Differential effect of iodination of ovotransferrin and its two half-molecule domains on binding to transferrin receptors on chick embryo red blood cells

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Iodination of the C-terminal half-molecule domain of ovotransferrin (OTF) causes a significant reduction in binding to transferrin receptors on chick reticulocytes when compared to the binding observed with holo-OTF or the N-terminal half-molecule domain. (In such studies binding of iodinated half-molecule is measured in the presence of equimolar unlabelled complementary half-molecule.) In particular iodination of the C-terminal half-molecule domain by the solid-phase reagent Iodogen resulted in half the binding found when ICl was used. The iodinated N-terminal half-molecule domain labelled by either Iodogen or ICl showed consistently higher binding than was observed with the C-terminal half-molecule or Fe₂OTF. Although the molecular basis for the reduced binding of these proteins relative to the N-terminal half-molecule has not been definitively established, the implication is that there is a Tyr in the C-terminal domain which is involved in receptor recognition and binding. Addition of one or more bulky iodine atoms to the Tyr interferes with the interaction. Tryptic peptide maps of unlabelled holo-OTF and half-molecule domains and of the half-molecule domains labelled by both ICl and Iodogen are presented. The maps indicate limited access of the tyrosine residues to iodination especially in the C-terminal half-molecule domain. Equilibrium binding experiments have been carried out to compare the K^*_d (the apparent dissociation constant for the interaction between OTF and the transferrin receptors on chick-embryo red blood cells) with the B_{max} (binding at infinite free-ligand concentration) for Fe₂OTF labelled using ICl, Iodogen, Enzymobeads and Chloramine-T. The effect of labelling Fe₂OTF by Bolton-Hunter reagent has also been assessed. These studies show that ICl appears to be the reagent of choice for labelling Fe₂OTF and its half-molecule domains.

INTRODUCTION

The transferrins are a group of homologous glycosylated iron-transport proteins which include serum transferrins from many species, ovotransferrin from egg white and lactotransferrin from mammalian milk (Aisen & Listowsky, 1980; Chasteen, 1983). The 80 kDa molecule of transferrin is divided into two approximately equal homologous globular domains each containing a specific binding site for ferric iron and a concomitant obligate anion (Aisen & Listowsky, 1980). Limited proteolytic digestion preserves the integrity of the two 'half-molecule' domains which can then be isolated (see below). Transferrin molecules bind to specific receptors found on the surfaces of virtually all actively proliferating cells (Newman *et al.*, 1982; Trowbridge *et al.*, 1984; Bomford & Munro, 1985). The only function which has been thoroughly documented for transferrin is delivery of iron to dividing cells (Brock & Mainou-Fowler, 1983).

Research from our laboratory has focused on establishing which regions of the transferrin molecule are involved in binding to the transferrin receptor. In pursuing this aim we have prepared and isolated N- and C-terminal half-molecule domains of ovotransferrin and have

shown that both domains must be present in the incubation medium to allow binding and iron donation to a suspension of chick-embryo red blood cells at physiologically relevant levels (Brown-Mason & Woodworth, 1984). Equilibrium binding studies of equimolar mixtures of the complementary half-molecule domains yielded convex Scatchard plots suggestive of positive co-operativity in binding. Gel filtration studies were used to show that the half-molecule domains associate with each other in solution and that this association appears to account quantitatively for the co-operativity observed when the half-molecules bind to cellular receptors (Mason *et al.*, 1987a). More recently monoclonal antibodies against holo-ovotransferrin and the isolated half-molecule domains have been used to further substantiate that both domains must be present for recognition by and binding to receptor. In these studies we have shown that antibodies specific to either half-molecule domain can block binding of ¹²⁵I-labelled diferric ovotransferrin or of the combined half-molecules to receptors on CERBCs. In addition an antibody which recognizes the N-terminal half-molecule domain but not holo-ovotransferrin blocks binding of the combined half-molecule domains to receptor presumably by preventing the noncovalent association described above

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Abbreviations used: TF, transferrin; OTF, ovotransferrin; Fe₂OTF, iron-saturated OTF; FeOTF/2N and FeOTF/2C, the iron binding domains or 'half-molecules' from the N-terminal and C-terminal halves of OTF; CERBC, chick-embryo red blood cells; EGAB, Earle's salts containing 6 mM-glucose, 2.5% (w/v) bovine serum albumin and 0.22% (w/v) NaHCO₃.

(Mason *et al.*, 1987b). As discussed in detail elsewhere (Mason *et al.*, 1987a) we feel that reports by others of individual half-molecules binding to cellular transferrin receptors are not well substantiated.

