Physiological Levels of Binding and Iron Donation by Complementary Half-molecules of Ovotransferrin to Transferrin Receptors of Chick Reticulocytes*

(Received for publication, May 9, 1983)

Anne Brown-Mason, and Robert C. Woodworth
From the Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405

Two fragments, each corresponding to approximately half of the ovotransferrin (OTf) molecule and containing an iron-binding site were produced by digestion with affinity bound trypsin and were purified by isoelectric focusing and gel filtration chromatography. The immunologically distinct "half-molecules" individually have little ability to bind to transferrin receptors on chick embryo red blood cells or to donate iron to them. Combining them, however, leads to both binding and iron donation approaching that found for holo-OTf. Furthermore, similar amounts of radiolabeled iron can be extracted into the putative heme molecules derived from Fe2OTf and from the various combined half-molecules. These findings conflict with those reported by Keung and Azari ((1982) J. Biol. Chem. 257, 1184-1188) for subtilisin-derived half-molecules of OTf examined in a similar system. They found that each half-molecule appeared to bind at a level of approximately one-third that of Fe2OTf and that the half-molecules competed with each other for binding sites.

In contrast, our equilibrium binding studies, in the presence of 2,4-dinitrophenol to prevent iron removal, led to the determination of $4.79 \times 10^4$ binding sites/cell for Fe2OTf, $4.44 \times 10^4$ for the NH2-terminal half-molecules in the presence of excess COOH-terminal halves of OTf and $4.17 \times 10^4$ for COOH-terminal halves of OTf in the presence of NH2-terminal half-molecules; apparent binding constants were estimated to be $3.29 \times 10^6$, $1.19 \times 10^6$, and $0.67 \times 10^6$ M$^{-1}$ for these same samples. Problems associated with equilibrium binding studies in which a narrow range of concentrations of ligand is used and/or iron is being removed are discussed.

Labeled combined half-molecules were half as effective as labeled Fe2OTf in competition with unlabeled Fe2OTf. These findings are consistent with the lower apparent binding constant found in the equilibrium binding studies. Equimolar apo-OTf had no effect on binding of either Fe2OTf or the combined half-molecules.

It seems apparent from our studies that the NH2- and COOH-terminal half-molecules each contain a recognition region both of which are necessary for binding to the transferrin receptor and iron donation to the chick embryo red blood cell.

The transferrins (siderophilins) are a group of homologous glycosylated iron transport proteins which include serum transferrin from a variety of organisms, ovotransferrin from egg white, and lactotransferrin from mammalian milk. Each protein molecule comprises a single polypeptide of about 80,000 Da, roughly divided into two similar domains, each containing a specific binding site for ferric iron and a concomitant obligate anion which physiologically may be carbonate or bicarbonate (1, 2). Transferrin binds to specific receptors on the surfaces of many different cells of animal origin (3) and is involved in iron transport to these cells.

Much research has been directed toward establishing the equivalence or nonequivalence of the two iron-binding sites. Confusion has come from the use of heterologous transferrin- reticulocyte systems. It now appears that in isologous systems of rabbit, human, and chicken, the sites are functionally equivalent (4-6). Most chemical probes, however, suggest that physical nonequivalence of the two sites (7-12).

One approach which has been taken with limited success is the use of proteolytically derived monoferric iron-binding fragments or half-molecules in an uptake system. Thus, it has been reported (13, 14) that OTf1 half-molecules are able to donate iron to rabbit reticulocytes, although no data have been presented to support the claim. In fact, it does not appear that even dimeric OTf can function in this heterologous system (15). Brock et al. (16) report that rabbit reticulocytes take up little iron from bovine half-molecules. In preliminary studies with human NH2-terminal half-molecules, there is no binding to Tf receptors on human placenta or rabbit reticulocytes (17). As mentioned above, however, evaluation of half-molecules derived from Tf of one species with reticulocytes from a different species is probably a poor experimental procedure. Furthermore, none of the studies cited reported the result of combining the half-molecules.

