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Reversible association of half-molecules of ovotransferrin in solution

Basis of co-operative binding to reticulocytes

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In the present paper, gel-filtration studies of diferric-ovotransferrin (Fe_2OTf), the individual half-molecules of ovotransferrin (OTf) and equimolar mixtures of half-molecules have been interpreted according to the Gilbert theory as developed by Ackers & Thompson [(1965) Proc. Natl. Acad. Sci. U.S.A. **53**, 342–349]. The data indicate that the half-molecules associate reversibly in solution and allow determination of a dissociation constant, $K'_d = 8.0(\pm 2.7)$ μM. Equilibrium binding studies have been performed using NH_4Cl to block removal of iron from equimolar differentially iodine-labelled half-molecules (^{125}I and ^{131}I), in order to evaluate the binding of each to chick-embryo red blood cells under identical conditions. The amount of associated half-molecules over a range of concentrations has been calculated using the constant derived from the gel-filtration experiments described above. A computerized non-linear least-squares regression analysis of the data leads to determination of K^*_d (the apparent dissociation constant for the interaction between OTf or half-molecules and the transferrin (Tf) receptors of chick-embryo red blood cells) and $B_{max.}$ (binding at infinite free-ligand concentration) for the half-molecules similar to those found for Fe_2OTf. Recent reports confirm that the two iron-binding domains of both OTf and human lactotransferrin associate non-covalently in solution. Our work shows that the isolated half-molecules of OTf are able to reassociate in solution and that this reassociation has *functional* significance by allowing the complex to be recognized by the Tf receptor.

INTRODUCTION

The transferrins (siderophilins) comprise a set of homologous 80 kDa glycoproteins that reversibly bind up to two ferric ions per protein molecule. Physiologically the transferrins derived from blood plasma are able to deliver their two bound ferric ions at equal rates to cultured cells from the isologous species (Williams & Woodworth, 1973; Harris & Aisen, 1975; Huebers *et al.*, 1981; Delaney *et al.*, 1982; Young, 1982). Such iron delivery is mediated by specific transferrin receptors found in the plasma membranes of target cells.

In order to segregate the two iron-binding domains of ovotransferrin (OTf) for study by high-resolution n.m.r. spectroscopy, we prepared 'half-molecules' comprising either the *N*- or *C*-terminal domain, by tryptic cleavage of the appropriate monoferric OTf species. In experiments designed to assess the physiological activity of these half-molecules, we found that neither half-molecule on its own was able to bind to or to donate iron to chick-embryo red blood cells (CERBC) (Brown-Mason & Woodworth, 1984). On addition of the complementary half-molecule, however, rapid binding of both species occurred and iron donation at rates approaching those found for intact Fe_2OTf ensued. Equilibrium binding studies of equimolar mixtures of *N*- and *C*-terminal half-molecules of OTf yielded convex Scatchard plots suggestive of positive co-operativity in binding. The present report seeks to rationalize this apparent positive co-operativity in terms of a reversible association of the two species of half-molecule in solution before their binding to the cellular transferrin receptor.

EXPERIMENTAL

Protein preparation

Ovotransferrin was prepared by modification of our previous method (Williams & Woodworth, 1973). Briefly, 120 fresh egg whites were gently blended with an equal volume of glass-distilled water. The resulting solution was dialysed for 24 h against cold running water and then against 5 mM-sodium phosphate buffer, pH 6.6. The sample was loaded on to either a CM-Sephadex or a CM-Sepharose (Pharmacia) column, pre-equilibrated in the same buffer. The column was developed stepwise with 5 mM buffer until all of the ovalbumin had been eluted, followed with 10 mM-sodium phosphate, pH 6.6, and finally with 50 mM-sodium phosphate buffer, pH 6.8, to effect elution of the OTf. The supernatant from a 50%-(w/v)-satd.-$(NH)_4SO_4$ precipitation of the eluted OTf was dialysed exhaustively against glass-distilled water. Final purification was achieved on an LKB electrofocusing column using 0.4% pH 4–9 ampholytes (Serva; Servalyt 4–9T). The *N*-terminal half-molecule, designated 'FeOTf/2N', was prepared as previously described (Brown-Mason & Woodworth, 1984). The *C*-terminal half-molecule, designated 'FeOTf/2C', was

Abbreviations used: Tf, transferrin; OTf, ovotransferrin; Fe_2OTf, iron-saturated OTf; FeOTf/2N (or N) and FeOTf/2C (or C) designate the iron-binding domains or 'half molecules' from the *N*-terminal and *C*-terminal halves of OTf; CERBC, chick-embryo red blood cells; EGAB refers to Earle's salts containing 6 mM-glucose, 2.5% (w/v) bovine serum albumin and 0.22% (w/v) $NaHCO_3$.