A disturbing aspect of all of our own studies is an apparent non-equivalence in the stoichiometry of the two half-molecule domains binding to receptor. Although there is no evidence in our experiments for a stoichiometry other than 1:1 for the half-molecules, data from equilibrium binding, kinetic and other studies consistently indicates approximately 25% fewer binding sites per cell for the C-terminal half-molecule domain when compared to the number of sites per cell found for the N-terminal half-molecule domain (in the presence of the complementary equimolar half-molecule). In the present paper an explanation for the discrepancy is offered. Iodination *per se* appears to have a differential effect on the two half-molecule domains. This observation has led to a series of equilibrium binding studies to test the ability of diferric ovotransferrin labelled by five different iodinating reagents to bind to transferrin receptors on CERBCs.

EXPERIMENTAL

Bio-Gel P-6DG desalting resin, AG1 X-8, Affigel-10 and Enzymobeads were obtained from Bio-Rad Laboratories. Na¹²⁵I was from Amersham (carrier free, 100 mCi/ml in NaOH solution). Iodogen was from Pierce. Bolton-Hunter reagent came in a kit from ICN. Earle's salts, tosylphenylalanylchloromethane ('TPCK')-treated trypsin and bovine serum albumin (Fraction V) were obtained from Sigma. Ampholytes 4-9, technical grade, came from Serva and Centricon microconcentrators were purchased from Amicon. ⁵⁹FeCl₃ (32.54 mCi/mg at 10 mCi/ml) was obtained from New England Nuclear.

Protein preparation

Ovotransferrin (OTF), the N-terminal half-molecule domain of ovotransferrin designated FeOTF/2N and the C-terminal half-molecule domain of ovotransferrin designated FeOTF/2C were prepared as described previously (Williams & Woodworth, 1973; Brown-Mason & Woodworth, 1984; Mason *et al.*, 1987a). Preparation of the half-molecule domains involved digestion of the appropriate monoferric species with Affigel-trypsin followed by electrofocusing on an LKB electrofocusing column using 0.4% pH 4-9 ampholytes and/or chromatography on Sephadex G-75. Protein concentrations were determined spectrophotometrically using the following molecular masses (Jeltsch & Chambon, 1982; Brown-Mason & Woodworth, 1984) and absorption coefficients (Mason *et al.*, 1987a) at 280 nm ($A_{1\%}^{1\text{cm}}$): Fe₂OTF, M_r 79882, $A_{1\%}^{1\text{cm}} = 13.95$; apo-OTF, $A_{1\%}^{1\text{cm}} = 11.53$; FeOTF/2N, M_r 38532, $A_{1\%}^{1\text{cm}} = 12.73$; apo-OTF/2N, $A_{1\%}^{1\text{cm}} = 10.61$; FeOTF/2C, M_r 41350, $A_{1\%}^{1\text{cm}} = 13.74$; apo-OTF/2C, $A_{1\%}^{1\text{cm}} = 10.87$. Portions of all ovotransferrin samples were subjected to both urea and SDS/polyacrylamide-gel electrophoresis to assess purity (Brown-Mason & Woodworth, 1984). Urea gels were used to evaluate the iron status of the OTF samples.

Radiolabelling procedures

McFarlane procedure. An amount of ICl (McFarlane, 1963) sufficient to give one iodine atom per molecule of protein was incubated for 10-15 min with 200 μ Ci of

Fig. 1. Elution profiles of tryptic peptides from digestion of reduced and alkylated OTF (a), OTF/2N (b) and OTF/2C (c) monitored at 280 nm

Approx. 400 μ g of protein in a volume of 100 μ l was loaded onto a PepRPC HR 5/5 column (Pharmacia) which was then developed as described in the Experimental section. The portions of the chromatogram shown correspond to 14.4-49.4% buffer B. No peaks are observed prior to 14.4% buffer B or after 49.4% buffer B.

¹²⁵I (as Na¹²⁵I), diluted by half with alkaline glycine buffer (0.8 M-glycine/0.2 M-NaOH, pH 9.0, containing 0.25 M-NaCl) and combined with 10 mg of protein which was in 0.4 ml of 50% alkaline glycine buffer. After a 10-20 min incubation at room temperature with occasional mixing, the sample was passed over a Bio-Gel P-6DG desalting column (8 ml of resin in the barrel of a 10 ml disposable syringe fitted with a 22 gauge needle and plugged with glass wool). The pink iron-containing OTF and half-molecules were collected by eye and were concentrated, washed, and exchanged into the buffer of choice on a Centricon microconcentrator (Centricon-30 for OTF; Centricon-10 for the half-molecules). If the washes revealed that substantial amounts of free radio-labelled iodine were still present, the sample was passed over an AG1 X-8 column (2 ml of resin in the barrel of a 3 ml disposable syringe) in 0.15 M-NaCl. The eluant was collected (2 ml), reduced on a Centricon and washed into the buffer of choice as described above.

