a conformational change in human transferrin, facilitating iron release (6, 48). Gel filtration studies have suggested that TbpA alone is capable of removing iron from human transferrin, albeit with reduced efficiency (23). Studies have also revealed that TbpB can bind peptides on the surface of human transferrin which wrap around both lobes (45), indicating that TbpB could also play a role in destabilizing the iron-binding sites of human transferrin to facilitate iron release. However, the precise nature of the Tbp complex is still unknown. Analysis of purified Tbps with gel filtration chromatography and photon correlation spectroscopy has revealed that isolated TbpA and TbpB form a 2:1 complex in solution (7, 9). Other groups have suggested that TbpA and TbpB may instead form a 1:1 complex (40, 42). Another study, with gonococci, has suggested that the complex may comprise one TbpA protein and several TbpB proteins (15).

These contradictions are likely to be the result of investigating membrane protein-ligand interactions in an aqueous phase, where proteins exist as detergent micelle complexes, which may give rise to artifacts. To overcome these limitations, we have developed a novel method whereby purified Tbps are reconstituted into liposomes and spontaneously fused to the surface of an HPA chip (BIAcore). A similar procedure has been used successfully for monitoring the Bacillus thuringiensis Cry1Ac toxin interaction with Maduca sexta aminopeptidase in a model membrane environment (14). With surface plasmon resonance techniques, the HPA chip system allowed us to study the human transferrin-Tbp interactions for the first time in a hydrophobic environment that more closely resembles the situation in vivo. This technique has been further developed to obtain kinetic data for human transferrin interactions with the components of the meningococcal receptor. Previous kinetic analysis of the interaction between human transferrin and Tbps was performed with whole-cell studies with 125I-labeled human transferrin (15, 39, 40, 50).

There are currently no published data on the kinetic rate constants for the human transferrin-TbpA-TbpB complex interaction or for the human transferrin interaction with individual TbpA and TbpB. To further characterize the nature of the TbpA-TbpB complex, chemical cross-linking experiments were performed with purified Tbps. Iron release experiments were carried out to further establish the role of this human transferrin-Tbp complex in facilitating iron removal. In the light of these studies, the nature of the human transferrin-Tbp complex will be discussed with respect to the role of TbpA and TbpB as the functional meningococcal human transferrin receptor.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Neisseria meningitidis* mutants N16T2K (TbpA− TbpB+) and N16T1E (TbpA+ TbpB−) (31) were kindly provided by A. B. Schuyers. The mutants and wild-type strain B16B6 were grown in Mueller-Hinton broth as described previously (25).

**Purification of human serum transferrin.** Human transferrin was extracted from human serum donated by Guy’s Hospital blood bank by the method described previously (20), with ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration. Purity was determined by the migration of a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (33). Iron-free human transferrin was prepared by overnight dialysis against multiple changes of 0.1 M sodium citrate buffer, pH 4.7. Difteric human transferrin was prepared by resuspending iron-free human transferrin in 50 mM ammonium bicarbonate buffer and adding an excess of iron nitritolactric acid, pH 7.0. Excess and nonspecifically bound iron was removed by passing the human transferrin sample through a Sephadex G-75 gel filtration column (Pharmacia). The iron-loaded status of both human transferrin preparations was assessed on 6% polyacrylamide–6 M urea gels by a method adapted from that of Evans and Williams (19), omitting EDTA from the gel and running buffers.

**Purification of transferrin binding proteins from *Neisseria meningitidis*.** TbpA and TbpB from strain B16B6, TbpA from strain N16T2K, and TbpB from strain N16T1E were solubilized from whole-cell extracts and affinity purified with a modified version of previously established protocols (1, 26). All steps were performed at 4°C, and chromatography flow rates were maintained at 1 ml/min. Briefly, membrane proteins were solubilized from whole cells with 2% (vol/vol) Eluent (Calbiochem) in phosphate-buffered saline (PBS) for 1 h. Following denaturation, the supernatant was applied to a human transferrin-Sepharose affinity column equilibrated with solubilization buffer. Following a wash step with 10 column volumes of solubilization buffer, proteins were eluted with 2% (vol/vol) Eluent–PBS–50 mM glycine, pH 2.0, and collected in 1-ml fractions containing 100 µl of 1 M Tris, pH 8.0, to neutralize. Tbp purity was assessed by SDS-PAGE, and human transferrin binding of purified Tbps was assessed by their ability to bind human transferrin-horseradish peroxidase (Jackson ImmunoResearch) with dot blots, following the methods described previously (7).