prepared from Fe_2OTf by a modification of our previously used protocol. Fe_2OTf at a concentration of approx. 6 mg/ml was diluted with an equal volume of 1.0 M-sodium acetate, pH 5.5, in a beaker with stirring at room temperature. The A_{280} and A_{465} of the sample were monitored at 5 min intervals until the A_{280}/A_{465} ratio changed from 20 to ~ 35 (20–30 min). At this time, a 3–5-fold excess of Desferal (desferrioxamine mesylate; Ciba) was added; the sample was made 20 mM with respect to $NaHCO_3$ and the pH was raised to 7.1 with 1 M-NaOH. The sample was placed into a 400 ml stirred cell (Amicon) fitted with a PM-10 membrane and exchanged into water by multiple concentrations and dilutions. The monoferric species isolated from an electrofocusing column was digested with Affigel–trypsin, and FeOTf/2C was further purified by electrofocusing with gel filtration on Sephadex G-75 as described previously (Brown-Mason & Woodworth, 1984).

Determination of absorption coefficient

Samples of approx. 10 mg of apo- and iron-saturated holo-OTf and half-molecules judged pure by urea/polyacrylamide- and SDS/polyacrylamide-gel electrophoresis, were freeze-dried, taken up in 0.5 ml of glass-distilled water and run over columns containing 6 ml of desalting gel (P6DG; Bio-Rad). Before addition of the samples, the columns were washed with 20 ml of 0.1 mM-Desferal in 0.1 M-sodium acetate, pH 5.2, containing 2 mM-EDTA. Each column was then washed with 50 ml of glass-distilled water, the sample was loaded, and 2 ml aliquots were collected in iron-free test tubes. The second 2 ml aliquot, which contained the sample, was freeze-dried and taken up in 1.0 ml of glass-distilled water. The A_{280} of triplicate 50 μl aliquots in 1.0 ml was read in a Cary 219 spectrophotometer. Triplicate 50 μl aliquots of the indiluted samples were placed on tared 25 mm Cahn aluminium pans, and dried to constant weight using a hot-plate covered with aluminium foil and set at 60 °C. Aliquots of the undiluted samples were subjected to urea/polyacrylamide-gel electrophoresis to check that no change in the iron status had occurred.

Radiolabelling procedures

Iodination by the McFarlane method with ^{125}ICl or ^{131}ICl was performed on iron-saturated OTf or half-molecules, the bound iron having been labelled with ^{59}Fe when appropriate; protocols for these procedures have previously been described (Brown-Mason & Woodworth, 1984). The specific radioactivity, expressed as c.p.m./nmol, was determined just before each experiment. For this purpose, 5 μl triplicates of each sample were removed with a 10 μl Hamilton syringe, placed into vials and diluted to 1 ml with normal saline solution (0.15 M-NaCl containing 20 mM-$NaHCO_3$ and 1 mM-$MgSO_4$). The radioactivity and the A_{280} were determined on the same aliquots. In the experiments in which both isotopes of iodine were present the radioactivity in the ^{125}I channel was corrected for 15.5% spill from the ^{131}I channel. Millimolar absorption coefficients for apo- and iron-OTf and half-molecules are provided in Table 1.

CERBC

Red blood cells were obtained from White-Leghorn-chick embryos after 14–15 days of incubation at 37 °C and 80% relative humidity. Incubations of cells were carried out in Earle's salts (Gibco), supplemented with 2.5 mg of bovine serum albumin (Sigma, Fraction V)/ml.

Table 1. Absorption coefficients for OTf and the half-molecules determined as described in the Experimental section

Data were calculated by using M_r values of 79882, 38532 and 41350 for OTf, OTf/2N and OTf/2C respectively, calculated from the sequence (Jeltsch & Chambon, 1982; Williams *et al.*, 1982). M_r values of 81216, 37127 and 40592 were determined by sedimentation equilibrium at pH 7.4 in 20 mM-Tri/HCl/150 mM-NaCl, a partial-specific-volume value of 0.720 cm^3/g being used.

Sample	$A^{1\%}_{1\,cm,\,280}$	ϵ_{280} (mM·cm^{-1})
Apo-OTf	11.53	92.09
Fe_2OTf	13.95	111.44
Apo-OTf/2N	10.61	41.93
FeOTf/2N	12.73	50.31
Apo-OTf/2C	10.87	44.94
FeOTf/2C	13.74	56.81

Uptake experiments

Experiments aimed at assessing the binding and iron uptake of OTf and its half-molecules to CERBC was as described previously (Brown-Mason & Woodworth, 1984), except that serum bottles were substituted for polypropylene tubes. Furthermore, the washing procedure was changed as follows: three 50 μl aliquots of cell suspension were each pipetted into 1.0 ml of ice-cold EGAB layered over 300 μl of dibutyl phthalate (Sigma) in 1.5 ml conical tubes (Sarstedt), a modification of a procedure reported elsewhere (Klausner *et al.*, 1983*a*,*b*). The samples were spun for 1 min in a Beckman Microfuge B. The supernatant and most of the dibutyl phthalate were aspirated, and either the tube walls and tops were wiped with absorbent paper wadding (Egyed, 1984) or the bottom of the tube containing the pellet of cells was cut off into a polystyrene tube by using a heated wire. Data from kinetic studies of binding of OTf and the half-molecules by CERBC were analysed as previously described (Woodworth *et al.*, 1982; Brown-Mason & Woodworth, 1984).