Iodogen procedure. Iron-containing OTF or half-molecules (10 mg) were lyophilized and taken up in 0.01 M-KPO₄, pH 7.4, containing 0.15 M-NaCl and 0.02 M-

Fig. 2. Elution profiles of tryptic peptides from digestion of reduced and alkylated OTF/2N (a) and OTF/2C (b) monitored at 214 nm

Approx. 200 µg of protein in a volume of 100 µl was loaded onto a PepRPC HR 5/5 column which was then developed as described in the Experimental section. Other details are as described in the legend to Fig. 1.

NaHCO₃. Typically 100 µl of sample (100–500 µg of protein) was pipetted into a 1.5 ml conical tube (Sarstedt) coated with 1 µg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril). After addition of 200 µCi of ¹²⁵I (as Na¹²⁵I) the sample was incubated for 2–5 min at room temperature with occasional agitation. The sample was removed with a 1 ml disposable syringe fitted with a 22 gauge needle and added to the remaining unlabelled protein. The entire sample was then passed over a Bio-Gel P-6DG column, an AG1 X-8 column or both as described above for the McFarlane procedure. Further handling of the sample was also as described. In some cases the entire 10 mg was exposed to the Iodogen reagent.

Bolton-Hunter procedure. Labelling of Fe₂OTF with Bolton-Hunter reagent followed the protocol described by ICN. Fe₂OTF (100 µg) was taken up in 10 µl of 0.05 M-sodium borate buffer, pH 8.5, and added to 1 mCi of reagent. After incubation and processing as described above the labelled sample was diluted with 10 mg of unlabelled Fe₂OTF.

Enzymobead procedure. Iodination using Enzymobeads (beads containing a mixture of lactoperoxidase and glucose oxidase) followed the protocols described in Bio-Rad Laboratories Technical Bulletin 1071. Either 100 µg of Fe₂OTF or FeOTF/2C in 20 µl of water or 10 mg of protein in 150 µl of water was placed into a 1.5 ml conical tube. In either case, 50 µl of 0.2 M-KPO₄, pH 7.2, 200 µCi of ¹²⁵I and 25 µl of 1 % β-D-glucose were pipetted into the protein solution and incubated with occasional vortexing for 30 min at room temperature. Further treatment of the iodinated protein followed the protocols described above for ICI and Iodogen labelled samples.

Table 1. Protocol for conducting equilibrium binding experiments with labelled diferric ovotransferrin and with equimolar combined half-molecule domains of ovotransferrin binding to chick-embryo red blood cells (CERBC)

For holo-ovotransferrin, samples 1–4, 3 × 100 µl of cell suspension was removed, washed and assayed for radioactivity; for samples 5–7, 50 µl aliquots in triplicate were taken; for samples 8–12, 20 µl aliquots in triplicate were taken. Nominal concentrations of OTF were 0.06–33.3 µM. Measured actual free concentrations were 0.06–50 µM. For the half-molecules, samples 1–3, 100 µl aliquots were taken in triplicate, for samples 4–7, 50 µl aliquots in triplicate and for samples 8–12, 20 µl aliquots in triplicate were removed, washed and assayed for radioactivity. Nominal concentrations of the half-molecule domains were 0.25–33.3 µM. Actual free concentrations corrected for association of the half-molecules (Mason *et al.*, 1987a) were 0.15–33 µM.

Fe ₂ ¹²⁵ I-Ovotransferrin					¹²⁵ I-Half-molecule domains				
Sample number	Concentration (nmol/ml)	Volume (µl)	Volume of buffer (µl)	Volume of CERBC (µl)	Sample number	Concentration (nmol/ml)	Volume (µl)	Volume of buffer (µl)	Volume of CERBC (µl)
1	2.5	15	185	400	1	5.0	30	140	400
2		30	170		2		60	80	
3		60	140		3		100	—	
4		100	100		4		15	70	
5	10.0	15	85	200	5	20.0	30	40	200
6		30	70		6		40	20	
7		50	50		7		50	—	
8	100.0	5	45	100	8	200.0	5	40	100
9		10	40		9		8	35	
10		15	35		10		10	30	
11		30	20		11		15	20	
12		50	—		12		25	—	

Chloramine-T procedure. Iodination using Chloramine-T was performed essentially as described by Keung & Azari (1982). Briefly, 10 mg of Fe₂OTF was taken up in 200 μ l of buffer (0.05 M-KPO₄, pH 7.6) to which 200 μ Ci of Na¹²⁵I and 20 μ l of Chloramine-T (5 mg/ml) were added. After a 5 min incubation at room temperature 20 μ l of KI (100 mg/ml) was added and the sample was further processed as described above for the ICl procedure.

The iodinated samples were stored at -20 °C for up to 1 month. They were routinely checked on urea gels to assure that they were completely iron-saturated both before and after the iodination procedures. The specific radioactivity (c.p.m./nmol) was usually determined prior to each experiment. For this purpose, 3-5 μ l in triplicate was removed and placed into a total volume of 1 ml. After determining the amount of radioactivity in each portion in a gamma counter, the absorbance at 280 nm was determined on the same diluted samples in a 1 ml black masked cuvette using a Cary 219 spectrophotometer.