**Tbp reconstitution into dimyristoyl phosphatidylcholine liposomes.** We dissolved 1 mg of dimyristoyl phosphatidylcholine (DMPC, Sigma) in 0.5 ml of chloroform and dried it to a thin film on a round-bottomed flask with a rotary evaporator. The lipid film was resuspended in 200 µl of either copurified TbpA–TbpB, TbpA, or TbpB solution and dialyzed with 0.5 ml of Slide-A-lyse cassettes (Pierce) against PBS for 24 h at 4°C with continuous stirring and frequent changes of buffer. Recombined TbpA–TbpB was prepared by resuspending the lipid film in 100 µl of Tbps and 100 µl of TbpB solution. The liposome solutions were then extruded 21 times through a 50-nm pore polycarbonate membrane with a Lipofer assembly (Creston).

**Formation of lipid monolayers.** The surface of an HPA chip was washed with 40 nM octylglucoside (Sigma) at a flow rate of 5 µl/min for 5 min in a Biacore X biosensor (Biacore AB). One flow cell was coated with the liposome–Tbp preparation at a flow rate of 2 µl/min for 30 min, and the other was coated with liposomes containing no Tbps (negative control); 50 mM NaOH was then injected over both flow cells at 5 µl/min for 30 s to remove any lipid blebs from the chip surfaces (13).
Human transferrin-Tbp binding assay with surface plasmon resonance. Dif-ferent human transferrin suspended in PBS at concentrations of up to 62.5 μg/ml was injected at a flow rate of 5 μl/min for 2 min over both of the flow cells. After the injection, bound human transferrin was removed with an injection of GentLe Ag/Ab elution buffer (Pierce) at a flow rate of 5 μl/min for 1 min. The temperature was maintained at 25°C throughout all experiments unless other-wise stated.

Stability of diferric human transferrin-TbpA-TbpB while immobilized in a DMPC monolayer. The effect of pH on the human transferrin-Tbp interaction in the DMPC monolayer was monitored over a pH range of 6.8 to 7.8. Human transferrin at 20 μg/ml in 0.1 M phosphate buffer–125 mM NaCl at the given pH was injected over the flow cells at a flow rate of 5 μl/min for 2 min. To determine the effects of temperature, human transferrin at 20 μg/ml in PBS was injected over the flow cells at a flow rate of 5 μl/min for 2 min over a temperature range of 20 to 40°C. In order to determine the stability of copurified TbpA and TbpB in the DMPC monolayer, human transferrin in PBS at 20 μg/ml was injected over the flow cells at a flow rate of 5 μl/min for 3 min at intervals over a period of 21 h. After the injection, bound human transferrin was removed with an injection of GentLe Ag/Ab elution buffer at a flow rate of 5 μl/min for 1 min.

Specificity of diferric human transferrin-TbpA-TbpB while immobilized in a DMPC monolayer. The specificity of copurified TbpA-TbpB for human transferrin was determined by separate experiments with diferric duck ovotransferrin and bovine, porcine, and sheep transferrins in PBS at concentrations of 50 μg/ml. These were injected at a flow rate of 5 μl/min for 2 min over both of the flow cells, with results taken as the mean of five injections ± standard error of the mean. Bovine serum albumin was used as a negative control to determine nonspecific binding. Human transferrin injected at the same concentration was also used in each experiment as a positive control. After the injection, bound human transferrin was removed with an injection of GentLe Ag/Ab elution buffer at a flow rate of 5 μl/min for 1 min.

Surface plasmon resonance data analysis. The association and dissociation phases were fitted separately with the Marquardt-Levenberg algorithm (Biae-valuation 3.0 software [5]), which minimizes the sum of the squared residuals to optimize the parameter values. The interaction between human transferrin and Tbps was assumed to be a pseudo-first-order reaction, and a simple 1:1 model was used in the analysis. To obtain $K_a$ and $K_d$ values, it was also assumed that during the dissociation phase, after initially binding to its ligand, the analyte did not rebind.

