The transferrin receptor of *Neisseria meningitidis* is composed of the transmembrane protein TbpA and the outer membrane protein TbpB. Both receptor proteins have the capacity to independently bind their ligand human transferrin (htf). To elucidate the specific role of these proteins in receptor function, isothermal titration calorimetry was used to study the interaction between purified TbpA, TbpB or the entire receptor (TbpA + TbpB) with holo- and apo-htf. The entire receptor was shown to contain a single high affinity htf-binding site on TbpA and approximately two lower affinity binding sites on TbpB. The binding sites appear to be independent. Purified TbpA was shown to have strong ligand preference for apo-htf, whereas TbpA in the receptor complex with TbpB preferentially binds the holo form of htf. The orientation of the ligand specificity of TbpA toward holo-htf is proposed to be the physiological function of TbpB. Furthermore, the thermodynamic mode of htf binding by TbpB of isotypes I and II was shown to be different. A protocol for the generation of active, histidine-tagged TbpB as well as its individual N- and C-terminal domains is presented. Both domains are shown to strongly interact with each other, and isothermal titration calorimetry and circular dichroism experiments provide clear evidence for this interaction causing conformational changes. The N-terminal domain of TbpB was shown to be the site of htf binding, whereas the C-terminal domain is not involved in binding. Furthermore, the interactions between TbpA and the different domains of TbpB have been demonstrated.

Meningococcal disease continues to be a worldwide health problem and can lead to death within several hours if untreated (1). There is currently no vaccine to prevent serogroup B meningococcal disease. The proteins that form the transferrin receptor of *Neisseria meningitidis* are promising candidates for inclusion in such a vaccine (2).

The receptor consists of two types of subunits, TbpA and TbpB (transferrin-binding proteins A and B), both of which have the capacity to independently bind their ligand, human transferrin (htf) (3). TbpA (100 kDa) is thought to be a porin-like integral membrane protein that is proposed to serve as channel for the transport of iron across the outer membrane. TbpA shares sequence similarities with FepA and FhuA (4). Both proteins have been shown to form an antiparallel β-barrel (5, 6), and TbpA is thought to have a similar structure (7). TbpB (65–85 kDa) is considered to be an outer membrane protein that is anchored to the membrane via the lipidated N-terminal part of the protein (8, 9), and an interaction between TbpA and TbpB has been demonstrated (10, 11).

A sequence alignment of all currently available *N. meningitidis* TbpB sequences reveals the presence of two different isotypes (12). Both isotypes differ in their molecular masses: isotype I proteins (such as strain B16B6) have molecular masses of ~68 kDa, whereas isotype II proteins (such as strain M982) are characterized by molecular masses of ~80–90 kDa (13). These differences are caused by large sequence inserts predominantly in the C-terminal part of isotype II proteins (4). TbpB sequences from other bacteria such as *Neisseria gonorhoeae* (8) or *Moraxella catarrhalis* (14) resemble the isotype II proteins found in *N. meningitidis*, indicating that this isotype is predominant. However, the physiological relevance for two isoforms in the meningococcus is not understood.

The immunological properties of both proteins have been studied extensively. Antibodies to TbpA and TbpB are detected in the sera of infected individuals as well as of healthy carriers (2). The receptor (TbpA + B) was shown to induce bactericidal antibodies in laboratory animals and is protective in a mouse infection model (15). TbpB was shown to be protective and to generate bactericidal antibodies (12, 16), whereas TbpA can confer protection in a mouse challenge model without a detectable bactericidal antibody response (17). The two isotypes of TbpB are also reflected in their immunogenic behavior, because it has been demonstrated that antibodies raised against one TbpB isotype do not have any cross-bactericidal activity toward meningococcal strains containing a TbpB that belongs to the other isotype (12). This finding is of importance for vaccine development because it implies that a TbpB-based meningococcal vaccine needs to contain at least a representative of each isotype to achieve broad cross-protection (12).

In contrast to the detailed knowledge concerning the immunogenicity of the receptor, a number of fundamental questions about the structure and function of the transferrin receptor remain to be answered. First, it is generally accepted that the...
receptor consists of TbpA and TbpB. However, the architecture of the receptor is unclear and reports on the TbpB/TbpA stoichiometry range from 1.2 (18) to 2.1 (19), 5:1 (3), and 9:1 to 15:1 (20). Second, the study of meningococcal and gonococcal mutants lacking the gene for either TbpA or B has shown that both proteins are required for the optimal uptake of hIft iron (21, 22). In contrast to TbpA, which forms a transmembrane pore permitting iron internalization, the role of TbpB in iron uptake remains unclear. Third, it needs to be elucidated whether hIf binding by TbpA and TbpB occurs in an independent or sequential fashion. Both motifs of hIf binding are supported by previous reports (3, 18), and the number of hIf-binding sites per receptor has been variously described. Fourth, little is known about the affinities with which the iron-free and iron-loaded forms of hIf are bound by the bacterial receptor and how these affinities compare with the human transferrin receptor. Fifth, based on antigenic and sequence differences, TpbBS can be divided into isotype I and II proteins (12, 13). It has not been established whether this classification is also reflected in differential hIf binding characteristics. These questions will be addressed in the first part of this article where the interaction of purified TbpA, TbpB, or the receptor complex with apo- and holo-hIf is analyzed by isothermal titration calorimetry (ITC) (23).

In the second part we present a protocol for the cloning, expression, and purification of active, recombinant TbpB as well as its individual N- and C-terminal domains. These proteins have been used to characterize the interaction of both domains with each other as well as with hIf and TbpA. Binding interactions were monitored by a combination of ITC, differential scanning calorimetry (DSC), and cd. In addition, DSC has shown utility in the characterization of vaccine antigens.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Recombinant TbpA—TbpB** was obtained as reported previously (24).

**Overexpression and Purification of N-terminally Lipidated TbpB (lip-TbpB)—Recombinant, N-terminally lipidated TbpB of strains B16B6 (B2a; P1.2) and M982 (B9; P1.9) was overexpressed in Escherichia coli as described previously (25).** The protein was purified by affinity chromotography on transferrin-Sepharose (26).