More recently, Keung and Azari (18) have published a report showing limited binding and iron-donating ability of subtilisin-derived half-molecules of ovotransferrin to chick embryo red blood cells. They found that binding of each half-molecule was equal but the total number of binding sites at equilibrium was approximately one-third of the binding sites found for holo-OTf. The NH2-terminal half-molecule appeared to be more efficient at donating iron to the cells than the COOH-terminal half-molecule but again both were con-

---

* This investigation was supported by United States Public Health Service Grants F32-HL06231 and HL-23752. The multi-user γ scintillation counter used for these studies was purchased with funds provided through the Research Advisory Council of the University of Vermont. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: OTf, ovotransferrin; Tf, transferrin; Fe2OTf, iron saturated OTf; FeOTf/2N and FeOTf/2C designate the iron binding domains or "half-molecules" from the NH2-terminal and COOH-terminal halves of OTf; NTA, nitrilotriacetate; DPC, dodecylphosphocholine; DNP, 2,4-dinitrophenol; CERBC, chick embryo red blood cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
siderably less efficient than FeOTf. Combining the half-molecules led to no enhancement of binding; in fact, binding was lowered, perhaps showing that the two half-molecules were competing for the same site. In an experiment in which FeOTf competed against each of the half-molecules at equimolar concentrations, only 5% of the half-molecule remained bound. These results conflict with our findings discussed below. As described herein, we have successfully made NH₂-
and COOH-terminal half-molecules which when combined are active at levels comparable to FeOTf.

**MATERIALS AND METHODS**

**Protein Preparation**—Ouotransferrin was prepared as described previously (6). The NH₂-terminal half-molecule designated FeOTf/2N was prepared from purified OTf, 45% saturated with iron nitro-lotriacetate. A 0.5 × 10⁴ M FeNTA solution, pH 7.1, was freshly made from a 0.50 M Fe(C10₄)₂ stock solution before addition to the protein, which was 20 mM with respect to NaHCO₃. The monomeric species was isolated on an electrofocus column (Servalytes, pH 4-9), dialyzed against glass-distilled H₂O, and digested with DPCC-treated trypsin (Sigma) attached to Affi-Gel-10 (Bio-Rad). Both the sample and the Affi-Gel-trypsin were 0.05 M Tris-HCl, 0.02 M CaCl₂, 0.02% sodium azide, pH 7.8, at 4°C. After incubation at the appropriate temperature on a Labquake (Fisher Scientific), the digested sample was reduced in volume and loaded onto a precolumn electrofocus column (pH 4-9) and focused for 34 h. Final purification of the half-molecule was achieved by chromatography on a Sephadex G-75 column (2.5 × 90 cm) equilibrated and run in 0.1 M NaHCO₃.

The carboxyl-terminal half-molecule designated FeOTf/2C was prepared from FeOTf by dialysis against 0.5 M Na acetate, pH 5.5, until the ratio of A₂₅₀/A₄₆₅ fell from 20 to about 6.0. Typically, the following 6 different samples were run: 1) Fe₅⁹Fe₁²⁵I-OTf/2C; 2) Fe₁²⁵I-OTf/2C; 3) Fe₅⁹Fe₁²⁵I-OTf/2C; 4) Fe₁²⁵I-OTf/2C + Fe₁⁰⁹⁺-OTf/2C; 5) Fe₁⁰⁹⁺-OTf/2C + Fe₁²⁵I-OTf/2C; and 6) FeOTf/2N + Fe₁²⁵I-OTf/2C. The counts/min in each aliquot of cells was divided by a factor obtained by dividing the specific activity in counts/min/mmol by 0.62 × 10⁴ to obtain counts/min/molecule. This number was corrected for the number of cells in the aliquot as determined by hematocrit (6). Hematocrits were generally between 15 and 25% indicating 1.1-1.7 × 10⁶ cells/ml. Samples which contained both half-molecules, but in which only one species was labeled, were considered only in terms of the specific activity of the labeled species. In other words, the nonradioabeled iron and protein were not considered and the concentration of radioanalyzed protein and iron is 10 μM. In terms of the theoretical equivalence of 59Fe taken up per cell compared to 59FeOTf, a 50% level would be maximum. In a sample in which both half-molecules were labeled with iron and iodine, the iron specific activity is taken as the average of the specific activities of the two species, whereas the iodine specific activity is obtained by adding the specific activities of the two half-molecules together. It is obvious that, if the specific activities of the two half-molecules are very different, the Fe atoms/cell and sites/cell will not accurately reflect the relative contributions of each.