Stripping procedure

To assess the amount of OTf on the surface of the cells as against that inside the cell, i.e. inaccessible to removal by acid, the following procedure was used: the total OTf, both inside the cell and on the cell surface, was determined by the dibutyl phthalate method described above. In addition, three 50 μl aliquots were each pipetted into 1 ml of ice-cold EGAB in 1.5 ml conical tubes and centrifuged briefly. After removal of the supernatant by suction, two additional 1 ml washes were performed. The pellet of cells was then treated with 1 ml of 0.25 M-acetic acid/0.25 M-NaCl (VanRenswoude *et al.*, 1982), gently mixed, centrifuged, and the supernatant was aspirated. The cell pellet was then assayed for radiolabel. The difference between the total radioactivity and that still cell-associated was assumed to be the amount of OTf on the surface of the cells.

Gel-filtration analysis of half-molecule association

A jacketted column of Sephadex G-75 (1 cm × 25 cm) in glucose-free Earle's salts (0.9 mM-$CaCl_2$/5.4 mM-KCl/1.1 mM-$MgSO_4$/116.3 mM-NaCl/10 mM-KH_2PO_4, pH 7.4) with or without 2.5% bovine serum albumin, was maintained at 37 °C with a Haake circulating-water bath. Buffers and sample were infused into the column at a constant rate of 0.20 (±0.01) ml/min by a Pharmacia P-500 precision pump. The A_{280} of the effluent was continuously monitored in a 1.0 cm flow cell (200 μl hold-up) by a Cary 219 spectrophotometer equipped with a digital interface port (Cary). A DEC MINC 11/23 (Digital Equipment Corp.) computer configured with digital input and output modular interfaces read the absorbance data once per s from the digital interface port, using the MINC system line-frequency clock. Samples were run in sequential sets consisting of Fe_2OTf, FeOTf/2N, FeOTf/2C or a mixture of the last two. For a given set the concentration (between 3 and 8 μM) of each sample was the same. In some cases various concentrations of the combined samples were run. Sample application comprised continuous infusion from a Super-Loop (Pharmacia) from zero time until the A_{280} reached a stable plateau. The column was then washed with buffer until the A_{280} returned to the baseline. The sigmoid chromatographic front was smoothed by using three passes of a weighted smoothing filter algorithm (Savitsky & Golay, 1984), derivatized by a cubic spline routine, and interpreted in terms of the Gilbert theory for a rapidly equilibrating system of associating macromolecules (Ackers & Thompson, 1965; Cann & Goad, 1970). The maximum of the first-derivative curve was taken as the centroid of the elution front, and was used to calibrate the column for the reversible dissociation of the half-molecules, N-C $\rightleftharpoons$ N + C, where N is FeOTf/2N and C is FeOTf/2C. The apparent dissociation constant (K'_d) was calculated from the relationship:

$$K'_d = \frac{\alpha^n [\text{N-C}]^{(n-1)}}{1-\alpha}$$

where α was calculated from the ratio of the difference between elution times for Fe_2OTf and *N* plus C divided by the difference between elution times for Fe_2OTf and the average of the elution times for N or C alone, [N-C] is the total molar concentration of N-C at zero dissociation and *n* is the number of associating subunits (two in this case). Fe_2OTf represented zero dissociation and either N or C alone represented infinite dissociation.

Equilibrium binding

Washed cells were incubated in EGAB at 37 °C twice for 15 min. After centrifugation and dilution 1:1 with EGAB, the cells were made 20 mM with respect to NH_4Cl to inhibit iron removal, incubated for an additional 10 min, and pipetted into tubes containing the radiolabelled samples. In some experiments the binding levels of the two half-molecules were evaluated simultaneously by labelling one half-molecule with ^{125}I and the other half-molecule with ^{131}I. Appropriate windows were selected in a Packard Auto-Gamma 500C scintillation counter to discriminate between these two isotopes. Three 50 μl aliquots were washed by the one-step procedure described above. Other details of the equilibrium binding experiments follow protocols previously described, including the correction for non-specific binding (Brown-Mason & Woodworth, 1984).

The observed amount of OTf or half-molecules bound per cell, corrected for non-specific binding, was defined to be the dependent variable (*B*) of the free concentration ([F]) in the following equation:

$$B = [\text{F}] \cdot B_{\text{max.}} / K^*_d + [\text{F}])$$

where $B_{max.}$ is the binding at infinite free ligand concentration [F] and K^*_d is the apparent dissociation constant for the interaction between OTf or half-molecules and the TF receptors of CERBC. The free concentration of the associated equimolar half-molecules was calculated from the measured free concentration of each half-molecule and the dissociation constant (K'_d) derived from the application of the Gilbert theory. This approach is justified if only the N-C dimers are recognized by the receptor. Values for $B_{max.}$ and K^*_d were determined from the equation above by using a derivative-free non-linear regression routine (Ralston, 1981).