**Purification of the Transferrin Receptor (TbpA + B) from N. meningitidis**—The transferrin receptor from N. meningitidis strain M982 (isotype II) was purified as described previously (27) with the exception that membrane proteins were extracted from N. meningitidis by over-night incubation at 4 °C using PBS containing 2% (v/v) Elugent (Calbiochem-Novabiochem Inc.).

**Cloning, Overexpression, and Purification of Histidine-tagged TbpB M982 (isotype II) and Its Individual Domains—** The gene sequences coding for His6-TbpB (amino acids 2–691), N-ter (amino acids 2–351), and C-ter (amino acids 352–691) of N. meningitidis strain M982 were cloned into the plasmid pET28a (Novagen, Madison, WI) and linearized by the restriction enzymes NdeI and BamHI (Invitrogen). In this way, the gene sequence corresponding to a hexahistidine tag was added to the N-terminal end of all three inserts. PCR amplification of the corresponding DNA sequences was undertaken using pT3G721 (4) as template. PCR amplifications were performed on a PTC-200 thermal cycler (MJ-Research, Merck Eurolab, Fontenay-sous-Bois, France) using an Expand Long Template PCR system (Roche Diagnostics, Meylan, France). The experimental conditions suggested by the supplier were used. For each of the three constructs, restriction sites for NdeI and for BamHI were introduced into the forward and reverse primers, respectively. Moreover, a stop codon was added into the reverse primer for the specific amplification of the N-terminal tpbB fragment. The following primers were used to amplify the full-length, N- and C-terminal fragments of tpbB: 5′-ggattcctattcgcggggggecgcg-3′ and 5′-ccgagatccggggggecgcg-3′ for the 5′-end of the fragment, 5′-ggattcctattcgcggggggecgcg-3′ and 5′-ggggggcgccggttgcgttcg-3′, 5′-ggattcctattcgcggggggecgcg-3′, and 5′-ggggggcgccggttgcgttcg-3′, respectively. The three PCR products were digested with NdeI and BamHI and cloned into pET28a, and E. coli BL21 (DE3) were transformed with the ligation mix. Plasmids from recombinant kanamycin-resistant clones were purified, and the identity of the ligated fragments was verified by DNA sequencing of the inserts and flanking regions. Cultures of transformed E. coli were grown at 37 °C on LB broth supplemented with kanamycin (25 μg/ml). Protein expression was induced at an A600 of 0.6 by the addition of isopropyl-β-D-thiogalactopyranoside (Q-BioGene, Illkirch, France) to a final concentration of 1 mM. The bacteria were harvested 1 h after protein induction by centrifugation.

The bacterial pellet resulting from a 1-liter culture was resuspended at room temperature in 95 ml of 50 mM Tris/HCl, 100 μM Pefablock (Interbiotech, Montlucon, France), 100 μg/ml lysozyme (Sigma-Aldrich), pH 8.0, and gently agitated for 30 min. Afterward, MgCl2 and benzonase (Merck) were added to final concentrations of 1 mM and 1 units/ml, respectively. The bacterial suspension was then placed on ice, and the DNA was achieved by lysing by treatments using a Branson-Sonifer 450. The resulting solution was then centrifuged at 18,000 × g for 25 min at 5 °C. Aliquots of the resulting supernatant and pellet were analyzed by SDS-PAGE, and recombinant proteins were detected by Western blot analysis using an anti-polyhistidyl monoclonal antibody (Novagen).

**Protein Purification from the Supernatant (His6-TbpB and C-ter)—** NaCl and imidazole were added to the supernatant to final concentrations of 0.5 and 10 mM, respectively. The resulting solution was then filtered using a 0.45-μm cut-off and loaded onto a 1-ml Hi-Trap chelating column (Amersham Biosciences) previously equilibrated with buffer A (50 mM Tris/HCl, 0.5 mM NaCl, 10 mM imidazole, pH 8.0). After washing with 10 ml of buffer A, protein elution was achieved using buffer A containing 500 mM imidazole. The protein was then dialyzed against PBS and stored at −45 °C.

**Protein Purification from the Pellet (His6-TbpB, N-ter)—** The pellet was washed with 100 ml of H2O at room temperature. The water was decanted, and the pellet was resuspended in 35 ml of buffer B (50 mM Tris/HCl, 0.5 mM NaCl, 6 M guanidinium hydrochloride, 10 mM imidazole, pH 8.0). After filtration using a 0.45-μm cut-off filter, the protein was loaded onto the Hi-Trap chelating column previously equilibrated in buffer B. The column was washed with 10 ml of buffer B and afterward with 10 ml of buffer C (50 mM Tris/HCl, 0.5 mM NaCl, 6 M urea, 10 mM imidazole, pH 8.0). Protein elution was achieved using buffer C containing 500 mM imidazole. The protein preparation was then lowered by serial dialysis against PBS containing 0.5 M arginine and 4, 2, and 0 M urea, respectively. Protein was then stored at −45 °C.

**Isothermal Titrination Calorimetry—** ITC measurements were performed on a VP-Microcalorimeter (MicroCal, Northampton, MA). The proteins were exhaustively dialyzed into the buffer system stated in the legend of each figure. The ligand concentration was determined using the Micro BCA protein reagent kit (Pierce). A typical experiment involved a single 2-μl injection and a series of 4-μl injections with an interval of 240 s. If not otherwise stated, the experiments were carried out at 25 °C. The mean of the enthalpies measured from the injection of ligand buffer was subtracted from raw titration data prior to curve fitting using ORIGIN software (MicroCal). Titration curves were fitted by a nonlinear least squares method to a function for the binding of a ligand to a macromolecule (23). From the curve fit, the parameters ΔH (reaction enthalpy), Kd (dissociation constant), and n (reaction stoichiometry) can be determined directly. From the values Kd and ΔH, the change in free energy (ΔG) and entropy change (ΔS) can be calculated using the equation: ΔG = −RTln(1/Kd) = ΔH − TΔS, where R is the universal molar gas constant, and T is the absolute temperature. The thermodynamic parameters of ligand binding reported are the mean values and standard deviations of at least two independent measurements.