**Equilibrium Binding Studies**—Washed cells were incubated in EGA or EGAB at 37°C for 15-30 min and washed three times to clear as much endogenous Fe as possible. The cells were then incubated for 15-30 min at 37°C in EGAB containing 0.75 mM 2,4-dinitrophenol to inhibit iron uptake. After further washing, the cells were diluted 1:1 with EGB/DNP and 0.4 ml aliquots were pipetted into Omni-vials (VWR Scientific, Inc.) containing various concentrations of 59Fe labeled samples in a 0.1-ml volume. The samples were placed in a standard CO² incubator on a rocking platform for 30 min, at which time three 50-μl aliquots were removed and washed as described above. An aliquot of the supernatant from the hematocrit tube was assayed for radioactivity in order to determine the exact concentration of free protein in each vial.

The data shown in the results for OTF and the half-molecules are for a given batch of cells on a given day, as some variability was found between cells on different days with respect to their uptake rates of iron.

**Preparation of Antibody**—Antibody to OTF was raised in New Zealand White rabbits by established protocols (20). Pooled specific antisera dried against phosphate-buffered saline were fractionated with 45% ammonium sulfate, dialyzed against phosphate-buffered saline, applied to an OTF affinity column (Affi-Gel-10, Bio-Rad), and eluted with HCl, pH 2.0, after extensive washing with phosphate-buffered saline. The specificity of the antibody was tested on Oupetransferrin-Sepharose.
Molecular weight standards were obtained from Bio-Rad.

Presentation of Data—Data from kinetic studies of binding of OTf and the half-molecules by CERBCs were fitted to a first order rate equation by the PAR biomedical computer program as previously described (26). This gives estimates of the first order rate constant, \( k \), initial and final values of the dependent variable and their standard deviations. The lines in figures in which values of \( k \) are reported are the computer fitted lines to the experimental points presented in these figures.

RESULTS

The half-molecules are shown to be distinct by their immunological and physiological properties including Ouchterlony double diffusion plates (Fig. 1), pl, molecular weight, and sequence at the NH₂ terminus (Table I) as well as their NMR spectra (27). The sequence determination of the trypsin-derived half-molecules is important in confirming the absence of significant internal nicking. SDS-gel electrophoresis (Fig. 2) reveals slight differences in molecular weight between the half-molecules and shows that disulfide bridges within each half-molecule appear to be intact since OTf and the half-molecules migrate differently in the presence and absence of the reducing agent dithiothreitol. Furthermore, it should be noted that there is a small amount of holo-OTf in the final half-molecule preparations in addition to a band running below OTf/2N which is therefore of slightly lower molecular weight.

Urea-gel electrophoresis (Fig. 3) shows that the apo- and Fe-half-molecules migrate to positions distinct from apo- and Fe₂OTf. The apo-forms of the two half-molecules can be distinguished from each other whereas the iron-containing forms migrate to the same position in the gel.

Addition of carbohydrate (49) to OTf and OTf/2C increases the calculated molecular weight to 79,882 and 41,350, respectively.

Sequences are from Ref. 27.

### Table I

| Protein | Treatment | \( pI \) | Apparent molecular weight* | Calculated molecular weight* | Sequence* |
|---------|-----------|---------|----------------------------|----------------------------|-----------|
| OTf     | None      | 6.7     | 75,000                     | 78,014                     | Ala-Pro-Pro-Lys-Ser |
| FeOTf/2N| Trypsin   | 6.0     | 37,000                     | 38,532                     | Ala-Pro-Pro-Lys-Ser |
| FeOTf/2C| Trypsin   | 5.4     | 36,000                     | 39,482                     | Glu-Asn-Arg-Ile-Gln |

*The molecular weight was estimated by SDS-PAGE.

