The structural allosteric and binding interface for the human serum transferrin (Tf)-transferrin receptor (TfR) complex were identified using radiolytic footprinting and mass spectrometry. We have determined previously that the transferrin C-lobe binds to the receptor helical domain. In this study we examined the binding interactions of full-length transferrin with receptor and compared these data with a model of the complex derived from cryoelectron microscopy (cryo-EM) reconstructions (Cheng, Y., Zak, O., Aisen, P., Harrison, S. C. & Walz, T. (2004) Structure of the human transferrin receptor-transferrin complex. Cell 116, 565–576). The footprinting results provide the following novel conclusions. First, we report characteristic oxidations of acidic residues in the C-lobe of native Tf and basic residues in the helical domain of TfR that were suppressed as a function of complex formation; this confirms ionic interactions between these protein segments as predicted by cryo-EM data and demonstrates a novel method for detecting ion pair interactions in the formation of macromolecular complexes. Second, the specific side-chain interactions between the C-lobe and N-lobe of transferrin and the corresponding interactions sites on the transferrin receptor predicted from cryo-EM were confirmed in solution. Last, the footprinting data revealed allosteric movements of the iron binding C- and N-lobes of Tf that sequester iron as a function of complex formation; these structural changes promote tighter binding of the metal ion and facilitate efficient ion transport during endocytosis. Molecular & Cellular Proteomics 4: 1959–1967, 2005.

The transferrin (Tf)1-transferrin receptor I (TfR) complex has received considerable attention because of its role in cellular iron uptake, which is crucial for a broad range of cellular processes (1–7). TfR is a homodimer and type II transmembrane glycoprotein capable of binding two molecules of transferrin, a bilobal iron-carrying glycoprotein. TfR serves as a gatekeeper in regulating cellular iron uptake due to its selective binding of iron-bearing Tf at the slightly basic pH of the cell surface; this binding is followed by endocytosis and iron release in the acidic endosome (1). Apotransferrin remains bound to TfR at endosomal pH and is transported back to the cell surface where the transition to a slightly basic pH triggers the dissociation of the complex. Although high resolution crystal structures of Tf (8) and TfR (6) have been available for some time, no crystallographic structure of any Tf-TfR complex has been reported. Recently Cheng et al. (3) obtained an atomic model of the human Tf-TfR complex by docking the crystal structures of human Tf ectodomain and iron-loaded N-terminal and C-terminal lobes of Tf into a 7.5-Å resolution density map generated by cryoelectron microscopy (cryo-EM) image reconstruction and single particle averaging techniques. Although the moderate resolution of the cryo-EM images (compared with atomic resolution x-ray data) does not permit identification of individual residues and side chains of the proteins, the contours in this case are sufficiently well defined to allow unambiguous orientations in docking of the crystal structures into the electron density map. Side-chain interactions are then inferred from the overlaid crystal structures, and these proposed interactions have guided mutagenesis studies (3). The resulting model (Fig. 1) indicates that the Tf C-lobe binds laterally at the helical domain of TfR, and the Tf N-lobe is sandwiched between the TfR ectodomain and the cell membrane. Specific predictions as to points of side-chain contact can be made from this model. In particular, potential ion pair interactions between acidic residues in the Tf-C lobe and basic residues in the TfR helical domain that might mediate both specific as well as high affinity C-lobe binding to the helical domain were suggested (3, 9–11). Protein/protein interfaces for heteromeric complexes frequently contain charged and polar residues especially compared with the cores of proteins (12–17). However, the cryo-EM data are just suggestive with respect to the specific side chains involved, and methods to confirm protein/ligand interfaces and their charge-charge interactions are essential for understanding molecular recognition involving the specific and dynamic interactions of biological assemblies. Oxidative footprinting coupled with mass spectrometry has been proven to be a powerful technique for examining protein structure and biological assemblies as well as ligand-dependent conformational changes (5, 18–26). The radiolytic oxidations of amino acid side chains (27–32) within the protein structure are examined by LC-MS and LC-MS/MS; ligand-de-
pendent alternations of reactivity are interpretable in the context of known three-dimensional structure information. Using radiolytic footprinting, we previously examined the interactions of isolated Tf C-lobe with TfR (5). This confirmed that the Tf C-lobe binds to the receptor helical domain and induces allosteric changes in TfR remote from the binding site. In the present study, we examined the binding of full-length Tf to TfR. These data were then compared with the cryo-EM model of the complex to test specific hypotheses related to the contact surfaces in solution.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The soluble TfR ectodomain expressed in a baculovirus system was provided by Dr. Peter Snow at the California Institute of Technology. Lyophilized human diferric transferrin (dTf) was purchased from Roche Applied Science and shown to be homogeneous by SDS-PAGE and indistinguishable in its spectroscopic properties from native dTf isolated in this laboratory from Cohn fraction IV-7 (33). The dTf/H18528TfR complex (2:2 stoichiometry) was prepared by incubating a 2-fold molar excess of dTf with about 10^8/H11002M TfR subunits for 1 h at 37 °C and then overnight at 4 °C. The complex was separated from the uncomplexed components by size exclusion chromatography (10, 34). The complex is well resolved and can be cleanly isolated from the uncomplexed proteins. Based on A280 = 1.2, final concentrations of the 2:2 complex taken for synchrotron irradiation was estimated to be approximately 10^{-5} M.