**Differential Scanning Calorimetry—** DSC experiments were performed using a MicroCal VP-DSC apparatus (MicroCal). Prior to analysis, the proteins were exhaustively dialyzed against 10 mM Na2HPO4/ NaH2PO4, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.0, and degassed. The dialysis buffer was used for base-line scans and was present as reference buffer for the protein scans. The system was allowed to equilibrate at 5 °C for 15 min and temperature was raised to a rate of 85 °C. Thermograms obtained were analyzed using the MicroCal version of ORIGIN. The standard deviation indicated for each parameter corresponds to the error of curve fitting. Details concerning the calculation of thermodynamic parameters and instrumentation can be found in Refs. 28 and 29.

**Circular Dichroism—** Far UV of hIf solutions were made at 25 °C using a Jasco J-810 spectropolarimeter (Tokyo, Japan) using cuvettes with a pathlength of 0.1 mm. The proteins were exhaustively dialyzed against 5 mM NaH2PO4/Na2HPO4, 10 mM NaCl, 0.05% (v/v) Tween 20, pH 7.0. The protein concentrations were between 5 and 7 mg/ml. All of the proteins were unfolded by treatment with 5 M urea and then dialyzed against 0.1 M NaCl. The far UV CD spectra were measured using 1-cm pathlength quartz cuvettes. Data were collected at 220 nm with a scan speed of 100 nm/min, and the average of four scans was calculated. Additionally, proteins were subjected to various denaturing conditions, e.g. 5 M urea, 0.5 M guanidinium hydrochloride, 2, 1, and 0 M urea, respectively. The CD spectrum of unfolded hIf was used as reference. The data were fitted to the ideal mean residue ellipticity using the software CDNN2 (20).
the protein spectra were corrected using the spectra of the dialysis buffer.

Surface Plasmon Resonance (BIAcore) Studies—Surface plasmon resonance studies were carried out using the BIAcore 2000 system (BIAcore, Uppsala, Sweden). The sensor chip CM5 and the amine coupling kit (containing N-hydroxysuccinimide, N-ethyl-N'-[3-diethylamino-propyl]-carbodiimide, and 1 M ethanolamine hydrochloride, pH 8.5) were also from BIAcore. The buffer used for sample dilution and analysis was 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% BIAcore surfactant, pH 7.4 (HBS buffer). TbpA was immobilized onto activated carboxylated dextran by a covalent linkage involving the amino groups of the protein using the protocol provided by the manufacturer. Solutions containing 150 pmol of each ligand (diluted to 1.5 μM in HBS buffer) were passed over the sensor chip at a flow rate of 10 μl min⁻¹. The TbpA-ligand complex was then washed for 6 min with HBS buffer, and the increase in resonance units with respect to the initial baseline was determined. The data were corrected using the change in resonance units observed for the binding of bovine serum albumin, and the reported data are the means of three experiments. The sensor chip was regenerated by an exposure to 200 mM glycine, pH 2.0.

RESULTS

Study of the Interaction between TbpA, TbpB, and TbpA + B with Apo- and Holo-transferrin

Calorimetric Titrations of TbpA with Apo- and Holo-h holf—Calorimetric titrations of TbpA with apo- and holo-holfs are shown in Fig. 1. The binding of both apo-holfs and holo-holfs to TbpA was driven by large enthalpy change and opposed by an unfavorable entropy change. The ΔH for apo-holf binding was substantially larger than the value obtained for the binding of holo-holf, which might indicate that the complex between apo-holf and TbpA is maintained by more or stronger interactions as compared with the complex between holo-holf and TbpA. This difference in ΔH is also reflected in differences in the binding constants. TbpA has an affinity ~20 times greater for apo-holf than holo-holf.

Calorimetric Titrations of TbpB Isotypes I and II with Apo- and Holo-holf—ITC titrations of isotype I and II TbpB with holo-holf (Fig. 2 and Table II) show that the mode of ligand binding is different for the two isotypes. The binding of holo-holf to isotype I TbpB (strain B16B6) is enthalpy-driven and opposed by an unfavorable entropy change. The ΔH of ~14.5 kcal/mol corresponds to an average value for a protein-protein interaction (30). In contrast to the isotype I TbpB (strain B16B6), the holo-holf binding to isotype II TbpB (strain M982) was driven by favorable entropy and enthalpy changes. Approximately 60% of the binding energy can be attributed to entropy changes and approximately 40% to enthalpy changes (Table II). The observed ΔH of ~4.0 kcal/mol was approximately four times smaller than for the isotype I protein and below the average for a protein-protein interaction. This difference in ΔH between both isotypes was also reflected in the binding constants for holo-holf. The affinity of TbpB of B16B6 for holo-holf was significantly higher than the M982 protein. In agreement with previous studies (31), the interaction of both TbpB isotypes with apo-holf was negligible. Furthermore, the number of binding sites (n) (Table II) in all of the experiments was found to be close to 1, confirming the previously suggested 1:1 stoichiometry for the binding of holf to TbpB (32).

Calorimetric Titration of the Transferrin Receptor (TbpA + B) Purified from N. meningitidis Strain M982 (Isotype II) with Apo- and Holo-holf—The entire transferrin receptor was purified from N. meningitidis strain M982 using holf-Sepharose affinity chromatography. The resulting protein was analyzed on a 4–20% polyacrylamide-SDS gel (Fig. 3A) and was found to be very pure, with the two bands identified as TbpA and TbpB.

Fig. 3B shows the calorimetric titration of the receptor with holo-holf. In contrast to the titration of separate TbpA or TbpB, two different binding events can be distinguished. Data analysis was carried using the experimentally determined molecular mass of 300 kDa/receptor complex (32). The data fitted very well to the two independent binding sites model provided by the ORIGIN software and the thermodynamic parameters obtained are listed in Table III. A high affinity binding site (Kd = 0.71 nM) can be distinguished from a low affinity site (Kd = 22.2 nM), and both sites appear to be independent. Most interestingly, the data show approximately one high affinity site and approximately two low affinity sites (Table III, n), indicating that in total three molecules of holo-holf are needed to saturate the receptor.