In some later studies the iodinated proteins were subjected to trichloroacetic acid precipitation as described by Goding (1983) to measure the amount of radioactivity specifically bound to protein. Greater than 95% of the labelled iodine was associated with the protein in all of the samples which were tested.

Preparation and separation of half-molecule tryptic peptides

Reduction and alkylation of OTF and the half-molecules was carried out as described by Tsao *et al.* (1974) except that iodoacetamide was substituted for iodoacetic acid as the alkylating agent. The lyophilized samples were trypsin-treated [1:50 (w/w)] in 0.1 M-acetate/1 mM-CaCl₂, pH 8.0. The resulting peptides were resolved on a PepRPC HR 5/5 column (Pharmacia). All peptides eluted in a 17.5 ml 0-60% gradient consisting of 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The flow rate was 0.7 ml/min. Samples were monitored at 214 nm or 280 nm using a flow cell with a 5 mm path length. The runs from different digests were found to be very reproducible. As deduced from the sequence of OTF (Jeltsch & Chambon, 1982) the theoretical yield of tryptic peptides would be 40 from OTF/2N and 47 from OTF/2C. Of these, 18 in the N-terminal half-molecules and 25 in the C-terminal half-molecules have five amino acids or less. When monitoring at 214 nm approximately 20 separate (but not completely resolved) peaks are observed for OTF/2N and 25 for OTF/2C. In theory any peptide containing Tyr and/or Trp residues would be observed when monitoring at 280 nm. There are 11 peptides in OTF/2N and 13 peptides in OTF/2C which have one or more Tyr or Trp residues. Typical chromatograms are shown in Figs. 1 and 2.

Chick-embryo red blood cell (CERBC) protocols

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 containing 0.026 M-NaHCO₃ and 2.5 mg of bovine serum albumin/ml (EGAB) and adjusted to pH 7.4 as described previously (Brown-Mason & Woodworth, 1984; Mason *et al.*, 1987a).

Kinetic experiments to assess the binding of OTF and

the combined half-molecule domains by CERBC involved adding a saturating amount of OTF (6 μ M) or combined equimolar half-molecules (12 μ M) to a suspension of cells and incubating at 37 °C under CO₂/air (1:19). Triplicate portions of cell suspension (50 μ l) were removed after 0, 3, 6, 9, 12, 15, 20, 25 and 30 min of incubation and processed using a one-step washing procedure in which cells are spun through 1 ml of ice-cold EGAB and 300 μ l of dibutyl phthalate (Mason *et al.*, 1987a,b). The calculation of both the binding sites per cell and the apparent first-order rate constant is described elsewhere (Woodworth *et al.*, 1982; Mason *et al.*, 1987a).

As the results of the current paper rely on the validity of the method used to conduct equilibrium binding experiments the protocol is described in detail. First endogenous ovotransferrin was removed from CERBC by incubating the cells in EGAB at a 5-fold dilution for 10 min at 37 °C in CO₂/air (1:19). The cells were spun down in a Beckman TJ-6 centrifuge, the supernatant was removed and the procedure was repeated twice. The cells were then incubated for an additional 10 min at a two-fold dilution of EGAB in the presence of 20 mM-NH₄Cl to inhibit removal of iron from ovotransferrin (Morgan, 1981; Harding & Stahl, 1983; Rao *et al.*, 1983; Klausner *et al.*, 1983; Mason *et al.*, 1987a) and pipetted into tubes (Omnivials, VWR Scientific) containing radiolabelled holo- and half-molecules of OTF according to the protocol in Table 1. All samples were placed in a standard CO₂ incubator on a Junior Orbital shaker (Fisher Scientific) for 30 min at 37 °C. At this time portions of cell suspension were removed as indicated in Table 1, washed by the one-step procedure above and assayed for radioactivity. Haematocrits were taken for each sample to determine the number of cells. In addition an aliquot (20 μ l) of the supernatant from each haematocrit tube was assayed for radioactivity in order to determine the exact concentration of free ligand. Correction for non-specific binding was made by taking the slope of the line at the four highest concentrations of the binding curve visualized by plotting the molecules of OTF or half-molecule bound versus the concentration of free ligand (Brown-Mason & Woodworth, 1984). A description of the computerized non-linear least-squares regression analysis used to calculate the results is given elsewhere (Mason *et al.*, 1987a).