Protein estimation. Protein concentrations were calculated by relating the absorbance of the sample at 280 nm to the predicted extinction coefficient of the protein (obtained from http://ca.expasy.org/tools/protparam.html).

Cross-linking of purified Tbps with RS5 and formaldehyde. Purified Tbps were dialyzed against 0.5% (vol/vol) Eluent-PBS buffer overnight in 0.5-mL Slide-a-lyze cassettes. Human transferrin was added to Tbps in a 2:1 molar ratio and incubated for 1 h at room temperature to enable complex formation. A 50-fold molar excess of BS3 [bis(sulfosuccinimidyl)suberate] in PBS was added to the Tbp and human transferrin-Tbp samples. The reaction was stopped after 30 min by the addition of glycine to a final concentration of 25 mM. The samples were then boiled for 10 min and resolved by SDS–10% PAGE. For the formaldehyde cross-linking, Formalin (Sigma) was added to the Tbp and human transferrin-Tbp samples at a concentration of 1% (vol/vol) and incubated for 30 min at room temperature. The sample was then divided into two aliquots; one was boiled and the other was incubated at 37°C for 10 min before resolving by SDS–10% PAGE.

Western blotting. Proteins resolved by SDS-PAGE were transferred to nitrocellulose sheets with a wet Western blot system (Bio-Rad) run at 60 mA overnight. Following blocking with 1% (wt/vol) skimmed milk powder (Marvel) in PBS–0.05% (vol/vol) TWEEN 20, the nitrocellulose sheet was then incubated with mouse anti-TbpA-TbpB serum (kindly provided by K. Reddin, HPA, United Kingdom) or anti-human transferrin sheep immunoglobulin G-horseradish peroxidase (Biogenesis). Both antibodies were diluted to 1:500 in PBS. Following washing with PBS–0.05% (vol/vol) TWEEN 20, blots were probed with mouse anti-TbpA-TbpB serum incubated with goat anti-mouse immunoglobulin G-horseradish peroxidase (ICN) at a 1:1,000 dilution in PBS. The blots were washed and developed with 4-chloro-1-napthol (Sigma).

Urea gel analysis. Samples were run on 6% polyacrylamide–6 M urea gels with a previously established protocol (19), omitting EDTA from the gel and running buffers, with a mini-Protean II system (Bio-Rad). Diferric human transferrin was mixed with copurified TbpA-TbpB in a 1:2 molar ratio and then added to 0.05 M HEPES–0.1 M NaCl–2% (vol/vol) glycerol, pH 7.4, to a final human transferrin concentration of 50 μg/ml. Diferric human transferrin on its own was used as a control; 0.02 M pyrophosphate was included in both reaction mixtures to act as an acceptor for mobilized iron, as utilized in studies of iron release from the human transferrin-human receptor complex (4). We took 10-μl aliquots at 0 h, 0.5 h, 1 h, 2 h, and 4 h and loaded them onto a 6% polyacrylamide–6 M urea gel running at 10 V. After the final aliquots were added, the gel was run at 90 to 100 V for 4 to 5 h. Protein bands were visualized with a Coomassie blue stain.

RESULTS

Tbp reconstitution into DMPC liposomes. Copurified TbpA-TbpB, recombinant TbpA-TbpB, TbpA, and TbpB were reconstituted into DMPC liposomes as described in Materials and Methods. The size of the extruded liposomes was determined with transmission electron microscopy (11), and the largest diameter was found to be approximately 50 nm (data not shown). SDS-PAGE showed that TbpA, TbpB, and recombinant TbpA-TbpB were successfully reconstituted into the liposomes (Fig. 1). Human transferrin binding with the immobilized dot blot method confirmed that the reconstituted Tbps retained their ability to bind human transferrin by using human transferrin-horseradish peroxidase (data not shown).