Production and Characterization of Recombinant Hexahistidine-tagged TbpB (His6-TbpB, M982) and Its Individual N- and C-terminal Domains

Production of Histidine-tagged TbpB (His6-TbpB) and Its Individual N- and C-terminal Domains—TbpB used for the above-mentioned studies corresponds to a recombinant, N-terminally lipidated form of the protein, which was purified from E. coli membranes (25, 26). This purification is rather tedious, and protein yields are relatively low. To verify whether a non-lipidated TbpB was comparable with its lipidated form, the protein was produced in fusion with a hexahistidine tag (His6-TbpB).

The sequence of the N-terminal half of TbpB can be aligned with its C-terminal half. This observation has led to the hypothesis that TbpB consists of two domains that are joined by a linker region, and it has been suggested that TbpB is a result of a gene duplication event (33–35). To characterize both domains and to study their implication in binding to holf and TbpB, the two domains were also produced as individual histidine-tagged proteins.

Following induction with isopropyl-β-D-thiogalactopyranoside, a high level of protein expression in E. coli was observed for all three recombinant proteins. After the centrifugation of
the E. coli cell lysate, the large majority of the C-ter was found to be present in the soluble fraction, whereas the N-ter was recovered from the insoluble fraction (Table IV). Isolated C-ter was soluble and stable in PBS, whereas purified N-ter was only stable in the presence of either detergent or a chaotropic agent (Table IV). In contrast, His6-TbpB was almost equally present in the soluble and insoluble fractions. Either protein was purified directly from the soluble fraction (His6-TbpB, C-ter), or protein recovered from the insoluble fraction was denatured, purified, and then renatured (His6-TbpB, N-ter). DSC profiles of His6-TbpB purified from the insoluble fraction and soluble fraction are shown in Fig. 4 (A and B), and the derived parameters are listed in Table V. Both profiles can be closely superimposed, and the differences observed for both sets of thermodynamic parameters are minor. This indicates that both protein preparations are comparable and that the renaturation process of His6-TbpB purified from the insoluble fraction is efficient.

Calorimetric Titration of the N-terminal Domain of TbpB with Its C-terminal Domain—Fig. 5A shows a calorimetric titration of N-ter with C-ter at 25 °C, and it is evident that both domains strongly interact with each other. For the titration at 25 °C, a $K_D$ of 47 nM ($K_A = 21.4 \times 10^6$ M$^{-1}$, $\Delta G = -10.0$ kcal/mol) was determined. Binding was driven by a large enthalpy change ($-\Delta H = 41.0$ kcal/mol), which was compensated by an also large unfavorable entropy change ($-\Delta S = 31.0$ kcal/mol), demonstrating a substantial loss in the degree of conformational flexibility upon domain interaction. Furthermore, saturation was achieved at a molar ratio of 1:1, indicating that the recombinant domains are homogeneous samples of correctly folded protein.

**Table I**

| htf form | $n$ | $K_D$ (nM) | $K_A$ ($M^{-1}$) | $\Delta H$ (kcal/mol) | $-\Delta S$ (kcal/mol) | $\Delta G$ (kcal/mol) |
|----------|-----|------------|-----------------|-----------------------|------------------------|---------------------|
| Holo     | 0.48 ± 0.03 | 68.9 ± 21 | (14.5 ± 4.2) $\times 10^6$ | -26.6 ± 2.7 | 16.8 ± 2.7 | -9.8 ± 0.17 |
| Apo      | 0.48 ± 0.07 | 3.7 ± 0.02 | (27.1 ± 0.2) $\times 10^7$ | -41.8 ± 3.0 | 30.4 ± 3.0 | -11.4 ± 0.01 |

**Table II**

| TbpB strain | htf form | $n$ | $K_D$ (nM) | $K_A$ ($M^{-1}$) | $\Delta H$ (kcal/mol) | $-\Delta S$ (kcal/mol) | $\Delta G$ (kcal/mol) |
|-------------|----------|-----|------------|-----------------|-----------------------|------------------------|---------------------|
| B16B6 (isotype I) | holo     | 0.76 ± 0.18 | 10.4 ± 3.6 | (9.6 ± 3.2) $\times 10^7$ | -14.5 ± 0.5 | 3.6 ± 0.5 | -10.9 ± 0.20 |
| M982 (isotype II) | holo     | 0.82 ± 0.10 | 78.1 ± 2.5 | (12.8 ± 0.4) $\times 10^6$ | -4.0 ± 0.8 | 5.6 ± 0.8 | -9.6 ± 0.02 |
| B16B6 (isotype I$^a$) | apo | 0.82 ± 0.10 | 78.1 ± 2.5 | (12.8 ± 0.4) $\times 10^6$ | -4.0 ± 0.8 | 5.6 ± 0.8 | -9.6 ± 0.02 |
| M982 (isotype II$^a$) | apo | 0.82 ± 0.10 | 78.1 ± 2.5 | (12.8 ± 0.4) $\times 10^6$ | -4.0 ± 0.8 | 5.6 ± 0.8 | -9.6 ± 0.02 |

$^a$ Very weak signal, no analysis possible.