RESULTS

In ongoing studies examining the interaction of holo-OTF and the isolated half-molecule domains with transferrin receptors on CERBC, the proteins in one instance were labelled in the presence of Iodogen rather than by the ICl procedure of McFarlane which has been routinely used. The Iodogen labelled FeOTF/2C showed impaired binding leading to a series of experiments comparing the effect of the two iodinating reagents on the binding of Fe₂OTF, FeOTF/2N and FeOTF/2C to CERBC. The results of various kinetic studies are presented in Table 2 and show that the method of labelling produced no significant differences in the binding of either Fe₂¹²⁵I-OTF or Fe¹²⁵I-OTF/2N (in the presence of FeOTF/2C). In contrast to these results binding of Fe¹²⁵I-OTF/2C (+ FeOTF/2N) labelled by Iodogen was 40% less than binding of McFarlane labelled FeOTF/2C. Since, as reported previously, the

Table 2. Comparison of the binding sites per cell found for Fe₂OTF and the two half-molecule domains of OTF labelled with ¹²⁵I by the ICI procedure of McFarlane as against Iodogen reagent

The results show the binding sites per cell found at 30 min for Iodogen-labelled samples divided by the binding sites per cell found at 30 min for McFarlane-labelled samples as determined in kinetic experiments performed and described in the experimental section using 6 μ M-Fe₂¹²⁵I-OTF and 12 μ M-combined labelled and unlabelled half-molecule domains. The numbers in parentheses are the S.D. values for n = the number of experiments.

	Sites per cell (Iodogen/McFarlane)
Fe ₂ ¹²⁵ I-OTF	109.6 % (± 11.3 %), $n = 7$
Fe ¹²⁵ I-OTF/2N (+ FeOTF/2C)	96.6 % (± 4.7 %), $n = 6$
Fe ¹²⁵ I-OTF/2C (+ FeOTF/2N)	62.8 % (± 6.0 %), $n = 6$

Fig. 3. Progress curves for the binding of Fe₂OTF and the iron-containing half-molecule domains of OTF to receptors on CERBC; comparison of samples labelled with ¹²⁵I by ICI as against Iodogen

Samples and calculated first-order rate constants ($\pm$ S.D.) are as follows. ICI catalysed samples: (1) (○) Fe₂¹²⁵I-OTF, 0.41 (± 0.07) min⁻¹; (2) (□) Fe¹²⁵I-OTF/2N (+ FeOTF/2C), 0.32 (± 0.03) min⁻¹; (3) (△) Fe¹²⁵I-OTF/2C (+ FeOTF/2N), 0.32 (± 0.06) min⁻¹. Iodogen catalysed samples: (1) (●) Fe₂¹²⁵I-OTF, 0.37 (± 0.06) min⁻¹; (2) (■) Fe¹²⁵I-OTF/2N (+ FeOTF/2C), 0.25 (± 0.03) min⁻¹; (3) (▲) Fe¹²⁵I-OTF/2C (+ FeOTF/2N), 0.34 (± 0.03) min⁻¹.

binding of Fe¹²⁵I-OTF/2C labelled by McFarlane was already approximately 25 % less than binding for Fe¹²⁵I-OTF/2N (Mason *et al.*, 1987a) this additional reduction means that the sites per cell found for Iodogen-labelled FeOTF/2C are half those measured for Fe¹²⁵I-OTF/2N. A typical kinetic experiment comparing the various samples is shown in Fig. 3. Thus iodination of the C-terminal half-molecule, particularly iodination catalysed

by Iodogen, interferes with the ability of this half-molecule to interact with the transferrin receptor. The interference may be direct or it may be the result of disrupted association of the half-molecule domains in solution. Such association has been shown to be a prerequisite for receptor recognition (Mason *et al.*, 1987a,b). As shown in Table 3, binding of Fe₂¹²⁵I-OTF is approximately 15 % lower than binding of the N-terminal domain for both McFarlane and Iodogen-labelled samples. Lower binding of Fe₂OTF relative to FeOTF/2N may be because Fe₂OTF contains the C-terminal domain. The exciting possibility exists that there are one or more tyrosines (or possibly a histidine) either in the region of the C-terminal half-molecule domain in contact with the receptor or in the region of FeOTF/2C involved in association with FeOTF/2N which when iodinated causes profound changes in the interaction.

To pursue this possibility samples of FeOTF/2C iodinated by the McFarlane and Iodogen methods were reduced and alkylated, digested with trypsin and run on a PepRPC HR 5/5 column (see the Experimental section). Within experimental error it appears that the iodinated tyrosine residue(s) reside in the same peptide(s) regardless of the procedure used to iodinate FeOTF/2C. In three different chromatograms the main peak of radioactivity was within a single tube (volume/tube = 0.35 ml) in the two samples. A representative chromatogram is shown in Fig. 4(a). Of interest is the apparent limited availability of Tyr to the iodination procedures. There are nine tyrosine residues in FeOTF/2C dispersed into eight peptides (assuming complete digestion) (Jeltsch & Chambon, 1982) but iodination appears to be largely confined to the tyrosine residue(s) in a single peptide(s) which elutes last in the chromatogram when monitored at 280 nm. This peptide(s) is thus the most hydrophobic of the A₂₈₀, i.e., Tyr- or Trp-containing, peptides. In the case of FeOTF/2N (which contains 12 Tyr residues dispersed into seven peptides) iodination followed by reduction and alkylation and trypsin digestion led to a chromatogram (Fig. 4b) showing two major and one minor peaks of radioactivity for the Iodogen-labelled sample and four peaks of radioactivity in the ICI-labelled sample. Two of the peaks in the ICI-labelled sample appear to correspond to the two major peaks in the Iodogen sample. Thus the radiolabelled iodine in FeOTF/2N appears to be more widely distributed than in the peptides from FeOTF/2C.