RESULTS

Coefficients

The millimolar absorption coefficients for OTf and the half-molecule in the absence and presence of iron are reported in Table 1. The ratio of ϵ_{280} (apo)/ϵ_{280} (ferric) for holo-OTf is 82.6%, for OTf/2N is 83.3% and for OTf/2C is 79.1%.

Comparison between EGAB and dibutyl phthalate wash procedures

Binding studies were conducted at 37 °C and at 4 °C with samples taken at 0, 3, 6, 9, 12, 15, 20 and 30 min and washed by two different methods. At 37 °C the number of binding sites/cell for multiple EGAB washes was 95% of the number found with the dibutyl phthalate washing technique. At 4 °C, multiple EGAB washes resulted in only 60% of the number of binding sites/cell found with the dibutyl phthalate wash. The latter procedure was adopted for all subsequent studies. In these same experiments, after incubation for 30 min at 4 °C, 14% of the OTf was inaccessible to removal by acid; at 37 °C, 85% of the OTf was inaccessible to removal by acid.

Effect of NH_4Cl on OTf uptake

Although 20 mM-NH_4Cl appeared to inhibit completely removal of iron from Fe_2OTf and the ferric half-molecules, it had no effect on the number of binding sites/cell in CERBC over a 30 min time course. Thus, in six different experiments, the numbers of binding sites/cell in the presence of NH_4Cl were 99.3 (±3.6)% of that in the absence of NH_4Cl. Similar results were found with the half-molecules. In an uptake study with $Fe^{131}I$-OTF/2N and $Fe^{125}I$-OTF/2C the number of binding sites/cell was within 5% in the presence or absence of NH_4Cl, indicating that the presence of 20 mM-NH_4Cl does not appear to have a significant effect on half-molecule association and subsequent binding. The binding profile and iron uptake in the absence of NH_4Cl, in the presence of NH_4Cl added to the cells at zero time and added to the cells 20 min before addition of the radiolabelled OTf are presented in Figs. 1(*a*) and 1(*b*). Samples taken at 30 min and treated with acetic acid

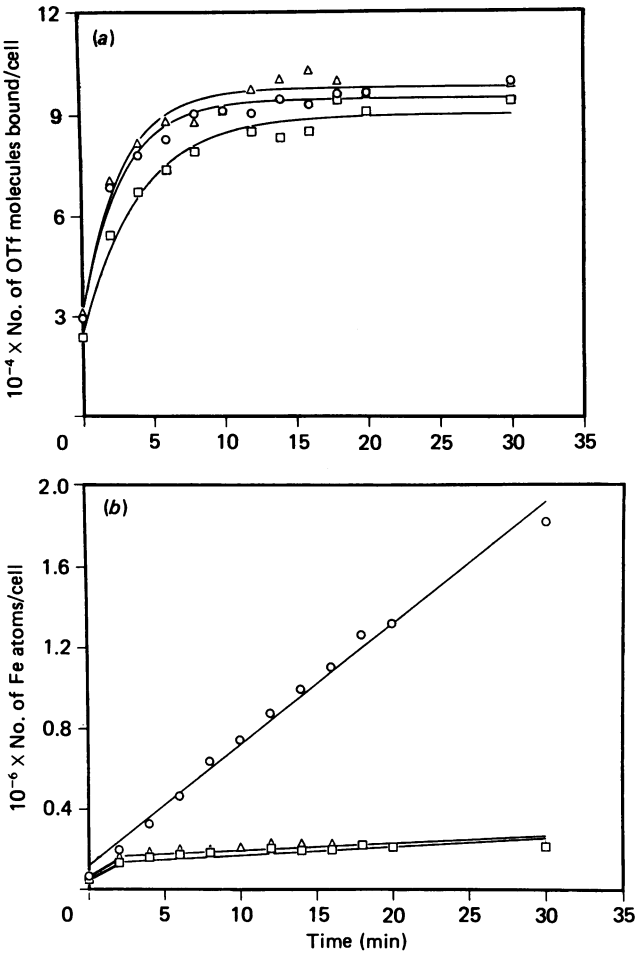

Fig. 1. Progress curves for the binding of OTf to CERBC in the absence and presence of 20 mM-NH_4Cl (*a*) and iron donation to them (*b*)

$^{59}Fe_2{}^{125}I$-OTf at 5.5 μM was added to each sample at zero time. Samples and first-order rate constants (±S.D.) are as follows: 1, control (○), no NH_4Cl, 0.35 (±0.04) min^{-1}; 2, 20 mM-NH_4Cl added at time zero (△), 0.36 (±0.05) min^{-1}; 3, 20 mM-NH_4Cl added to the cells 20 min before addition of labelled OTf (□), 0.25 (±0.03) min^{-1}.