**Solvent-accessible Surface Area (SASA) Calculation**—The VADAR 1.2 program (Protein Engineering Network of Center of Excellence, University of Alberta, Edmonton, Alberta, Canada) was used to calculate the solvent SASA of all side chains (Å²) from human dTf C-lobe, N-lobe, and TfR dimer using a Protein Data Bank file of iron-loaded Tf C-lobe (provided by Dr. Harmon Zuccola) and Protein Data Bank codes 1N84 and 1CX8, respectively. For comparison, the protected side chains as a function of complex formation were also predicted from the cryo-EM model (Protein Data Bank 1SUV) (3) using Surface Racer 1.2 (35). In this calculation TfR was removed for calculating the SASA of isolated Tf, whereas Tf was removed for calculating the SASA of isolated TfR.

**Synchrotron X-ray Radiolysis and LC-MS Analysis**—Prior to radiolysis, dTf, Tf, and their complex were dialyzed against 20 mM sodium cacodylate buffer (pH 7.0) at 4 °C overnight (5). Samples were exposed to the synchrotron x-ray beam at the X28C beamline of National Synchrotron Light Source at Brookhaven National Laboratory (Upton, NY) for 0–300 ms in accordance with our established protocols (5, 20, 36). The irradiated samples were then reduced with 10 mM DTT (Roche Diagnostics) and denatured by adding 15% acetonitrile and heating at 95 °C for 25 min followed by cooling on ice. Digestion was performed using sequencing grade modified trypsin (Promega Biosciences, Madison, WI) and Asp-N (Roche Diagnostic) at 37 °C for 15–18 h. Peptide mixtures were separated by reverse phase HPLC using a C18 column and examined by ESI-MS and MS/MS, and data analysis was carried out according to our published procedures (5, 20).

**RESULTS**

**Protections on Full-length Transferrin upon Receptor Binding**—Human transferrin (1) consists of two globular lobes, the N-lobe and the C-lobe, joined by a flexible peptide strand (Fig. 2A). Each lobe is further divided into two domains (C1, C2, N1, and N2) separated by a deep interdomain cleft, which houses a high affinity yet reversible iron binding site. Tf C-lobe is the primary receptor recognition lobe, accounting for most of the binding energy of the Tf-TfR complex (3, 11). The sequences and rate constants for peptides of full-length Tf and TfR that experienced protection upon complex formation are listed in Table I. Dose-response curves for two selected peptides, Tf peptide 366–380 and TfR peptide 648–658, are displayed in Fig. 3, while those for other peptides from Table I are shown in Supplemental Fig. 1. Many other peptides from Tf, TfR, and their complex were examined as well; none of them showed significant changes in oxidation rate constants as a function of complex formation and are not reported.