**Fig. 2.** Isothermal titration calorimetry data for the binding of human holo-transferrin to lip-TbpB of *N. meningitidis* strain B16B6, an isotype I protein (A), and to lip-TbpB of *N. meningitidis* strain M982, an isotype II protein (B). The proteins were in 10 mM Na$_2$HPO$_4}$/NaH$_2$PO$_4$, 0.05% (w/v) Zwittergent 3-14, pH 7.0. The derived thermodynamic parameters are listed in Table II.
This titration was repeated at different temperatures, and
the enthalpy changes as a function of the temperature are
shown in Fig. 5B. For these titrations, changes in free energy
($\Delta G$) were found to be similar, indicating the compensation
of $\Delta H$ by the $-T\Delta S$ term. From the slope of the line in Fig. 5B,
the parameter $\Delta C_p$ (change of heat capacity) can be calculated.
Negative changes in $\Delta C_p$ can be correlated to the burial
of apolar surfaces (36). The $\Delta C_p$ for the interaction of both
domains of TbpB was found to be $-3.16$ kcal mol$^{-1}$ K$^{-1}$ ($R^2 =
0.98$). This $\Delta C_p$ change was significantly larger than the
average value identified for a protein-protein ligand interaction
(30) ($-0.2$ to $-0.7$ kcal mol$^{-1}$ K$^{-1}$). To our knowledge,
such a large value for $\Delta C_p$ has not been reported in the
literature and might indicate that the nature of protein-
protein ligand interaction is rather different to interdomain
interactions.

Circular Dichroism Studies—cd spectra of both individual
TbpB domains, a stoichiometric mix of both domains, and the
were moved arbitrarily on the y-ometric mix of N-ter and C-ter (E/H9004 content of the cooperatively folding unit. For instance, a transition), thermodynamic parameters are listed in Table V. C-ter (D), and a stoichiometric mix of N-ter and C-ter (E). For clarity reasons, the traces were moved arbitrarily on the y axis. The proteins were in 10 mM Na2HPO4/NaH2PO4, 150 mM NaCl, 0.05% Tween 20, pH 7.0. The thermodynamic parameters are listed in Table V.

full-length His6-TbpB are shown in Fig. 6. The molecular ellipticity at 220 nm can be regarded as a measure of the secondary structure content of a protein (37). Interestingly, both the N-ter and C-ter were found to contain very little secondary structure. Using the procedure of Taylor and Kaiser (38), a secondary structure content below 10% was estimated for both proteins, with C-ter being slightly more structured than N-ter. This is consistent with a large part of both proteins being present in a coil conformation. However, the secondary structure content of a stoichiometric mix of both domains was found superior to that of both domains on their own (Fig. 6). This is evidence that the domain interaction is accompanied by structural rearrangements. The observed conversion of disordered parts of the domains into structured regions is likely to be related to the loss of rotational and translational freedom expressed by the very unfavorable entropy change (−TΔS = 31.0 kcal/mol; see above).

DSC Studies—His6-TbpB and its individual domains have been studied by DSC (Fig. 4 and Table V). This technique permits the determination of the thermodynamic parameters of the thermal unfolding, such as Tm (midpoint of protein unfolding transition), ΔHcal, the enthalpy change on protein denaturation, which corresponds to the integrated peak area, and ΔHvis (change in van’t Hoff enthalpy), expressed by the peak width, which compared with ΔHcal provides information on the content of the cooperatively folding unit. For instance, a ΔHcal′/ΔHvis ratio of 2 is, in simple cases, an expression of two protein monomers present in the cooperatively folding unit (for further details see Ref. 39).

The denaturation of both the N-ter and C-ter was characterized by two separate unfolding transitions (Fig. 4 and Table V). DSC profiles showing two separate unfolding transitions have been observed in the past for proteins containing two separate domains (40, 41). The C-terminal domain was found to be fairly thermostable with a Tm beyond 80 °C, which is astonishing considering the relatively low amount of secondary structure determined by cd spectroscopy (Fig. 6). N-ter was found to be much less thermostable with transitions centered around 33 and 58 °C. Both the DSC thermograms of His6-TbpB and the stoichiometric mix of N-ter with C-ter show three transitions. Furthermore, studies to evaluate the reversibility of protein unfolding have been carried out, and the obtained data are shown in Table V.

Calorimetric Titrations of His6-TbpB, N-ter, and C-ter with htf—The presence of at least 100 mM NaCl was an absolute requirement for the solubility of His6-TbpB. However, this ionic strength leads to precipitation of the lip-TbpB (isotype II) used for the experiments presented above (Fig. 2). Therefore, experiments with the lipidated and histidine-tagged forms of TbpB had to be carried out in different buffer systems, and the data need to be compared with caution.

The calorimetric titration of His6-TbpB with holo-htf is shown in Fig. 7A, and the derived data are listed in Table VI. Although holo-htf bound with an approximately 10-fold higher affinity to His6-TbpB as compared with lip-TbpB of isotype II (Tables II and VI), the overall mode of htf binding to His6-TbpB and lip-TbpB was similar. Binding was characterized by a favorable entropy change, a moderate negative ΔH, and a TbpB:htf stoichiometry of 1:1. This indicates that His6-TbpB, which can be obtained more easily and in larger amounts than the lipitated form, is suitable material for the study of this protein.

Holo-htf bound to N-ter with a very similar affinity to His6-TbpB (Fig. 7B and Table VI). However, the enthalpy change (Table VI) for htf binding to N-ter was about three times larger than the corresponding value for the binding to His6-TbpB. No interaction between holo-htf and C-ter was observed (Fig. 7C).

Study of the Interactions between TbpA and TbpB—Calorimetric titrations of TbpA with His6-TbpB were not successful. This failure was due to the aggregation of TbpB at concentrations above 1 mg/ml (in the buffer system used to study TbpA), which are necessary for the ligand in the ITC syringe. Therefore, surface plasmon resonance technology (BIAcore) was chosen as alternative technique to study the interaction.

An excess of TbpA was immobilized onto the BIAcore sensor chip, and 150 pmol of ligand were brought into contact with this sensor chip. The increase in resonance units was observed, and after each test the sensor chip was regenerated by a wash at low pH. Initial experiments were aimed at verifying that this washing procedure did not alter ligand binding, and very good

| TbpB | Localization of recombinant protein after centrifugation of cell lysate<sup>a</sup> | Protein yield | Protein stable in |
|------|-------------------------------------------------|---------------|------------------|
|      | Soluble fraction | Insoluble fraction | mg/liter of cell culture |    |
| His<sub>6</sub>-TbpB | 35 | 65 | 7 and 10<sup>b</sup> | PBS, 0.5 M arginine |
| N-ter | 10 | 90 | 7 | PBS, 0.5 M arginine |
| C-ter | 90 | 10 | 15 | PBS |

<sup>a</sup> Based on the quantification of bands on SDS gels. Bands corresponding to recombinant proteins were identified by Western blots analysis using an anti-polyhistidine monoclonal antibody.