show that the distribution of OTf was identical in the presence or absence of NH_4Cl; i.e., at 37 °C, approx. 85% of the total OTf bound is internalized. The major effect of NH_4Cl appears to be that it slows down the return of Fe_2OTf to the external milieu. Thus, after uptake of radiolabelled OTf by CERBC followed by a chase with unlabelled OTf, the $t_{\frac{1}{2}}$ for disappearance of radiolabelled OTf from control cells is 2.7 min, whereas that for disappearance from NH_4Cl-treated cells is 10 min.

Determination of K_d by gel filtration

Results for a set of gel-filtration experiments designed to assess the equilibrium constant for the system N-C⇌N+C, at 37 °C and at pH 7.4, are shown in Fig. 2. In this typical data set, the times of inflection are noted in the legend to the Figure. K'_d values were calculated for FeOTf/2N+FeOTf/2C from five full sets. In three sets, a single concentration of FeOTf/2N+FeOTf/2C was compared with Fe_2OTf and the half-molecules alone.

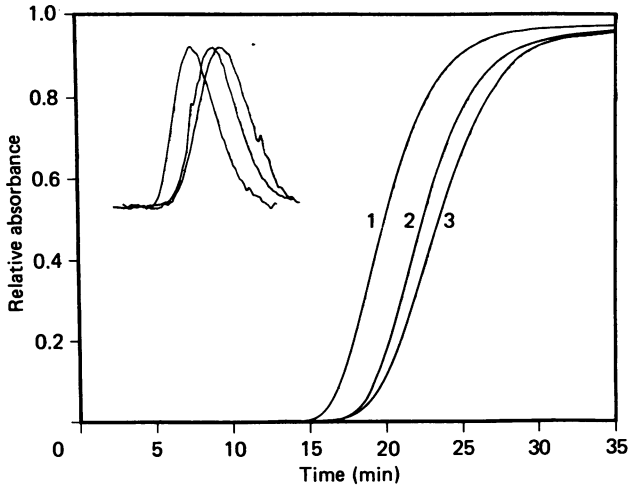

Fig. 2. Gel-filtration elution profiles of Fe_2OTf, individual half-molecules and the combined half-molecules and their first derivatives

The absorbance units shown are the measured absorbance as a fraction of the absorbance at the plateau. Only the leading edges of the profiles have been displayed. Samples include: 1, Fe_2OTf; 2, FeOTf/2N+FeOTf/2C; and 3, FeOTf/2N. The FeOTf/2C trace was very similar to trace 3. The first derivatives of these curves are shown in the inset. Data points were taken every second. Centroids are 19.1 min for curve 1, 21.6 min for curve 2 and 22.8 min for curve 3.

In two sets, concentrations of FeOTf/2N+FeOTf/2C ranging from 3 to 8 μM were assessed. We report the mean K'_d (±S.E.M.) as 8.0 (±2.7) μM, $n = 14$. This constant is relevant to the equilibrium binding experiments with CERBC reported here, and has not been evaluated at various temperatures, pH values or iron loadings. The presence of bovine serum albumin in the buffer did not change the results.

Equilibrium binding studies

Equilibrium binding studies were conducted with preincubated cells (cells incubated for 2 × 15 min, 37 °C) adjusted to 20 mM-NH_4Cl just before addition to 14 or 15 concentrations of sample, ranging from about 0.06 to 70 μM-OTf or half-molecules. The samples included the following: (1) $^{59}Fe_2{}^{125}I$-OTf; (2) $Fe^{125}I$-OTf/2N alone; (3) $Fe^{131}I$-OTf/2C alone; and (4) equimolar $Fe^{125}I$-OTf/2N+$Fe^{131}I$-OTf/2C. In a different experiment the FeOTf/2N was labelled with ^{131}I and the FeOTf/2C with ^{125}I. As previously shown, this range of concentrations is sufficiently large to produce a roughly sigmoid-shaped plot when the amount bound is plotted against the logarithm of the free concentration (Brown-Mason & Woodworth, 1984).