![Cryo-EM model of diferric Tf-TfR (ectodomain) complex. A, face-on view. B, bottom view.](image-url)
Table I shows the residue numbers and sequences for selected peptides from Tf or TfR; all the peptides shown are derived from trypsin digestions except two at the bottom of the table generated by Asp-N cleavages. The probe residues (e.g. the ones oxidized in the footprinting experiments) for each peptide were identified using tandem MS and are bold-
faced. These probe sites are the reporter groups in the footprinting experiments and may experience increases, decreases, or no changes in oxidation rates as a function of complex formation. The SASAs of the specific probe side chains as calculated from the crystal structures of the isolated proteins are shown underneath the peptide sequences. Three
peptides within the Tf C-lobe and two peptides within the N-lobe experienced significant protections upon complex formation (Table I). The backbone segments of these peptides are colored in green on the structure of full-length Tf in Fig. 2A, and the peptide residue numbers are labeled as well to illustrate the positions of these peptides on the structure. The actual probe side-chain residues are colored in purple. Within the C-lobe, the protected peptides include sequences 366–380 and 381–401 from the C1 subdomain and peptide 457–470 from the C2 subdomain. In our previous analysis of C-lobe binding to TfR, we observed protections upon complex formation for peptides 381–401 and 457–470, indicating that the binding mode of isolated C-lobe is very similar to that seen for the C-lobe of full-length Tf. The present studies provide new data for the N-lobe in which peptides 51–88 and 89–102 experienced protections upon complex formation.

**Oxidative Decarboxylation of Acidic Residues in Tf Is Suppressed upon Complex Formation**—Dose-response curves of peptide 366–380 in the C1 domain are shown in Fig. 3A. This peptide experienced a ~2-fold decrease in the observed oxidation rate constant upon complex formation when the +14- and +16-Da oxidation products of valine were quantitated (Fig. 3A, open versus closed circles, and Table I). The peptide contains four acidic residues; recently we determined that solvent-accessible acidic residues are subject to oxidative decarboxylation. The decarboxylation is accompanied by aldehyde formation and results in a characteristic mass shift of ~30 Da (29).

We examined the LC-MS spectra of trypsin-digested Tf for ~30-Da mass changes in peptide 366–380, represented as the fraction unmodified for each exposure time as described previously (5, 20). For isolated Tf a first order loss of the peptide 366–380 was accompanied by proportional increases of products shifted by ~30 Da. The modification rate constant corresponding to the ~30-Da products was 0.50 ± 0.02 s⁻¹ for isolated Tf (Fig. 3A, closed squares) and was reduced over 80% to 0.08 ± 0.02 s⁻¹ (Fig. 3A, open squares) in the case of the full-length Tf-TfR complex (Fig. 3A). Thus, based on both qualitative and quantitative mass spectrometry measures, we could specifically distinguish the oxidation of the acidic residues (based on the appearance of the ~30-Da products) from oxidation of the other reactive site chains in the peptide (based on appearance of the +14- and +16-Da products, Table I).

Tandem MS was carried out on the +16-Da and ~30-Da products of peptide 366–380 to determine the amino acids that experienced these mass changes as a function of oxidation (5, 20, 37). The tandem MS data indicate that Val-369 is the major contributor to the +16-Da modification (data not shown). However, the ~30-Da oxidations derive from multiple residues throughout the peptide; thus Glu-367, Glu-372, Glu-375, and Asp-376 are indicated to suffer decarboxylation in isolated Tf (Supplemental Fig. 2) (29). Side chains of residues whose oxidation is suppressed by complex formation are explicitly indicated and colored in purple on the structure of Tf in Fig. 2A. The acidic residues experienced ~6-fold protection, whereas Val-369 was modestly protected. Based on this result, we reanalyzed our previously collected data from the C-lobe complexed to TfR (5) and found similar oxidative decarboxylation of peptide 366–380 for isolated C-lobe that was reduced over 80% in the complex. The reduced oxidation of these acidic residues within the C-lobe for both isolated C-lobe and full-length Tf are consistent with the interface predicted by cryo-EM studies (3). These data demonstrate that footprinting can detect the protection of acidic residues as a function of complex formation.