Binding of human transferrin to Tbps embedded in a DMPC-supported monolayer determined by surface plasmon resonance. Following Tbp reconstitution into liposomes, the preparation was layered onto one flow cell surface of an HPA sensor chip. As a negative control, liposomes containing no Tbps were used to coat the other flow cell surface. Following injection of the Tbp-liposome preparation, a typical surface plasmon resonance response was approximately 5,000 resonance units (RU) after 20 s. A rapid increase in the surface plasmon resonance signal was observed for the negative control liposomes, which reached a lower plateau of 3,000 RU after 60 s. This difference can be attributed to the effects of the Tbps. A subsequent injection of 50 mM NaOH reduced the baselines obtained with the Tbp-liposome preparation and negative control liposomes, typically by 1,000 RU. This step was shown to be crucial for obtaining reliable surface plasmon resonance sensograms, as lipid blebs cause distortions that make it impossible to follow any binding events.

Specific diferric human transferrin binding to copurified TbpA-TbpB in the DMPC monolayer was observed, whereas
very little binding was seen with the DMPC monolayer surface alone. In order to elute bound human transferrin, a number of different regeneration buffers were used (5), with Gentle Ag/Ab elution buffer providing the best results. This produced a return to the baseline without reducing the level of subsequent binding events. The binding response of human transferrin to copurified TbpA-TbpB appeared to be linear (Fig. 2A) over a human transferrin concentration range of 2.5 to 40 μg/ml. Human transferrin binding to recombined TbpA-TbpB and TbpA was higher than the negative controls and was also dose dependent, although overall levels were lower than human transferrin binding to copurified TbpA-TbpB (Fig. 2B and 2C). As a further control, the last injection of human transferrin was at the same concentration as the first injection of a run to ensure that the level of binding had not diminished. If there was a large discrepancy, this indicated a significant loss of Tbps from the monolayer, and the results could not be used with any degree of confidence.

Human transferrin initially bound to TbpB, although TbpB failed to remain within the monolayer after regeneration. For each TbpB-liposome preparation, only one human transferrin binding event could be measured, as the subsequent regeneration step appeared to strip TbpB from the monolayer. Human transferrin binding to TbpB was higher than that of the control and proportional to the human transferrin concentration, but no dose curve could be obtained.

Copurified TbpA-TbpB stability and specificity for diferric human transferrin while immobilized in a DMPC monolayer. Several different phospholipids (DMPC, dimyristoylphosphatidylethanolamine, egg yolk lecithin, and phosphatidylcholine) were assessed for the Tbp reconstitution step. In all cases, reconstitution of Tbps into the liposomes was successful. However, only with DMPC was a stable monolayer formed on the HPA chip. Similar results were obtained in a previous HPA study (13). pH, temperature, and time parameters were then assessed to optimize the stability of the Tbp-human transferrin interaction in the DMPC, as described in Materials and Methods.

Human transferrin binding to copurified TbpA-TbpB was optimal at pH 7.4, with a decrease in binding at pHs above and
below this point. Human transferrin binding to copurified TbpA-TbpB was optimal at 25°C. The level of binding decreased with increasing temperature after this point. Temperatures of 30 and 37°C resulted in 50 and 80% reductions in the level of human transferrin binding, respectively. Very little human transferrin binding was observed at temperatures above 37°C, concomitant with a large variability in the data points. The stability of the Tbps in the monolayer was examined over time, and the level of human transferrin binding to the Tbps was undiminished after 5 h. Despite a steady loss in human transferrin binding after this period, it remained substantially higher than control levels, even after 21 h. To eliminate variation, an arbitrary threshold of 10% binding reduction compared to the first binding event was set, after which no further data points were collected.

The specificity of copurified TbpA-TbpB for human transferrin was established with binding studies of nonhuman transferrins. Bovine serum albumin failed to bind to the Tbps, as expected. Copurified TbpA-TbpB was shown to bind human transferrin, while the level of binding to other animal transferrins was less than 5% of that observed with human transferrin (data not shown).