The results of one such experiment are presented in Fig. 3. Plots of the actual free concentration of each half-molecule (corrected for non-specific binding) and the free concentration calculated by using the K'_d from the gel-filtration studies described above against the amount bound are shown for concentrations below 10 μM. It is evident that the half-molecules alone show only non-specific binding. The half-molecules, when combined and the amount plotted against the free concentration uncorrected for association, show a

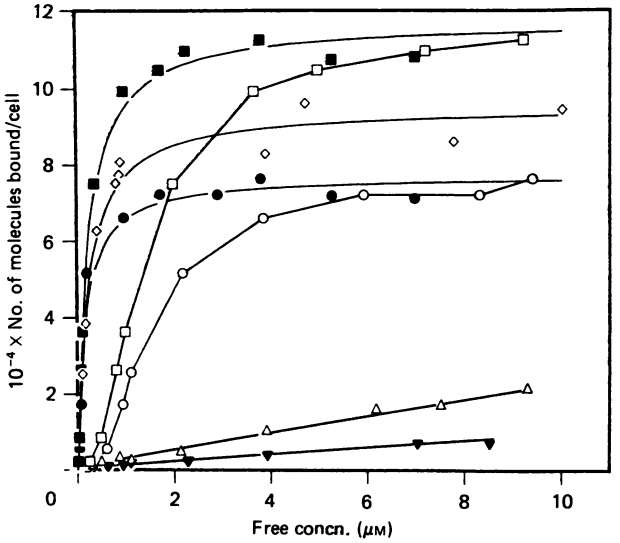

Fig. 3. Plots of the amount of bound ligand (OTf or half-molecules) per cell against the concentration of free ligand, both uncorrected and corrected for the association of the half-molecules

All samples were incubated at 37 °C for 30 min with CERBC in the presence of 20 mM-NH_4Cl. Samples include: (1) $^{59}Fe_2{}^{125}I$-OTf (◇); (2) equimolar $Fe^{131}I$-OTf/2N (□)+$Fe^{125}I$-OTf/2C (○) uncorrected for association; (3) the same samples, $Fe^{131}I$-OTf/2N (■) and $Fe^{125}I$-OTf/2C (●) corrected for association as described in the Results section; and (4) the half-molecules alone: $Fe^{131}I$-OTf/2N (△) and $Fe^{125}I$-OTf/2C (▼). In the case of (2), the data points are connected to form the roughly sigmoid-shaped curves. In the case of (1) and (3) the actual data points are shown with the computer-generated fits. The amount of bound ligand is corrected for non-specific binding, except in the case of (4).

sigmoid curve, as would be expected for an associating system showing positive co-operativity. Correction for the associated half-molecules leads to a hyperbolic curve similar to that seen for diferric OTf. The data from the equilibrium binding studies, corrected both for non-specific binding and for the association of the half-molecules, were used to calculate $B_{max.}$ and K_d^*. The results for two different sets of experiments are presented in Table 2. Note that specific activities for each sample are given in the legend to this Table.

A curious finding from the equilibrium binding data is the discrepancy in $B_{max.}$ for the two half-molecules. Thus $B_{max.}$ for the *C*-terminal half-molecule was 67.0% and 55.6% of the $B_{max.}$ for the *N*-terminal half-molecule in the two data sets (Table 2). Similar results were obtained from two other types of experiments. Binding studies at 37 °C with equimolar (~8 μM) amounts of the half-molecules labelled with the two iodine isotopes led to C/N ratios of 77.6, 70.9 and 77.0%. In other binding experiments in which ^{125}I-labelled half-molecules were incubated with unlabelled equimolar complementary half-molecule, the C/N sites/cell ratio was 79.9±3.9% ($n = 6$). In a third type of experiment, after incubation of the combined dual-labelled half-molecules for 30 min with CERBC, the cells were lysed and the OTf–Tf-receptor complex was solubilized with 0.5% Triton X-100. A portion (0.5 ml) of the supernatant from a 30 min ultracentrifugation (4 °C, 100000 *g*, Ti-70 rotor) containing solubilized receptor and associated half-molecules was run over a Sephacryl-300 column (1 cm × 26 cm), in 0.1 M-Tris/citrate (pH 5.0)/0.1% Triton X-100. Fractions (0.32 ml each) were collected and assayed for radioactivity. Under these conditions, 5.09 pmol of *C*-terminal half-molecule and 7.03 pmol of *N*-terminal half-molecule were found associated with receptor, giving a ratio of 72.4%.

Table 2. $B_{max.}$ and K_d^* calculated for OTf and the half-molecules from two different experiments

Numbers in parentheses are S.D. values. The $B_{max.}$ and K_d^* were derived by fitting the observed binding (*B*), corrected for non-specific binding and the measured free concentration ([F]) to the equation

$$B = [F] \cdot B_{max.}/K_d^* + [F]$$

using a non-linear least squares analysis and values of [F] below 10 μM. K_d^* is the apparent dissociation constant for the interaction of OTf or the combined half-molecules and the Tf receptors on CERBC. The free concentration of the *associated* half-molecules was calculated from the measured free concentration of each half-molecule and the dissociation constant (K_d') derived from the application of the Gilbert Theory.