Tandem MS was carried out on the other Tf peptides (data not shown), indicating that Met-382 and Met-389 are the primary probe sites in peptide 381–401 (Fig. 2A). Met-389 is close to the iron-binding residue Asp-392 and is slightly accessible from the interdomain cleft (Fig. 4). Peptide 457–470 corresponds to a helix near the hinge running from the surface toward the iron binding site (Fig. 4). Trp-460 and Met-464 were determined to be the probe sites in this peptide. Probe residues in N-lobe peptide 51–88 include Val-60, Tyr-68, Tyr-71, Leu-72, and Pro-74, whereas those in N-lobe peptide 89–102 include Phe-94 and Tyr-96.
Protection of Helical Domain of TfR upon Tf Binding—The TfR homodimer consists of four regions: a large extracellular ectodomain, a stalk of about 30 Å between the ectodomain and the cell membrane, a transmembrane segment, and an N-terminal cytoplasmic domain. The transferrin-binding ectodomain (6) is comprised of three distinct subdomains: a protease-like domain proximal to the membrane, a helical domain responsible for dimer interface contacts, and a membrane-distal apical domain. Table I shows six peptides in the domain responsible for dimer interface contacts, and a membrane-protease-like domain proximal to the membrane, a helical ectodomain (6) is comprised of three distinct subdomains: a N-terminal cytoplasmic domain. The transferrin-binding domain consists of four regions: a large extracellular domain, a stalk of about 30 Å between the extracellular domain and the cell membrane, a transmembrane segment, and an N-terminal cytoplasmic domain.

Protection of Arginine Residues in TfR upon Complex Formation—Multiple arginine residues seen in the TfR helical domain include Arg-623, Arg-629, Arg-646, Arg-651, and Arg-655; Arg-646 is part of the highly conserved RGD sequence (646–648) essential for TfR binding (3B). All of these arginine residues are within highly protected peptide sequences in the Tf-TfR complex. Recently using model peptides, we demonstrated that solvent-accessible arginine residues are susceptible to oxidation at the α-position of the side chain leading to loss of the guanidine moiety and a characteristic mass change of −43 Da (28). Oxidation at the β or γ positions of the side chain results in alcohol or carbonyl formation (mass change of +16 or +14 Da) (28). Loss of the positive charge would impede the action of trypsin; thus peptides that suffer deguanidination should be refractory to trypsin cleavage at the oxidized sites. We thus digested TfR with endoproteinase Asp-N, revealing two peptides within the helical domain that exhibited significant protections as a function of complex formation (Table I). When the +16- and +32-Da modification products of peptide 631–647 were quantified using LC-MS, the peptide was observed to have a rate constant of modification of 2.6 ± 0.1 s⁻¹ for isolated TfR compared with 0.2 ± 0.1 s⁻¹ in the complex. These rate constants of modification are nearly identical to that observed from (partially overlapping) peptide 634–646 generated from a trypsin digestion. However, no −43-Da products were observed for this peptide, e.g. oxidation of Arg was not detected. Like peptide 631–647, peptide 648–658 showed protections upon Tf-TfR complex formation when +16- and +32-Da products were examined. A modification rate of 1.6 ± 0.1 s⁻¹ was observed for isolated TfR (Fig. 3B, closed circles) compared with 0.23 ± 0.09 s⁻¹ in the complex (Fig. 3B, open circles). These data are consistent with the results seen for the partially overlapping peptide 647–651. This peptide also exhibited characteristic −43-Da products that appeared with a rate constant of 0.83 ± 0.08 s⁻¹ for isolated TfR (Fig. 3B, closed squares) that was reduced over 90% to 0.05 ± 0.08 s⁻¹ in the complex (Fig. 3B, open squares).

It appears that the single arginine in peptide 631–647 (Arg-646) was not sufficiently reactive compared with other nearby reactive residues to be oxidized, or the δ-CH₂ of the Arg-646 side chain is not sufficiently accessible for modification. In contrast, peptide 648–658, which contained two highly solvent-accessible arginine residues with a total SASA of over 220 Å², was relatively easily oxidized. The specific protection of these arginine residues as a function of complex formation is consistent with the interface predicted by cryo-EM (3). These data demonstrate that footprinting can detect the protection of Arg as a function of complex formation.

DISCUSSION

The cryo-EM model of the Tf-TfR complex provided by Cheng et al. (3) allows quantitative predictions for specific side-chain interactions in the Tf/TfR interface. Using this cryo-EM data, we carried out solvent accessibility calculations of the Tf-TfR complex as well as for the Tf and TfR coordinates used to construct the model (see “Experimental Procedures”). These calculations are illustrated in Fig. 2, C (for Tf) and D (for TfR), where residue side chains that experience SASA decreases >15% upon complex formation are colored in purple. In the following sections, we compare these model predictions with the footprinting data shown in Fig. 2, A and B, to evaluate the contacts predicted by the model and to identify regions of potential allosteric change as a function of complex formation.