Comparison of human transferrin binding to components of the meningococcal receptor. Human transferrin binding to copurified TbpA-TbpB produced a sensogram with a smooth association phase followed by a shallow dissociation phase (Fig. 3A). An almost identical human transferrin binding sensogram was obtained with recombinant TbpA-TbpB (Fig. 3C). Human transferrin binding to isolated TbpA or TbpB produced a similar association phase curve, but the dissociation phase curve was markedly steeper (Fig. 3B and 3D).

Association and dissociation rate constants for the purified components of the meningococcal receptor. There was no change in the association rate constant at flow rates between 2 and 20 μl/min, and therefore, under the experimental conditions employed in this study, there was no evidence to suggest that the system was limited by mass transport. Flow rates greater than 20 μl/min resulted in Tbp loss from the monolayer, which manifested itself as a downward drift in the baseline. A human transferrin concentration of 50 μg/ml was used since this produced surface plasmon resonance signals for the human transferrin-Tbp interactions not exceeding 200 RU.

To obtain $K_a$ and $K_d$ values, two assumptions were made with a computer model of 1:1 binding. First, it was assumed that this was a homogenous first-order reaction. The second was that during the dissociation phase, after initially binding to its ligand, the analyte did not rebind. Kinetic analysis revealed that the human transferrin–copurified TbpA-TbpB and human transferrin–TbpA interactions had similar $K_a$ values, whereas the interaction with TbpA alone displayed a 10-fold increase in $K_d$ (Table 1). TbpB alone had a slightly raised affinity for human transferrin in this system compared to copurified TbpA-TbpB, but the $K_d$ value was similar to that of human transferrin binding to TbpA. When individual TbpA and TbpB

![Figure 3](http://iai.asm.org/)

**FIG. 3.** Sensograms showing human transferrin binding to Tbps embedded in a DMPC monolayer. Human transferrin was injected over flow cells containing copurified TbpA-TbpB (A), TbpA (B), recombinant TbpA-TbpB (C), and TbpB (D) embedded in a DMPC monolayer for 2 to 3 min at a flow rate of 5 μl/min.
were recombined, a restoration in the $K_a$ and $K_d$ values was observed, albeit with a higher standard error of the mean.

**Cross-linking of purified Tbps.** SDS-PAGE of formaldehyde-cross-linked isolated TbpA-TbpB revealed that a very large complex was formed which could not penetrate the stacking and resolving gel (Fig. 4, lane 9). Following heating at 100°C, the formaldehyde cross-links were broken, and two bands at 98 and 65 kDa, corresponding to TbpA and TbpB, respectively, were resolved (Fig. 4, lane 8). The addition of human transferrin to the TbpA-TbpB sample produced similar results, with a small amount of TbpB remaining un-cross-linked (Fig. 4, lane 11). Following heating at 100°C, the formaldehyde cross-links were broken, and three bands at 98, 80, and 65 kDa, corresponding to TbpA, human transferrin, and TbpB, respectively, were resolved (Fig. 4, lane 10).

Isolated Tbp-human transferrin samples were also cross-linked with BS3. SDS-PAGE of BS3-cross-linked isolated TbpA-TbpB revealed that a very large complex was formed which could not penetrate the resolving gel (Fig. 4, lane 2). This was accompanied by the disappearance of TbpA monomers and a reduction of TbpB monomers within the sample. Upon the addition of human transferrin, a minor complex of approximately 150 kDa was formed, as was a major complex of more than 207 kDa, including some which did not penetrate the gel (Fig. 4, lane 3). Western blots were probed with anti-TbpA-TbpB mouse serum (Fig. 4, lanes 4 to 6) or anti-human transferrin antibody (Fig. 4, lane 7). Lanes 4, 5, and 6 correspond to lanes 1, 2, and 3, respectively, and show the same cross-linking profile. Lane 7 revealed that some human transferrin remained un-cross-linked, as shown by an immunoreactive band at 80 kDa, with an additional band resolved above 207 kDa.

**Iron removal from the human transferrin-Tbp complex.** Iron loss from human transferrin and the human transferrin-TbpA-TbpB complex was investigated by 6 M urea gel analysis over a time period of 4 h. At 0 h, the control (human transferrin only) was shown to contain approximately equimolar quantities of diferric and monoferric human transferrin. Over a 4-h period, gradual iron loss from human transferrin was observed, resulting in the appearance of iron-free human transferrin (data not shown). An identical pattern was observed with the human transferrin-Tbp complex. There was clearly no increase in the rate of iron removal from human transferrin in the presence of TbpA-TbpB.