Expt.	OTf	$10^{-4} \times B_{max.}$ (sites/cell)	K_d^* (μM)	MSE*
1†	$Fe_2{}^{125}I$-OTf	9.55 (0.27)	0.24 (0.03)	0.24
	$Fe^{131}I$-OTf/2N + $Fe^{125}I$-OTf/2C	11.73 (0.23)	0.23 (0.02)	0.17
		11.63‡ (0.19)	0.20 (0.02)	0.12
		7.75 (0.27)	0.18 (0.03)	0.27
2†	$Fe_2{}^{125}I$-OTf	7.88 (0.24)	0.13 (0.02)	0.33
	$Fe^{125}I$-OTf/2N + $Fe^{131}I$-OTf/2C	9.37 (0.30)	0.16 (0.03)	0.31
		9.40‡ (0.29)	0.17 (0.03)	0.29
		5.20 (0.25)	0.14 (0.04)	0.23

* MSE, mean square error.

† In the first experiment the specific radioactivity of $Fe^{131}I$-OTf/2N was 407034 c.p.m./nmol and that of Fe^{125}-OTf/2C was 1113299 c.p.m./nmol. In the second experiment the specific radioactivity of $Fe^{125}I$-OTf/2N was 814143 c.p.m./nmol and that of $Fe^{131}I$-OTf/2C was 545485 c.p.m./nmol.

‡ The $B_{max.}$ and K_d^* reported were calculated by using the measured free concentration of FeOTf/2N as the concentration of both half-molecules.

DISCUSSION

The present investigation was stimulated by the previous finding that OTf half-molecules alone showed no specific binding to CERBC, whereas an equimolar mixture of FeOTf/2N and FeOTf/2C showed specific binding with a convex Scatchard plot. In addition, a mixture of one radiolabelled half-molecule with a constant (12 μM) amount of non-labelled complementary half-molecule gave a linear Scatchard plot with a slope less than that for Fe_2OTf (Brown-Mason & Woodworth, 1984). The question which arose was whether the half-molecules were associating in solution before being recognized by, and bound to, the Tf receptor. To address this question we applied the Gilbert theory for rapidly associating macromolecules as adapted and elaborated upon by Ackers & Thompson (1965) for gel filtration. The Gilbert theory is applicable to systems in which fast equilibrium occurs relative to the time of separation. In our previous work we showed that preincubation of CERBC with either radiolabelled half-molecule led to little binding or iron donation. Addition of complementary unlabelled half-molecule led to immediate binding and iron delivery. In these experiments the $t_{\frac{1}{2}}$ for binding was estimated to be less than 1 min (Brown-Mason & Woodworth, 1984). In the present study, correction for the total associated half-molecule gives rise to K'_d values for binding of half-molecules to the receptor that are close to those found for holo-OTf (Table 2). In calculating the K_d^* and $B_{max.}$ for OTf and the half-molecules, we have adopted a computerized non-linear least-squares regression analysis. The advantages of such an approach are summarized by Bürgisser (1983).

The present study involved an attempt to establish mathematically whether the interaction of half-molecules in solution can account for the observed binding to cells. Half-molecules alone bind to cells at very low levels ($< 10\%$ of combined half-molecule). The gel-filtration studies show that there is significant interaction of *N*- and *C*-terminal half-molecules in solution. The change in shape from sigmoidal to hyperbolic of the cell-binding curve using the dissociation constant derived from the gel-filtration studies (Fig. 3) and the K_d^* values obtained from these curves (Table 2) appear to support the hypothesis that the interaction of half molecules in solution is important in receptor recognition.

Three fairly recent publications are of interest in regard to our findings. The first, by Ikeda *et al.* (1985), describes preparation of Fe_2-OTf nicked at the hinge region by trypsin at elevated temperature. The two domains were shown to remain bound to each other non-covalently and to be stabilized with respect to denaturation by either 2 M-guanidinium chloride or temperature. The second, by Evans *et al.* (1985), also reports the presence of extensive non-covalent interactions between the two domains of OTf. The third, a preliminary report by Legrand *et al.* (1985), describes limited trypsin digestion of diferric lactoferrin into two parts, which remain associated at neutral pH. In this case, the 30 kDa *N*-terminal domain and 50 kDa *C*-terminal domain can be separated by gel filtration in the presence of 10% acetic acid. At neutral pH the domains apparently reassociate. C.d. studies on the individual and associated domains suggest that there are significant conformational changes induced by the interaction of the two halves.

If the non-covalent association of iron-containing half-molecules has functional significance, then individual half-molecules should not bind to Tf receptors. This is the case with bovine (Brock *et al.*, 1978) and human half-molecules (Lineback-Zins & Brew, 1980). In fact, none of the reports of individual half-molecules binding to reticulocytes and donating iron to them is well substantiated. As mentioned in detail previously (Brown-Mason & Woodworth, 1984), we are sceptical of the findings of Keung & Azari (1982), because of the inability of their OTf half-molecules to compete effectively with holo-OTf. Furthermore, the narrow range of concentrations used in their equilibrium binding studies under conditions where iron would be removed make the results questionable. A report of a $\sim$ 40 kDa monosited Tf from the sea-squirt *Pyura stolonifera* binding to rat reticulocytes is not completely convincing either (Martin *et al.*, 1984). Much confusion has resulted from studies in which Tf from one species is evaluated with cells from a different species. The absence of albumin in the incubation medium and the limited binding data make it difficult to evaluate whether non-specific binding could account for the limited binding observed. The uptake of iron from the *Pyura* protein should be directly compared with the uptake of iron from rat Tf in order to evaluate its effectiveness as an iron donor.