<sup>b</sup> Values are for the soluble and insoluble fractions, respectively.

Fig. 4. Differential scanning calorimetry data of His6-TbpB purified from the insoluble fraction (A), His6-TbpB purified from the soluble fraction (B), N-ter (C), C-ter (D), and a stoichiometric mix of N-ter and C-ter (E). For clarity reasons, the traces were moved arbitrarily on the y axis. The proteins were in 10 mM Na2HPO4/NaH2PO4, 150 mM NaCl, 0.05% Tween 20, pH 7.0. The thermodynamic parameters are listed in Table V.
reproducibility for a large number of consecutive cycles was noted (data not shown).

Ligands tested for interaction with TbpA included His$_6$-TbpB, N-ter, C-ter, and a stoichiometric mix of N-ter and C-ter. In addition, heat-denatured forms of these ligands were tested. Denaturation was carried out by consecutive heating and cooling of protein in a DSC apparatus, and samples were removed when no residual enthalpy changes were noted. Controls included bovine serum albumin as well as native and denatured forms of htf and bovine transferrin.

His$_6$-TbpB, N-ter, and C-ter were found to bind TbpA (Table VII). Denatured forms of TbpB and N-ter showed similar binding, whereas denatured C-ter did not show any binding affinity. The binding of His$_6$-TbpB or N-ter to TbpA appeared to be independent of the ligand conformation but specific for TbpA, because denatured N-ter or His$_6$-TbpB did not bind to control proteins such as bovine serum albumin (data not shown).

**DISCUSSION**

Study of the Interaction between TbpA, TbpB, and TbpA + B with Apo- and Holo-transferrin—ITC experiments were performed to characterize the interaction between purified TbpA, TbpB, and TbpA + B with iron-loaded (holo) and iron-free (apo) htf. This allowed an assessment of the contribution of each receptor protein in htf binding. The advantages of ITC over alternative techniques are that both ligands are unmodified.

**Table V**

| Sample                           | $T_m$  | $\Delta H_{cal}$ | $\Delta H_{ot}$ | $\Delta H_{ot}/\Delta H_{cal}$ | Reversibility$^a$ |
|---------------------------------|--------|-----------------|-----------------|-------------------------------|-------------------|
| His$_6$-TbpB (purified from insoluble fraction) | 47.7 ± 0.08 | 58 ± 1.3 | 103 ± 2.8 | 1.77 | 32$^b$ |
|                                 | 62.0 ± 0.05 | 119 ± 1.4 | 94 ± 1.4 | 0.79 | 90$^b$ |
|                                 | 81.3 ± 0.06 | 56 ± 1.1 | 150 ± 3.8 | 2.68 | 83 |
| His$_6$-TbpB (purified from soluble fraction) | 47.7 ± 0.07 | 57 ± 1.3 | 96 ± 2.3 | 1.68 | ND$^c$ |
|                                 | 61.4 ± 0.04 | 116 ± 1.2 | 92 ± 1.2 | 0.79 | ND |
|                                 | 80.9 ± 0.05 | 59 ± 1.0 | 138 ± 2.7 | 2.34 | ND |
| N-ter                           | 32.6 ± 0.09 | 35 ± 0.7 | 71 ± 1.8 | 2.02 | 0 |
|                                 | 58.0 ± 0.04 | 49 ± 0.6 | 111 ± 1.8 | 2.26 | 32 |
| C-ter                           | 70.5 ± 0.48 | 46 ± 2.8 | 52 ± 2.7 | 1.13 | >80$^b$ |
|                                 | 80.6 ± 0.04 | 67 ± 2.3 | 134 ± 3.2 | 2.00 | 81 |
| N-ter and C-ter                 | 43.2 ± 0.05 | 53 ± 0.6 | 82 ± 1.1 | 1.54 | 0 |
|                                 | 58.9 ± 0.06 | 56 ± 0.7 | 58 ± 1.1 | 1.03 | 0 |
|                                 | 80.3 ± 0.03 | 38 ± 0.5 | 124 ± 1.9 | 3.26 | 85 |

$^a$ The percentage of reversibility was defined as $\Delta H_2/\Delta H_1 \times 100\%$, where $\Delta H_2$ is the change of enthalpy from the second up-scan and $\Delta H_1$ is the change of enthalpy from the first up-scan of the same protein sample.

$^b$ Interference with the appearance of misfolded protein resulting from unfolding transitions with a higher $T_m$.

$^c$ ND, not determined.

**Fig. 5.** Interaction between the N- and C-terminal domain of TbpB from N. meningitidis M982. A, ITC data for the binding of the C-ter to the N-ter. B, temperature dependence of the enthalpy change for the binding of the C-terminal domain to the N-terminal domain of TbpB. The proteins were in 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 150 mM NaCl, 0.05% Tween 20, pH 7.0.

The binding of His$_6$-TbpB or N-ter to TbpA appeared to be independent of the ligand conformation but specific for TbpA, because denatured N-ter or His$_6$-TbpB did not bind to control proteins such as bovine serum albumin (data not shown).
and in solution and that the heat change measured is a direct signal of a molecular interaction. ITC is the only technique that permits the determination of the complete set of thermodynamic parameters of ligand binding (42). Apart from the binding constant (K), the obtained changes in enthalpy (ΔH), and entropy (ΔS) provide valuable information for the study of protein interaction. In general, negative contributions to ΔH can be attributed to the formation of hydrogen bonds, van der Waals' forces, or electrostatic interaction (42). Positive entropy changes are mainly due to the displacement of ordered water molecules (associated with a protein) into the bulk water, whereas a negative ΔS reflects a loss of conformational flexibility as a consequence of ligand binding (43, 44). Furthermore, the stoichiometry of ligand binding (n) can be determined by ITC.