*The molecular weight was calculated from the amino acid sequence of OTf (48). Addition of carbohydrate (49) to OTf and OTf/2C increases the calculated molecular weight to 79,882 and 41,550, respectively.

*Sequences are from Ref. 27.

---

**FIG. 1.** Immunodiffusion pattern of Fe₂OTf and trypsin-derived FeOTf/2N and FeOTf/2C half-molecules. The center well contains anti-Fe₂OTf IgG. The outer wells contain the following: 1, Fe₂OTf; 2, FeOTf/2N + FeOTf/2C; 3, FeOTf/2N; 4, FeOTf/2C; 5, Fe₂OTf; and 6, FeOTf/2N + FeOTf/2C.

**FIG. 2.** Sodium dodecyl sulfate-polyacrylamide gradient gel (4–12% acrylamide) of OTf and the half-molecules. Lanes 1 and 8 contain low molecular weight standards (Bio-Rad). The molecular weights \( \times 10^{-5} \) are indicated next to each standard in Lane 8. Lanes 2 and 3 contain trypsin-derived FeOTf/2N with and without dithiothreitol, respectively; Lanes 4 and 5 contain trypsin-derived FeOTf/2C with and without dithiothreitol; and Lanes 6 and 7 contain Fe₂OTf with and without dithiothreitol. The samples contain about 25 \( \mu \)g of protein each.

**FIG. 3.** The electrophoresis of OTf and its half-molecules in a 6 M urea, Tris, borate, EDTA, pH 8.4, system. Samples are as follows: Lane 1, Apo-OTf; Lane 2, Fe₂OTf; Lane 3, Apo-OTf/2N; Lane 4, FeOTf/2N; Lane 5, Apo-OTf/2C; Lane 6, FeOTf/2C; Lane 7, Apo-OTf + Apo-OTf/2N + Apo-OTf/2C; and Lane 8, Fe₂OTf + FeOTf/2N + FeOTf/2C. The samples contain about 12 \( \mu \)g of protein each.
is about 20% of theoretical. The purified iron-containing half-molecules characteristically have $A_{280}/A_{465}$ ratios of about 20, the same ratio found for FeOTf.

**Binding and Iron Uptake of $^{59}$Fe$^{125}$I-labeled OTf and Half-molecules by CERBCs**—A typical binding profile and iron uptake is presented in Fig. 4, A and B. It is clear that the trypsin-derived half-molecules alone show little binding or ability to donate iron to CERBCs. Combining them, however, leads to binding and iron uptake approaching that of holotf. Addition of the unlabeled complementary half-molecule, whether from the start of the experiment or at 30 min, leads to rapid binding of the labeled species. This type of experiment has been repeated a number of times with different batches of cells and different preparations of radiolabeled half-molecules. Table II presents results from four experiments in which the Fe atoms/cell and sites/cell found at 60 min for the various combinations of half-molecules are expressed as the per cent of Fe atoms/cell and sites/cell found for $^{59}$Fe$^{125}$I-OTf.

**Of possible significance is the observation that the Fe atoms/cell donated by $^{59}$Fe$^{125}$I-OTf/2N to which FeOTf/2C was added after 30 min of incubation (Fig. 4B, sample 4) reaches only about half of the Fe atoms/cell after 60 additional min of incubation as are found for the same combination of samples incubated for 60 min from the beginning of the experiment (Fig. 4B, sample 3). In contrast, there is no significant difference between the Fe atoms/cell found after a 60-min incubation for $^{59}$Fe$^{125}$I-OTf/2C whether the FeOTf/2N is present at the beginning of the experiment or is added after 30 min (Fig. 4B, samples 5 and 6). The experiment shown was done in EGA. In two subsequent experiments using the EGAB-CO$_2$ buffering system, the Fe atoms/cell after 60 min of incubation, whether from the start of the experiment or after the initial 30-min period, were identical for a given sample.