Footprinting Supports Transferrin Binding Sites Suggested by Cryo-EM Data—Peptide 366–380 corresponds to one β-strand and one α-helix on the surface of the C1 domain. In the cryo-EM model, the side-chain residues ranging from 367 to 372 are buried nearly 50% in the formation of the Tf-TfR complex including protected footprinting probe residues Glu-367, Val-369, and Glu-372 (Table I and Fig. 2C). Cryo-EM and mutagenesis data also indicate that peptide 349–365 is involved in TfR binding (3); unfortunately no MS signal was observed for this peptide in our experiments. Overall the data indicate a TfR binding region within the Tf C-lobe that includes sequences 349–380 (two helices and a β-strand). This region contains multiple charged residues on the Tf surface.
Fig. 5. Binding model of Tf C-lobe/TfR interface. Shown are the open-book view of the complementary structural epitopes on the TfR helical domain (A) and the Tf C-lobe (B). A complex charge-charge interaction network exists between the positively charged TfR helical domain and the highly negatively charged Tf C-lobe. The predicted contact regions are linked, and the residues are colored according to their physicochemical properties (blue for Arg; red for Asp and Glu; green for Lys and His; yellow for Asn, Ser, and Gln; brown for Val, Leu, Ile, and Gly; purple for Trp, Tyr, and Phe; and magenta for Met).

Within the Tf N-lobe, footprinting indicates that the probe residues within peptides 51–88 and 89–102 experienced conformational change as a result of complex formation (shown in green in Fig. 2A). The sequence protection pattern is consistent with the cryo-EM model (3), which predicts that side-chain residues 50–75 are within the contact interface. Specifically, the five footprinting probe residues identified within peptide 51–88 (Val-60, Tyr-68, Tyr-71, Leu-72, and Pro-74) experience 65% reductions in solvent-accessible surface area in the formation of the complex. Meanwhile, the cryo-EM model suggests that the N-lobe contacts the receptor between residues 123 and 126. We analyzed a tryptic peptide of sequence 122–128 (with probe residues of Tyr-123 and Trp-124) from this region of TfR (Table I and Fig. 2B) and found that the rate of oxidation was reduced about 40% upon complex formation, consistent with this contact as well. On the other hand, none of the residues within Tf peptide 89–102 corresponding to the hinge connecting the N1 and N2 subdomains of Tf are predicted in the cryo-EM model to be protected in the formation of the complex. Note that the backbone segment behind the N-lobe iron atom is colored green in Fig. 2A but not colored in Fig. 2C; these residues appear to be buried as a result of ligand-induced conformational change.

**Allosteric Effects of Complex Formation Sequester Iron**—Peptides 381–401 and 457–470 from the C-lobe are not predicted to be part of the binding interface; the probe residues within these two peptides experience allosteric conformational change as a result of complex formation (Fig. 4). All the probe residues in peptide 381–401 are within the sequence 381–392; thus only this part of the peptide sequence is colored in Fig. 4. Residues 381–392 correspond to the last turn of a helix near the binding site with a β-strand running from the surface to the protein core in the C1 domain. Peptide 457–470 (with Trp-460 and Met-464 as probe residues) in the C2 domain is on the opposite side of the C-lobe relative to the predicted interacting surface and corresponds to one helix running from the surface toward the iron binding site. The iron binding site is located close to the hinge, which consists of two antiparallel β-strands running behind the iron binding site connecting the C1 and C2 domains. One β-strand forming the hinge includes peptide 415–433, which has probe residue Phe-427 adjacent to iron ligand Tyr-426. This peptide is significantly protected in experiments where isolated Tf C-lobe is bound to TfR (5); unfortunately this peptide experiences ion suppression in the LC-MS experiments with full-length Tf. The observed protections, as well as protections observed for Met-389 near the iron-binding residue Asp-392, suggest subdomain movements and a tightening of the structure adjacent to the hinge resulting in sequestration of the iron atom.