Previous studies have shown that TbpB preferentially binds holo-htf, whereas TbpA does not discriminate between apo- and holo-htf (32). In this study, we have determined that TbpA has a much greater affinity for apo-htf than the holo form (Table I). This observation, in combination with the fact that in vivo the iron-free state is the predominant form of htf, indicates that in the absence of TbpB, iron acquisition from htf is slowed down by a quasi-saturation of TbpA with apo-htf. These data explain previous reports of TbpB-defective mutants of N. meningitidis, N. gonorrhoeae, Haemophilus influenzae, and M. catarrhalis, which showed either slower growth on htf-containing medium or reduced iron uptake capacity (21, 22, 45–47). The large unfavorable entropy change observed for the binding of both htf forms by TbpA is most likely to be the consequence of a loss of conformational flexibility upon htf binding, which might also be related to the conformational changes in TbpA following ligand binding as demonstrated in N. gonorrhoeae by Cornelissen et al. (48).

Cornelissen and Sparling (3) have studied htf binding to whole cells of N. gonorrhoeae using solid phase and liquid phase binding assays. They concluded that there are two independent htf-binding sites with Kd values of 0.8 and 16 nM. The calorimetric titration of purified receptor with holo-htf provides evidence for there being two independent binding sites with Kd of ≈0.7 and ≈22 nM (Table III). Considering that the technique used by Cornelissen and Sparling is entirely different from our approach, the similarity of both sets of data is remarkable. Furthermore, these values indicate that the neisserial receptor can compete successfully with the human transferrin receptor, which was shown to bind holo-htf with affinities between 5 and 20 nM (49). However, the technique employed by Cornelissen and Sparling did not allow the attribution of obtained Kd values to either TbpA or TbpB. This has been achieved by ITC. The iron-free form of htf is known to bind exclusively to TbpA but not to TbpB (32). This implies that the signal observed in the titration of the entire receptor with apo-htf corresponds to binding to TbpA. An ITC titration of the TbpA + B receptor with apo-htf at a ligand concentration similar to that used with holo-htf is shown in Fig. 3C. The same experiment with a 3-fold lower concentration of apo-htf (Fig. 3D) was used to determine the binding parameters. In both cases, there was only approximately one binding event, and the number of binding sites was found to be 1 (n = 0.81; Table III). This n value was close to the corresponding value of the high affinity binding site seen in the titration with holo-htf (n = 0.74; Table III), which identifies the high affinity site as binding to TbpA. The fact that the low affinity binding site corresponds to binding to TbpB was also confirmed by the similarity of binding parameters of this low affinity site present in the receptor and the parameters for the titration of TbpB (isotype II) alone with holo-htf (Tables II and III).

As stated above, the experimentally determined molecular mass of 300 kDa/receptor complex (32) was used for data analysis. Under these conditions we provide evidence that the receptor has approximately one high affinity site at TbpA and approximately two low affinity sites at TbpB. This indirect information on the receptor stoichiometry needs to be validated by a direct analysis of the TbpA-TbpB stoichiometry. The receptor protein (TbpA + TbpB) analyzed in this study was purified from N. meningitidis M982 grown under iron-limiting conditions. We have studied the expression of tbpB and tbpA genes of N. meningitidis M982 grown under iron-limiting conditions using TaqMan real time quantitative reverse transcriptase-PCR and have observed a ratio of 2:1 for the expression of tbpB.
and tbpA genes, respectively. Using a similar approach, a ratio of 2:1 has been reported for the tcpB tcpA gene expression in *N. gonorrhoeae* (19). The data of the tcpB tcpA gene expression are consistent with the ITC data reported in this article.

**New Insights Obtained on the Structure and Function of the Receptor**—The model of the transferrin receptor as suggested by Boullon et al. (18) contains a single htf-binding site. It was proposed that initial htf binding occurs at TbpB, which then triggers conformational changes in htf and binding to TbpA. This proposition was based on the observation by surface plasmon resonance technology that the affinities of TbpA and TbpB for htf are comparable (18). Here we show that holo-htf binding to the TbpA + B receptor occurs with an approximately 30 times higher affinity at TbpA than TbpB (Table III; *K*<sub>D</sub> of 0.71 nM versus 2.2 nM for binding at TbpA and TbpB, respectively), which does not suggest an initial binding event at TbpB. Our data seem to indicate that htf binds directly to the TbpA component of the receptor. In addition, we have provided evidence that there are approximately three binding sites for htf per receptor. TbpA has been identified as the high affinity site, whereas the other two binding sites represent binding at TbpB. Both binding sites appear to be independent, and no evidence for cooperative binding was obtained.

We have demonstrated that TbpA (as a component of the receptor) binds holo-htf with an ~3-fold greater affinity than apo-htf (Table III; *K*<sub>D</sub> of 0.71 nM versus 2.0 nM for the binding of holo- and apo-htf, respectively). In strong contrast, TbpA alone had a strong ligand preference for apo-htf (Table I; *K*<sub>D</sub> of 68.9 nM versus 3.7 nM for the binding of holo- and apo-htf, respectively). We now propose that within the receptor complex, TbpB shifts the ligand specificity of TbpA toward the iron-loaded form of htf. This proposition is consistent with the data from Cornelliessen and Sparling (3), who demonstrated that the interaction between TbpB and TbpA in *N. gonorrhoeae* is accompanied by conformational changes in both proteins. We also suggest that the specific binding of holo-htf by the TbpB component of the receptor increases the local concentration of free holo-htf in the proximity of TbpA. As a consequence, this reduces apo-htf binding by TbpA, increasing the efficiency of iron uptake from htf. Thus, we propose a double function for TbpB: to shift the ligand specificity of TbpA toward holo-htf and to increase the local concentration of holo-htf in the proximity of TbpA.