**Competition of the Combined Half-molecules were Equimolar FeOTf and Apo-OTf**—Table III presents the results of an experiment involving competition of the various combinations of half-molecules with equimolar amounts of either unlabeled FeOTf or apo-OTf. FeOTf at 10 $\mu$M concentration reduced the Fe atoms/cell to about 15% of the control values, a slightly greater effect than was observed on the binding. Apo-OTf had little effect on Fe uptake from the combined half-molecules.
Equilibrium Binding of OTf and the Half-molecules—Initial experiments with \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\) under aerobic conditions in EGA or EGAB showed that all of the radiolabeled iron was removed from the protein at concentrations below about 2 \(\mu\)M during the 30-min incubation period. Since apo-OTf exhibits significantly different binding than \(\text{Fe} \text{OTf}\), the results are meaningless (see "Discussion"). Subsequently, 0.75 \(\mu\)M DNP was used to inhibit the removal of iron. It was found that a 30-min preincubation of cells with DNP was necessary to prevent iron uptake completely, as indicated by obtaining a ratio of 2 Fe atoms/site for \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\). The presence of glucose in the incubating medium did not overcome the effect of DNP. It must be noted that a significantly lower amount of binding was found with DNP-treated cells. In a dozen different uptake experiments in which cells were treated with DNP or not and incubated with 10 \(\mu\)M \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\) for 30 min at 37 \(^\circ\)C, there were an average of 24.1 \(\pm\) 5.8% fewer sites/cell for the DNP-treated cells than for the untreated cells.

A potential source of error, particularly in dealing with the half-molecules and DNP-treated cells, is the presence of endogenous OTf in the system. It was found that with \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\)-loaded, DNP-treated cells that simple incubation at 37 \(^\circ\)C in EGA did not cause much OTf to be removed. Removal of labeled OTf was achieved after a chase with unlabelled \(\text{Fe} \text{OTf}\), indicating that binding is reversible. We therefore attempted to strip the cells prior to treatment with DNP. Although it has been reported (18, 28) that a 15-min incubation at 37 \(^\circ\)C in EGA removes endogenous OTf, our results in which cells were loaded with labeled \(\text{Fe} \text{OTf}\) and then incubated at 37 \(^\circ\)C for different periods of time indicate that about 40% of the OTf remains associated with the cells after a 15-min incubation. In contrast, incubation in EGA for 15 min removes all but 13% of the original OTf. If the cells are then reincubated with labeled \(\text{Fe} \text{OTf}\), the sites/cell found in the original "uptake" are found.

In view of these considerations, equilibrium binding studies were conducted using stripped cells treated with 0.75 mM DNP in the presence of 12 concentrations of sample ranging from about 0.06 to 60 \(\mu\)M. The samples included the following: 1) \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\); 2) \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{N}\) alone; 3) in the presence of equimolar \(\text{Fe} \text{OTf}/2\text{C}\); 4) in the presence of excess (about 12 \(\mu\)M) \(\text{Fe} \text{OTf}/2\text{C}\) across the range of concentrations; 5) \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{C}\) alone; 6) in the presence of equimolar \(\text{Fe} \text{OTf}/2\text{N}\); and 7) in the presence of excess \(\text{Fe} \text{OTf}/2\text{N}\).

Fig. 5A shows the amount of protein bound or sites/cell plotted against concentration for free ligand for labeled \(\text{Fe} \text{OTf}\) and the three samples containing labeled \(\text{Fe} \text{OTf}/2\text{N}\). Labeled \(\text{Fe} \text{OTf}/2\text{C}\) alone and in the presence of unlabeled \(\text{Fe} \text{OTf}/2\text{N}\) gives very similar looking curves. Fig. 5B depicts the sites/cell plotted against the log of the free concentration for labeled \(\text{Fe} \text{OTf}\) and the various combined half-molecules. A plot of this type for the half-molecules alone predictably yields a long flat curve which bends upward at the higher concentrations (not shown).