Although further observations relevant to these findings are made in the Discussion one additional consideration which may be important should be mentioned. Estimates of incorporation of added ¹²⁵I into the proteins showed somewhat higher levels using the McFarlane procedure: for Fe₂OTF, 73.8 % (± 16.6 %), $n = 4$ of the radiolabel is protein bound, for FeOTF/2N, 80.2 % (± 14.7 %), $n = 6$ and for FeOTF/2C, 91.7 % (± 11.5 %), $n = 4$. The Iodogen-catalysed incorporation is less in all cases: for Fe₂OTF, 53.1 % (± 14.2 %), $n = 4$, for FeOTF/2N, 65.8 % (± 17.5 %), $n = 5$ and for FeOTF/2C, 34.0 % (± 12.5 %), $n = 5$. Obviously the largest discrepancy occurs in the case of Iodogen-catalysed incorporation of ¹²⁵I into FeOTF/2C. The resulting radiolabelled half-molecule has a specific activity which is 2–3 times lower than the specific activity of the FeOTF/2C prepared by the McFarlane procedure. These findings imply that there is more limited access to residues which can be iodinated.

Table 3. Comparison of the binding sites per cell for Fe₂OTF and for FeOTF/2C compared with those found for FeOTF/2N using proteins labelled by both the ICI and Iodogen procedures

The results for the half-molecule domains are those found in the presence of equimolar complementary half-molecule. The equilibrium binding and kinetic experiments were conducted as described in the Experimental section. In both cases incubation of CERBC was for 30 min at 37 °C.

	Experimental protocol	Binding sites per cell (%)	
		McFarlane	Iodogen
Fe ₂ OTF ÷ FeOTF/2N	Equilibrium binding	85.1 (±2.2 %), <i>n</i> = 3	80.3 (±2.7 %), <i>n</i> = 3
	Kinetic	84.6 (±2.9 %), <i>n</i> = 3	89.1 (±5.4 %), <i>n</i> = 5
FeOTF/2C ÷ FeOTF/2N	Kinetic	81.6 (±7.3 %), <i>n</i> = 7	42.9 (±7.0 %), <i>n</i> = 6

Fig. 4. Elution profiles of tryptic peptides from digestion of ¹²⁵I-labelled reduced and alkylated (a) C-terminal half-molecule domain and (b) N-terminal half-molecule domain of OTF

Incorporation of ¹²⁵I into the half-molecule domains was catalysed by ICI (open symbols) or by Iodogen reagent (closed symbols). An aliquot of tryptic digest containing 300 000 c.p.m. was loaded onto a PepRPC HR 5/5 column which was developed as described in the Experimental section. Fractions of 0.35 ml were collected. The portion of the chromatogram shown corresponds to 12–53.8 % buffer B. Tube 20 corresponds to an elution time of 10 min.

Meanwhile these studies led us to pursue other iodination procedures in the hope of finding one which would not have an adverse effect on the binding of FeOTF/2C. The results derived from equilibrium binding experiments in which Fe₂¹²⁵I-OTF labelled in the presence of the McFarlane reagent, Iodogen, Enzymobeads and Chloramine-T was incubated with CERBC are presented in Table 3. A computerized non-linear least-squares regression analysis of the hyperbolic curve obtained by plotting the OTF bound per cell against the concentration of free ligand, yields estimates of *B*_{max} (binding at infinite free-ligand concentration) and *K*_d^{*} (the apparent dissociation constant for the interaction between OTF and the OTF receptors on chick-embryo red blood cells). Each of the two representative sets in Table 4 was done on a different day and shows variation in

*B*_{max} and *K*_d^{*} typically found with different batches of CERBC. The conclusion of these studies with the four different iodinating reagents is that they all yield approximately the same results. ICI reagent leads to slightly higher *B*_{max} and over many experiments seemed to give the most consistent results. Again further reflections on these results and what they mean are included in the Discussion section.

Bolton–Hunter reagent yielded iodinated ovotransferrin which was compromised with respect to binding. In a typical kinetic uptake experiment Fe₂OTF labelled by ICI, by Iodogen and by Bolton–Hunter reagent gave apparent first-order rate constants of 0.43 (±0.05), 0.47 (±0.05) and 0.32 (±0.09) min⁻¹ and OTF molecules bound per cell values of 1.26 (±0.15) × 10⁵, 1.33 (±0.16) × 10⁵ and 0.86 (±0.03) × 10⁵ respectively. Thus

Table 4. The results of two sets of equilibrium binding experiments using Fe₂OTF labelled by the iodination procedures indicated below

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

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

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 Fe₂OTF and the TF receptors on CERBC. $B_{\max}$ is the number of OTF molecules bound per cell, usually defined as being equivalent to the number of receptor sites per cell.