For the N-lobe as well, footprinting data suggest closure around the iron atom as a function of receptor binding. Asp-63, one of the iron-binding ligands, is located at the same helix as protected probe residues Val-60, Tyr-68, Tyr-71, Leu-72, and Pro-74 (Fig. 2A). In addition, sequence 89–102 corresponds to one of the two β-strands of the N-lobe hinge; the primary probe residues Phe-94 and Tyr-96 are next to iron-binding ligand Tyr-95. The footprinting data point to a range of allosteric effects of receptor binding that stimulates increasing closure around the iron atoms beyond what is already present in the iron-bearing free protein; this helps provide a molecular explanation as to why receptor-bound diferric Tf releases iron significantly more slowly than free diferric Tf at the slightly alkaline extracellular pH (39, 40).

**TfR Helical Domain Binding**—The cryo-EM data indicate significant decreases in solvent-exposed surface area for Leu-619 (50%), Arg-623 (93%), Arg-629 (58%), Gln-640 (65%), Tyr-643 (83%), Arg-646 (91%), Phe-650 (77%), and Arg-651 (43%) as a function of complex formation. These residue side chains are colored purple in Fig. 2D. These predicted decreases in surface area are consistent with the protections for specific probe residues indicated in Table I. Helix 3 contains the RGD motif (residues 646–648) established to be critical for Tf binding (38). This binding site on TfR is also consistent with that found for hereditary hemochroma-
tosis protein binding to TfR, which competes with Tf for TfR binding (7, 41, 42). Mutagenesis studies also implicate this interface as mutations L619A, R629A, Y643A, and F650A decrease Tf binding (9, 41), whereas R651A and G647A abolish it (38). Protection at the flexible C-tail peptide 733–760 due to complex formation may involve changes in the dimer interface; the cryo-EM model does predict Tf contacts with TfR residues from 757 to 760. Although mutations such as F760A do not affect dTf binding at pH 7.4 (9), rearrangement of the flexible C-tail may assist in Tf binding by excluding solvent from the Tf-TfR binding area.

**Charge-Charge Interactions in the TfR Binding Site of C-lobe**—The most striking feature observed in the interface of the C-lobe within the TfR helical domain is the clustering of charged and polar residues on both sides at the interface. The footprinting data demonstrate the burial of acidic and basic residues within the C-lobe and the TfR helical domain, respectively. Fig. 5 is an “open-book” view of the Tf C-lobe/TfR interface. There are five positively charged arginine residues (Arg-623, Arg-629, Arg-646, Arg-651, and Arg-655), one negatively charged aspartate residue (Asp-648), and several other polar residues present within the TfR helical domain. Within the Tf C-lobe binding site, there are many negatively charged residues (Asp-356, Glu-357, Glu-367, Glu-372, Asp-376, and Glu-385) and four positively charged residues (Lys-343, His-349, Arg-352, and Lys-380) proximal to the Tf C-lobe binding site. The hydrophilic, charged nature of the interface strongly implies a cooperative charge-charge interaction network between the two proteins. Such networks involving two or more ion pairs are more common at complex interfaces and contribute more to protein stability than isolated ion pairs, which predominate in intramolecular interactions (43). The electrostatic interactions are enhanced by solvent exclusion and contacts of hydrophobic residues such as Leu-619, Tyr-643, and Phe-650 of Tf; these are implicated by footprinting to be buried in the interface. Based on the cryo-EM, hydrophobic residues such as Leu-353, Val-360, and Val-369 in Tf are likely buried as well, enhancing the exclusion of solvent.

**Conclusion**—The structural allostery and binding interface for the human serum Tf-TfR complex were identified using radiolytic footprinting and mass spectrometry. This work extended the use of such footprinting approaches to the examination of conformational changes for charged residues, and we confirmed a predicted ionic interaction between the Tf C-lobe and the receptor helical domain. In addition, the overall binding surfaces for C-lobe and N-lobe of transferrin and the corresponding interactions sites on the transferrin receptor predicted from cryo-EM were confirmed in solution. The footprinting data also revealed allosteric movements of the iron-binding C- and N-lobes of Tf that sequester iron as a function of complex formation resulting in tighter binding of the metal ion and promoting efficient ion transport during endocytosis.

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§ Both authors made equal contributions to this work and share the first authorship.

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