Fig. 6, A and B shows the results of plotting the data, uncorrected for nonspecific binding, according to the method of Scatchard (29) for the two sets of half-molecules. Fig. 7 presents the Scatchard plots for \(\text{Fe} \text{OTf}\) and for the half-molecules per cell against the \(\mu\)M concentration of free ligand (\(F\)), \(A\), rectilinear plot; \(B\), semilogarithmic plot. Samples include: 1, \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}\); 2, \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{N}\) (0); 3, \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{N}\) in the presence of equimolar \(\text{Fe} \text{OTf}/2\text{C}\) (■); and 4, \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{N}\) in the presence of 12 \(\mu\)M \(\text{Fe} \text{OTf}/2\text{C}\) at each of the concentrations (○). In addition, in B are shown \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{C}\) in the presence of equimolar \(\text{Fe} \text{OTf}/2\text{N}\) (A) and \(^{59}\text{Fe}^{125}\text{I}-\text{OTf}/2\text{N}\) in the presence of 12 \(\mu\)M \(\text{Fe} \text{OTf}/2\text{N}\) at each of the concentrations (L). The amount of bound ligand is corrected for nonspecific binding. The curves are fitted by eye. Note that the curves are sigmoidal in shape.
divided by the free concentration of ligand
cell
units.

specific binding. The presence of equimolar FeOTfIPC
specific binding ranges between 2.5 and 51.3% for Fe,OTf, be-
tween 2.8 and 48.1% for FeOTfl2N in the presence of excess
FeOTfl2C, and 4.9 and 55.6% for FeOTfl2C in the presence of excess FeOTfl2N.

From the intercepts, the number of binding sites/cell was found to be 4.79 \times 10^4 for FeOTf/2C, 4.44 \times 10^4 for FeOTf/2N in the presence of excess FeOTf/2C, and between 4.9 and 55.6% for FeOTf/2C in the presence of excess FeOTf/2N.

The studies described demonstrate for the first time binding and iron donation in a "reconstituted" system which approach those found for diferric transferrin. It is clear from our results that both half-molecules are necessary to effect specific binding to the receptor and the donation of iron. Both uptake and equilibrium binding experiments indicate that the nature of the binding of the half-molecules alone is nonspecific or nonsaturable. This is further confirmed by the apparent inability of the half-molecules alone to donate iron to CERBCs. Whether the combined complementary half-molecules associate prior to binding to the receptor or whether the binding takes place at the receptor site is an important question which we are currently investigating. In either case, the association appears to be very rapid reaching half-maximal within a minute after addition of the unlabeled complementary half-molecule (see Fig. 4A, samples 4 and 6).

Some interesting differences (Table II) in binding and iron donation are observed depending on which half-molecule bears the radiolabel. Although relatively more of the NH2-terminal half-molecule appears to bind, iron donation by the COOH-terminal half-molecule is almost twice that of the NH2-terminal half-molecule. The reasons for these differences, particularly in the ability of the half-molecules to donate iron, are not understood. It may be that production of the half-molecule in some way affects the way in which iron is held in the NH2-terminal half-molecule which in turn interferes with iron donation. There are a number of indications that the iron in the NH2-terminal half-molecule is more labile than that in the COOH-terminal half-molecule. Thus, we have observed that iron is more readily removed from FeOTf/2N than FeOTf/2C by dialysis against phosphate buffer containing NTA and EDTA. Also, in the EGA medium, the level of iron donation falls in the sample incubated for a longer period of time (Fig. 4B, sample 4) although this effect is not observed.

using it to correct the experimental points for nonsaturable
binding. At concentrations between 1 and 60 \mu M, the nonspecific binding ranges between 2.5 and 51.3% for FeOTf/2N, between 2.8 and 48.1% for FeOTf/2N in the presence of excess FeOTf/2C, and between 4.9 and 55.6% for FeOTf/2C in the presence of excess FeOTf/2N.