Our results using NH_4Cl essentially agree with those reported by others (Morgan, 1981; Klausner *et al.*, 1983*a*,*b*; Harding & Stahl, 1983; Rao *et al.*, 1983). Although some authors report either slightly more or less binding in the presence of NH_4Cl, our results indicate no significant difference between control and NH_4Cl-treated cells. If NH_4Cl is added at the same time as radiolabelled OTf, the binding profile is indistinguishable from that obtained in the absence of NH_4Cl (Fig. 1*a*). If the cells are preincubated with NH_4Cl, the binding profile is changed; $B_{max.}$ remains approximately the same, but takes longer to reach. If NH_4Cl is removed from the medium, iron uptake is resumed (results not shown). The distribution of radiolabelled OTf is the same in the presence or absence of NH_4Cl, indicating that internalization of OTf is not blocked. The major effect of NH_4Cl in our system appears to be a 4-fold decrease in the rate of release of $^{59}Fe_2$-^{125}I-OTf from the cells after incubation, followed by a chase with unlabelled OTf. Harding & Stahl (1983) reported a similar, albeit less dramatic, effect of NH_4Cl on Tf release from cells. They present evidence that the decrease is due to a slower dissociation of externalized diferric Tf from the receptor rather than an effect on the externalization of the Tf–receptor complex. This observation is consistent with the high affinity of diferric Tf for its receptor at pH 7.4 (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983*a*,*b*; Morgan, 1983). Our data do not distinguish between the externalization of the OTf and its dissociation from the surface.

As detailed previously (Brown-Mason & Woodworth, 1984), we feel it is important to inhibit the removal of iron in order to obtain meaningful data from equilibrium binding experiments. In previous studies we used 2,4-dinitrophenol, but found that it decreases total binding by 25%. Because NH_4Cl added at zero time yields the identical binding profile, we feel it is the compound of choice with which to conduct equilibrium binding studies at 37 °C. It is difficult to evaluate the report (Morgan, 1981) that the presence of NH_4Cl increases the apparent affinity relative to a control, since

in the control cells iron is being removed and equilibrium cannot be attained. As in our previous work, what is of interest is the comparison of binding of OTf with that of the half-molecules under identical conditions.

We report the $A_{1\,cm,\,280}^{1\%}$ to be 11.53 for apo-OTf and 13.95 for Fe_2OTf. Glazer & McKenzie (1963) reported values of 11.3 and 14.8 for these same proteins at pH 6.0. This close agreement for holo-OTf lends some confidence that the values obtained for the half-molecules using the same technique are correct. Furthermore, the similar ratios of apo to iron forms of the three samples are reassuring. The stoichiometry of *C*-terminal half-molecule to *N*-terminal half-molecule in the cell experiments is peculiar and not readily explained. In uptake, equilibrium-binding and gel-filtration studies we observe approx. 25% fewer binding sites/cell for FeOTf/2C as against FeOTf/2N (in the presence of complementary equimolar half-molecule). In examining what we know about the association of the half-molecules in solution there is no evidence for a stoichiometry other than 1:1. Thus, in gel-filtration studies, the elution of the combined half-molecules is between holo-OTf and the individual half-molecules. In addition the N/C ratio is observed whether iron is being removed or not, in the presence of cells under equilibrium or uptake conditions and with isolated receptor. An error in the absorption coefficient used to calculate the amount of half-molecule would be consistent with all of the results. We estimate, however, that such an error could not be greater than 10%, and thus would not account for the observed discrepancy. For example, data from sedimentation-equilibrium studies on the model E ultracentrifuge, presented in the legend to Table 1, indicate that the M_r values for OTf and the half-molecules are within 4% of those calculated from the sequence (Jeltsch & Chambon, 1982; Williams *et al.*, 1982).

More recently we have obtained evidence (A. Brown-Mason & S. A. Brown, unpublished work) that the discrepancy is due to a differential effect of iodination on the two half-molecules which causes a decrease in the amount of 'functional' FeOTF/2C. In spite of the discrepancy found in the number of binding sites/cell for the half-molecules, we believe that the overall conclusions remain valid. The K'_d determined for the half-molecules in the gel-filtration studies involved non-radiolabelled samples. As shown in Table 2, recalculation of the results using the free concentration of the *N*-terminal half-molecule for both half-molecules leads to insignificant differences in $B_{max.}$ and K_d^*. Thus we contend that, within experimental error, the conclusions of the study are valid; the isolated half-molecules associate in solution, and the measured association accounts for the observed binding of the half-molecules to the TF receptors on CERBC.

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