**DISCUSSION**

This study used novel techniques to provide useful information on the specific roles of components of the meningococcal human transferrin binding system along with the nature of their interactions. The results obtained from the cross-linking experiments with purified Tbps indicate that, in solution, TbpA forms a variety of multimeric complexes in addition to dimers, as described previously (9). Purified TbpA and TbpB were

![Fig. 4. Cross-linking of purified TbpA-TbpB.](http://iai.asm.org)
seen to associate in the absence of human transferrin, with either cross-linking agent, to form complexes that were too large to enter the resolving gel. Regardless of the size of these complexes, BS$^3$ was observed to cause the majority of TbpA monomers to become cross-linked, while a significant amount of TbpB remained in monomeric form. Given that both proteins were present in the initial sample in equal quantities, this can be explained either by TbpA multimerization or by the idea that the complexes that formed between the two species possess a TbpA:TbpB ratio higher than 1:1. The addition of human transferrin to purified Tbps prior to BS$^3$ cross-linking resulted in two additional complexes that were evident after Coomassie staining. The band at approximately 150 kDa corresponds most closely to the predicted mass of a TbpB-human transferrin heterodimer. The faintness of this band also suggests that this complex is transitory and goes on to form larger complexes with TbpA, possibly resulting in the denser band seen above 207 kDa.

In addition, it can be seen from the Western blot in Fig. 4 that the presence of human transferrin in the incubation mix appeared to slightly enhance complex formation by TbpB in the presence of BS$^3$. This was associated with a small decrease in TbpA cross-linking, evident as an increase in the density of the band corresponding to 98 kDa. This suggests that TbpB has a greater affinity for human transferrin than TbpA under the conditions employed, as would be expected in a model where TbpB facilitates the initial interaction with human transferrin. Caution must be taken when interpreting these results since multiple cross-links could form between adjacent Tbp complexes, possibly accounting for aggregates that were too large to enter an SDS gel. In order to overcome this limitation, the concentration of the cross-linking agent was reduced. The same results were observed until a critical concentration was reached, where no cross-linking at all was observed (data not shown). In light of these results, a possible model for the meningococcal receptor is that TbpA forms multimeric complexes in the membrane, initially dissociated from TbpB, which may exist as discrete monomers. Human transferrin binding to TbpB in a 1:1 molar ratio results in a human transferrin-TbpB complex, which is then able to interact with the TbpA complex, presumably as a result of conformational changes.

The use of purified Tbps in detergent solutions was a limitation of this study, since the human transferrin-Tbp complex(es) which forms in artificial systems may differ from the one(s) which forms when the Tbps are embedded in the meningococcal outer membrane. Predictions of the stoichiometry of the human transferrin receptor complex from these results are also highly speculative since discrimination between TbpA and TbpB within each complex was not possible with the antisera used in the Western blot. Future work will ideally make use of monoclonal antibodies which are specific for individual TbpA and TbpB, enabling better evaluation of the TbpA-TbpB complex stoichiometry.

Human transferrin binding to the Tbp complexes formed in this system did not result in an increased iron release rate, as determined by urea gel analysis. This observation contradicts speculations from other groups that TbpA and TbpB on their own are sufficient to facilitate iron release from human transferrin (23, 48). Components of the meningococcal human transferrin iron uptake system that are not present in our studies may be required for successful removal of iron from human transferrin. An obvious candidate is the energy-transporting molecule TonB, which has recently been demonstrated to associate with TbpA in vivo (32). Alternatively, again as a consequence of using an artificial system, it is possible that the Tbp complex is unable to bring about the necessary conformational changes in human transferrin that are required to induce iron release from the iron-binding site.