From the intercepts, the number of binding sites/cell was found to be 4.79 \times 10^4 for FeOTf/2C, 4.44 \times 10^4 for FeOTf/2N in the presence of excess FeOTf/2C, and between 4.9 and 55.6% for FeOTf/2C in the presence of excess FeOTf/2N.

Extraction of 59Fe into Heme—By following the protocol of Teale (30) as described by Keung and Azari (18), the amount of 59Fe incorporated into heme was determined after a 30-min incubation with 10 \mu M of the various labeled samples. In the heme containing 2-butaneone extract, 80.7, 97.9, 96.0, and 88.8% of the total cell-associated 59Fe cpm were found for 59Fe125I-OTf, 59Fe125I-OTf/2N + 59Fe125I-OTf/2C, 59Fe125I-
when EGAB is used. A speculative explanation for the results is that, although iron from both half-molecules can be removed, the removal may be ordered, i.e., iron may be taken from the COOH-terminal half-molecule first. Preliminary experiments (not shown) in which apo-half-molecule is substituted for the iron-containing species point in this direction.

Competition experiments demonstrate that the combined half-molecules are about half as effective as $^{55}$Fe$_2$OTf in competing with equimolar unlabeled Fe$_2$OTf. This is a significant finding when compared to the results of Keung and Azari (18) in which equimolar Fe$_2$OTf reduced binding of a half-molecule to 5% of the control. In fact, virtually all of our findings are in conflict with those reported by these authors. In contrasting our results with theirs, we find numerous differences including methods used to prepare the proteins, radiolabeling procedures, experimental procedures, and presentation of results. First, and probably most significant, is their use of subtilisin-derived half-molecules. Furthermore, in presenting their work, they do not show uptake studies in which both binding and iron donation in the same cells were examined. It is interesting to note that significant iron uptake from the half-molecules is found in their experiments at concentrations of protein which are higher than the maximum shown in their binding studies. Iron incorporation from half-molecules into heme does not level off even at a concentration of 37.5 $\mu$M which is 15 times higher than the highest concentration shown for binding. In view of these results, there is some question as to whether iron from the half-molecules might be transported into the cells via residual endogenous OTf. Our experiments show that with the procedure followed by Keung and Azari to eliminate endogenous OTf there may still be a considerable amount remaining. In our own experiments, we feel that the failure of the half-molecules alone to transport iron serves as a control for the combined half-molecule results. We are quite confident that the combined half-molecules are not operating by interacting with OTf endogenously present in the system. Furthermore, in our experiments, similar amounts of radiolabeled iron can be extracted into the putative heme fraction from Fe$_2$OTf and the various combined half-molecules. Therefore, the iron taken up by the cells under these circumstances is specifically available for heme synthesis.

Our first goal in setting up equilibrium binding studies was to try to establish conditions under which the system is at equilibrium. Most past studies have been satisfied with what is described as a "steady state." Recent articles (31, 32) have pointed out that data from such studies are inherently incorrect. Most equilibrium studies, in addition, fail to include a large enough range of concentrations of free ligand to render the resulting plot meaningful (31). Two additional points deserve comment. It is now well established that apotransferrin and diferric transferrin have significantly different binding properties (33-38). Furthermore, there is mounting evidence that, once bound to the receptor, diferric transferrin is internalized by endocytosis, iron is removed in "endosomes," and apotransferrin still associated with the receptor is returned to the surface (39-44). Although there is not complete agreement that internalization takes place (26, 38, 45), if it does, the situation may be too complex to be described by Scatchard analysis. Regardless of the details of the process, the use of DNP in our equilibrium binding studies prevents the removal of iron (and probably endocytosis (46)). Under these conditions, a Scatchard plot is appropriate because binding is reversible and equilibrium can be attained. Although DNP reduces apparent binding by 25% and may have other unknown effects, we believe that relative numbers for OTf versus the half-molecules are valid. The Scatchard analysis is important in yielding estimates of nonspecific binding which is approximately the same for OTf and the combined half-molecules. If the combined half-molecules had significantly higher nonspecific binding than OTf, there would be concern about the data obtained in the uptake experiments. The apparent binding constants derived from the Scatchard plots are of interest. Again, although the absolute numbers may not be correct, the relative numbers are probably valid.