Experiment number	Labelling procedure	$10^{-6} \times$ Specific radioactivity (c.p.m./nmol)	$10^{-5} \times B_{\max}$ (sites/cell)	K_d^* (μM)
1	McFarlane	3.19	1.42 (0.07)	0.50 (0.08)
	Iodogen	2.24	1.20 (0.08)	0.41 (0.09)
	Enzymobead	1.84	1.14 (0.10)	0.48 (0.14)
	Chloramine-T	0.29	1.16 (0.10)	0.55 (0.15)
2	McFarlane	2.52	1.32 (0.07)	0.45 (0.08)
	Iodogen	1.65	1.19 (0.07)	0.40 (0.08)
	Enzymobead	1.46	1.29 (0.05)	0.68 (0.09)
	Chloramine-T	1.56	1.39 (0.07)	0.75 (0.12)

binding of Bolton–Hunter-labelled Fe₂OTF was 68 % compared to ICl-labelled material and 65 % compared to Iodogen.

FeOTF/2C was labelled with Enzymobeads and compared to McFarlane-labelled FeOTF/2C in uptake experiments. The Enzymobead preparation gave slightly higher specific binding than material labelled by McFarlane reagent but also higher non-specific binding (binding observed in the absence of equimolar unlabelled FeOTF/2N). Both samples exhibited approximately 20 % fewer binding sites/cell than FeOTF/2N labelled by McFarlane, Iodogen or Enzymobeads, all of which gave comparable results (not shown).

DISCUSSION

The present studies were stimulated by our previous work in which a stoichiometry of 1:1 was not found for the binding of equimolar ovotransferrin half-molecule domains to transferrin receptors on CERBCs (Brown-Mason & Woodworth, 1984; Mason *et al.*, 1987*a,b*). In the current paper we present evidence that the discrepancy in stoichiometry is due to an effect of iodination *per se* on the C-terminal half-molecule domain. In particular iodination using the solid-phase reagent Iodogen caused a 50 % reduction in the binding of FeOTF/2C (in the presence of equimolar unlabelled FeOTF/2N) when compared to binding observed with ICl-labelled FeOTF/2C (Table 3). Several observations must be made in regard to this work. In carrying out the ICl procedure as described by McFarlane (1963) an average of one iodine molecule is bound per protein molecule. The protein thus obtained is modified as indicated by an alteration in the R_F value observed after urea/polyacrylamide-gel electrophoresis; the iodinated proteins migrate further on these gels than the unmodified proteins. In the Iodogen method no carrier iodine has been added and no change in R_F on urea-gel electrophoresis is observed. It follows from these considerations that changes in the peptide patterns could occur such that the apparent co-migration of the ¹²⁵I-peptides from

the tryptic digest of Iodogen and McFarlane-labelled C-terminal half-molecule domains may be fortuitous. Addition of iodine to Tyr residues in cytochrome *c*, for example, has been shown to give a peptide which is more hydrophobic than the equivalent peptide from unmodified cytochrome *c* (Osheroff *et al.*, 1977). Important differences between the Iodogen and ICl iodination procedures include both pH and access of the protein to the iodinating reagent. The pH at which the Iodogen procedure is carried out is 7.4 as against 8.5 for the ICl procedure. More of the phenolic hydroxyl groups (pK_a 10.1) of Tyr would be ionized at the higher pH. In addition, since the Iodogen reagent is immobilized on the surface of the reaction tube, only the Tyr residues on the surface of the protein would be expected to be susceptible to iodination. This may account for the 2–3 times lower incorporation of ¹²⁵I into FeOTF/2C when catalysed by Iodogen as against ICl. Limited reactivity of Tyr (or in some cases of His residues) to iodination using a number of different reagents has been reported for many proteins including insulin (Springell, 1961) α -chymotrypsin (Dube *et al.*, 1964), ribonuclease A (Covelli & Wolff, 1966), basic trypsin inhibitor (Sherman & Kassell, 1968) and cytochrome *c* (McGowan & Stellwagen, 1970; Stellwagen & McGowan, 1970; Pal *et al.*, 1975; Osheroff *et al.*, 1977). It is obvious from our work that further studies will be needed to definitively establish whether the residues iodinated by Iodogen as against ICl are the same and whether they play a role in receptor recognition or in half-molecule interaction (or both). Studies must also address whether di- or tri-iodination of susceptible Tyr residue(s) could contribute to the reduction of measurable binding.