This study also describes a novel technique that is potentially applicable to the study of a wide range of membrane-bound receptor-aqueous analyte interactions. By inserting functionally active Tbps into an artificial lipid membrane on an HPA chip surface, surface plasmon resonance analysis can be used to collect useful kinetic data about their binding to human transferrin. The fluid nature of the HPA chip surface presumably resembles the situation found in vivo, in which both components of the Tbp complex are laterally mobile and free to move independently of one another until the correct succession of binding events leads to the formation of the functional receptor. It is also assumed that the lipid membrane, by exerting lateral pressure on the reconstituted proteins, encourages them to adopt their native conformations. Thus, only the epitopes that are exposed on the meningococcal outer membrane are exposed to human transferrin in the HPA system, reducing nonspecific binding by hydrophobic interactions. All of these advantages were deemed important for the collection of reliable kinetic data. The observation that the Tbps retained their species specificity for human transferrin lends credence to the reliability of the system, given the close structural homology that exists between most transferrins.

Having obtained simple ranking data for human transferrin binding by the Tbps with the HPA system, this was optimized to obtain real-time kinetic data for the interactions, taking into consideration problems with mass transport (30, 36). From the surface plasmon resonance and HPA studies, it was clear that there were differences between the $K_d$ and $K_a$ values for the interactions between human transferrin and the components of the meningococcal human transferrin receptor. The greatest differences were observed in the $K_d$ values for the individual Tbps compared to the TbpA-TbpB complex. The $K_d$ values obtained for the interaction of human transferrin with either copurified TbpA-TbpB or recombined TbpA-TbpB (Table 1) are in the nanomolar range and closely resemble those calculated from previous studies with whole cells.

Kinetic analyses with gonococci have enabled the determination of $K_a$ values for the wild-type receptor, ranging from 1 to 16 nM (18) to 23 nM (40). The wild-type meningococcal receptor has been shown to have a $K_a$ value of 30 nM in a similar study (50). Additionally, the $K_d$ value obtained for the human transferrin-TbpB interaction in this study (59.2 nM) is almost identical to the $K_d$ value of 62 nM obtained with a TbpA-deficient gonococcal mutant (40). The same studies also used a TbpB-deficient gonococcal mutant to obtain $K_d$ values for the human transferrin-TbpA interaction ranging from 2.3 to 3.8 nM, suggesting that TbpA has a greater apparent affinity for human transferrin than does TbpB. In this study, the $K_d$ value for the human transferrin-TbpA interaction also lies in the nanomolar range, but TbpA is calculated to possess a $\pm 75$-fold-lower affinity for human transferrin. This discrepancy is most likely due to the fact that the meningococcal homo-
logue of TbpA is under examination in this study as well as variations caused by the experimental conditions.

Dot blots with horseradish peroxidase-labeled human transferrin showed that the TbpB-liposome sample retained the ability to bind human transferrin. This binding ability was abolished after one round of regeneration with the HPA-surface plasmon resonance system, indicating that TbpB was not sufficiently anchored in the DMPC monolayer in the absence of TbpA. To test this hypothesis, TbpA from strain N16T2K and TbpB from strain N16T1E were recombined, and a restoration in the $K_d$ and $K_L$ values close to the values obtained for copurified TbpA-TbpB was observed (Table 1). The fact that regeneration of the chip surface had little effect on the reproducibility of the kinetic data obtained indicates again that TbpA and TbpB form a receptor complex following human transferrin binding. The restoration of optimal $K_L$ values upon recombining TbpA and TbpB suggests that the role of TbpB is primarily to act as an initial binding site to hold human transferrin in place long enough for iron to be removed from the human transferrin-TbpA-TbpB complex. The data also suggest that the role of TbpA in the human transferrin-TbpA-TbpB interaction may have more to do with iron removal than with holding human transferrin to the receptor complex (as shown by the high $K_d$ value for TbpA on its own).

The data obtained in this study suggest that iron removal from human transferrin relies on a succession of conformational changes. Elucidation of epitopes within the Tbps that are important for complex formation can now be studied with site-directed mutants of a recombinant TbpA that has previously been expressed and purified (37) or by performing TbpB peptide inhibition studies. The HPA-surface plasmon resonance system, indicating that TbpB was not sufficient after one round of regeneration with the HPA-surface plasmon resonance system, allowing Kd values to be obtained for the transferrin-binding proteins. J. Bacteriol. 178:1437–1444.