Fig. 5B, in which the amount of ligand bound is plotted against the log of the concentration of free ligand, is included to show that roughly sigmoidal curves, which pass well through an inflection point at approximately half the number of sites found at saturation, are obtained. This is the expectation for a set of $n$ identical binding sites (31). The corrected data, when plotted according to the method of Scatchard (29) for radiolabeled FeOTf/2N in the presence of excess unlabeled FeOTf/2C or labeled FeOTf/2C in the presence of excess unlabeled FeOTf/2N, yield straight lines (Fig. 7). The number of binding sites are 92.7 and 87.0% of the number of sites/cell found for Fe$_2$OTf. The lower slopes for the combined half-molecules reveal somewhat lower binding constants consistent with the competition experiments. Furthermore, the presence of excess unlabeled complementary half-molecules appears to facilitate increased binding of the labeled species. The shape of the curves found in the case of equimolar amounts of the two half-molecules is suggestive of positive binding cooperative (47). It is not until concentrations of about 3 $\mu$M that the curve meets that found for the labeled half-molecule in the presence of excess unlabeled species. The molecular basis for this apparent cooperativity is presently under investigation.

In conclusion, we feel that further studies with the half-molecules may yield important insights into the interaction of transferrin with its receptor. By differential labeling of the two half-molecules with for example $^{55}$Fe and $^{57}$Fe more information could be obtained as to the flow of iron into the cell. Furthermore, preliminary experiments with apo-half-molecules have yielded interesting results in regard to both iron uptake and effects on binding. The interacting complementary half-molecule provide a unique opportunity to dissect the effects of each iron-binding domain on the other.

Acknowledgments—We are indebted to Joanne Olszewski for providing excellent technical assistance and to Robert Shaw for his typing and editing skills.

REFERENCES

1. Aisen, P., and Listowsky, I. (1980) Annu. Rev. Biochem. 49, 357-390
2. Woodworth, R. C., Virkaitis, L. M., Woodbury, R. G., and Fava, R. A. (1975) in Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R., ed) pp. 39-50, North-Holland, Amsterdam
3. Neuwald, R., Schneider, C., Sutherland, R., Vodineich, I., and Greaves, M. (1982) Trends Biochem. Sci. 7, 397-400
4. Delaney, T. A., Morgan, W. H., and Morgan, E. H. (1982) Biochim. Biophys. Acta 701, 295-304
5. Harris, D. C., and Aisen, P. (1975) Nature (Lond.) 257, 821-823
6. Williams, S. C., and Woodworth, R. C. (1973) J. Biol. Chem. 248, 5548-5553
7. Morgan, E. H. (1974) in Iron in Biochemistry and Medicine (Jacobs, A., and Worwood, M., eds) pp. 29-71, Academic Press, New York
8. Chasteen, N. D. (1977) Coord. Chem. Rev. 22, 1-36
9. Harris, D. C. (1977) Biochemistry 16, 560-564
10. Aisen, P., Leibman, A., and Zweier, J. (1978) J. Biol. Chem. 253, 1930-1937
11. Huebers, M., Huebers, E., Linck, S., and Rummel, W. (1977) in Proteins of Iron Metabolism (Brown E. B., Aisen, P., Fielding, ...
Physiological levels of binding and iron donation by complementary half-molecules of ovotransferrin to transferrin receptors of chick reticulocytes.
A Brown-Mason and R C Woodworth

J. Biol. Chem. 1984, 259:1866-1873.

Access the most updated version of this article at http://www.jbc.org/content/259/3/1866

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/3/1866.full.html#ref-list-1