**Production and Characterization of Recombinant Hexahistidine-tagged TbpB (His<sub>6</sub>-TbpB, M982) and Its Individual N- and C-terminal Domains—His<sub>6</sub>-TbpB, N-ter, and C-ter have been used to determine the intramolecular architecture of TbpB. N-ter and C-ter were shown by ITC to largely interact, with saturation at a 1:1 molar ratio and a large negative change in heat capacity (*ΔC*<sub>P</sub>) of ~3.1 kcal mol<sup>−1</sup> K<sup>−1</sup> (Fig. 5). The magnitude of the *ΔC*<sub>P</sub> value suggests that extensive apolar surface area is buried upon domain interaction. This domain interaction is accompanied by secondary structure changes as observed by circular dichroism spectroscopy (Fig. 6). In addition, the cd spectra of *His<sub>6</sub>*-TbpB was almost superimposable upon the spectrum of the stoichiometric mix of both domains (Fig. 6), indicating that the observed domain interaction corresponds to the interaction of both domains in the full-length TbpB. The role of these structural changes in the function of TbpB remains to be elucidated.

The thermal unfolding of both the N-ter and C-ter are characterized by two transitions (Fig. 4 and Table V), which leads to the hypothesis that both TbpB domains contain two subdomains. This is a structural organization also found in transferrin (50). The DSC profile of *His<sub>6</sub>*-TbpB (Fig. 4A) was fairly similar to the profile of the stoichiometric mix of both domains (Fig. 4E), confirming the cd data, which indicated a close structural resemblance between the full-length protein and the stoichiometric mix of both domains. The first two transitions in both profiles might be attributed to the N-terminal domain, whereas the transition centered at approximately 80 °C corresponds to the denaturation of the C-terminal domain. The most striking difference between the DSC profiles of the stoichiometric mix of both domains and the profiles of the individual domains was the up-shift of the first transition of N-ter by approximately 11 °C accompanied by an increase in Δ*H* from 35 to 53 kcal/mol (Table V). This observed stabilization of one subdomain of N-ter on interaction with C-ter is thought to account for a large part of the strong exothermic signal observed for the ITC titration of N-ter with C-ter. These data indicate an involvement of one N-terminal subdomain in inter-domain interaction. The cd and DSC data presented do not confirm the data of vonder Haar et al. (51), who described the N-terminal domain of *N. meningitidis* TbpB as highly structured and extremely stable. However, the study of vonder Haar et al. was based on Western blot analysis of SDS-treated protein samples, and the different techniques employed might explain the observed discrepancies.

Information obtained from DSC can show insight into the structural arrangement of a protein but can also be used to predict long term stability of a protein. An increased *T*<sub>m</sub> and an elevated degree of the reversibility of protein denaturation indicate favorable long term stability of proteins (52, 53), which
is a very desirable property for any vaccine component. The thermal denaturation of C-terminal domain either as a separate protein, in complex with N-ter, or as part of the full-length protein showed a reversibility of 80–85%, which is above the average for a protein, whereas the denaturation of the N-terminal domain was much less reversible. The observed thermostability of C-ter and its high degree of reversibility predict increased long term stability of this domain. These biochemical and thermodynamic properties, together with its immunological properties (12, 34) suggest considerable potential for C-ter as a vaccine antigen.

Previous studies, mainly using solid phase htf-TbpB binding assays, have indicated that htf-binding occurs primarily at the N-terminal domain of TbpB (51, 54), but the participation of the C-terminal domain in ligand binding has also been demonstrated (34, 35). We found by isothermal calorimetric studies that C-ter did not bind htf, whereas N-ter bound the ligand with a similar affinity as the full-length protein (Fig. 7 and Table VI). However, the modes of htf-binding by N-ter and His6-TbpB are very different. Htf binding by the N-ter was enthalpy-driven, with an \( \Delta H \) largely superior to the full-length protein, and counterbalanced by an unfavorable entropy change. In contrast, htf binding of full-length TbpB was dominated by a relatively large and favorable entropy change, which compensates for the low enthalpy change. As mentioned above, enthalpy changes are often related to the creation of molecular interactions, and one might hypothesize that there is an optimal structural fit between N-ter and htf (large \( \Delta H \)), resulting in a loss of conformational flexibility (unfavorable entropy change). For the interaction of the full-length TbpB with htf, this optimal fit might not be achieved (maybe because of a steric hindrance caused by the presence of C-ter), and consequently a reduced enthalpy change and a favorable entropy change is observed. Interestingly, the apparent stoichiometry between N-ter and htf (large \( \Delta H \)) was found to be 2:1, whereas a 1:1 stoichiometry was observed for the experiment with the full-length protein (Fig. 7 and Table VI). This is consistent with a dimer of N-ter binding one molecule of htf. As stated above, N-ter and C-ter share sequence homologies that might be reflected in a similar structure, and N-ter dimerization might occur in a similar fashion as N-ter/C-ter association. Further evidence for N-ter dimerization was obtained by the DSC analysis, where for both unfolding events of the N-ter a ratio of approximately 2 was observed (Table V). Such a ratio is observed when the cooperatively folding unit contains a homodimer (55).

Biochemical evidence for a conserved interaction between TbpA with TbpB has been obtained by Fuller et al. (10). We have confirmed the existence of this interaction using surface plasmon resonance technology (Table VII). Furthermore, we show that both recombinant domains of TbpB are able to bind to TbpA. The binding of N-ter to TbpA was independent of its conformation because native and denatured forms bound to TbpA. In contrast, only native C-ter but not its denatured form bound to TbpA, indicating that this interaction is conformation-specific.

**New Insights Obtained on the Structure and Function of the Receptor**—The data presented confirm that TbpB contains two domains that can be obtained as individual, active, recombinant proteins. The two domains were shown to strongly interact with each other. This interaction is accompanied by an increase in secondary structure. Surface plasmon resonance data indicate that both TbpB domains interact with TbpA, but only the N-terminal domain of TbpB is involved in htf binding. DSC data suggest that both domains contain two subdomains, a structural organization also found in htf.
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Insight into the Structure and Function of the Transferrin Receptor from *Neisseria meningitidis* Using Microcalorimetric Techniques
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