Meanwhile a paper by Williams *et al.* (1985) suggests an alternative explanation for our results. There is apparently a disulphide bridge between residues 478 and 671 in the C-terminal portion of OTF which seems particularly sensitive to reduction by dithiothreitol under non-denaturing conditions. This same bridge could conceivably become oxidized during iodination leading to an irreversible change in the three-dimensional

structure of protein. A preliminary experiment indicates that Iodogen may be causing such structural damage to FeOTF/2C. Ordinarily the Iodogen procedure involves incubating 0.5 to 1 mg of protein in 100 μ l of pH 7.4 buffer, 200 μ Ci of Na¹²⁵I and 1 μ g of Iodogen reagent coated on the surface of a small tube. After 2 min the labelled sample is removed and added to the remaining 10 mg of unlabelled sample which is passed over a AG1 X-8 and/or a P-6DG desalting column. On one occasion the entire 10 mg of C-terminal half-molecule was incubated with Na¹²⁵I and Iodogen. The resulting Fe¹²⁵I-OTF/2C showed 30 % less specific binding (incubated in the presence of FeOTF/2N) and 30 % greater non-specific binding (incubated alone) to CERBC than the sample prepared in the ordinary manner. By contrast FeOTF/2N had identical specific and non-specific binding regardless of the procedure followed. An argument against the explanation is the fact that ⁵⁹Fe-labelled OTF/2C exposed to Iodogen reagent did not appear to be altered with respect to its ability to donate iron to cells when compared to ⁵⁹FeOTF/2C not exposed to Iodogen. Experiments are under way to follow up on these observations. It should be noted that human TF possesses an analogous disulphide bond linking residues 474 and 665 (MacGillivray *et al.*, 1982). Human TF thus might be expected to show similar behaviour to OTF, if destabilization of this bond turns out to cause reduced binding to receptor.

Equilibrium binding studies are probably the most valid way to evaluate binding of TF to its specific receptor. As detailed previously (Brown-Mason & Woodworth, 1984) such studies are fraught with both theoretical and practical problems. An important consideration not addressed previously is the possible adverse effect of iodination itself or the by-products of the iodination procedure (i.e. oxidation) on the protein. There does not appear to be any completely satisfactory test (or tests) for measuring the physiological competency of the iodinated protein. The pioneering work of Katz (1961) and of Jandl & Katz (1963) attempted to address the issue. They concluded on the basis of visible extinction, iron binding capacity, ultracentrifuge data and immunological testing that lightly iodinated diferric human TF was indistinguishable from non-iodinated protein. They also showed that ⁵⁹Fe₂ ¹²⁵I-TF delivered iron *in vivo* at the same rate as ⁵⁹Fe₂ TF alone. Similar turnover studies *in vivo* have since been used to compare various TF samples labelled by different procedures (Hemmaplardh & Morgan, 1976; Regoeczi, 1983). On the basis of such studies, in addition to iron and protein uptake studies with isolated reticulocytes, Hemmaplardh & Morgan (1976) concluded that neither ICl nor lactoperoxidase iodinations altered the physiological behaviour of rabbit TF. More recently Tsavaler *et al.* (1986) reported that lactoperoxidase-catalysed iodination of bovine TF destroys or drastically reduces its binding to human TF receptors. Given the extremely low affinity of bovine TF for human receptor (Penhallow *et al.*, 1986) this conclusion may not be warranted. It is necessary to examine binding of labelled TF to cells from the isologous species to prove that lactoperoxidase-labelled protein is really compromised with respect to binding. Susceptibility to adverse effects of iodination could well differ in transferrins from different species.

Iodination *per se* involves many variables any or all of which could determine the ultimate physiological rele-

vance of the final iodinated product. These include choice of buffer, pH, the level of iodine incorporated, the length of exposure to the iodinating reagent, and the processing of the sample after iodination to eliminate free iodine. Accurate measurement of the specific activity of the iodinated sample is also critical. An important consideration which is too often ignored is the biological competence of the starting material which may have been compromised during the isolation procedure. No systematic study has been done to examine the many parameters involved although Regoeczi (1983) presents a detailed description of the Iodogen-catalysed iodination of human and rabbit transferrins. The current study was not intended to provide an exhaustive study of the effects of iodination on OTF as indicated by binding to CERBC TF receptors. It was undertaken to attempt to find a procedure which would yield the most physiologically relevant samples as defined by the binding of both holo-OTF and the isolated combined half-molecule domains. Using the protocols exactly as presented in the Experimental section we conclude that iodination by Bolton-Hunter leads to OTF which is compromised with respect to binding to CERBC receptors. Further proof of the physiological incompetence of the Bolton-Hunter labelled Fe₂OTF derives from equilibrium binding studies (not shown) in which very high levels of nonspecific binding were found. Iodination of Fe₂OTF with ICl, Iodogen, Enzymobeads and Chloramine-T following our protocols led to B_{max} and K_d^* values which were not strikingly different (Table 4). Since Fe₂OTF labelled by the ICl method gave slightly more binding sites per cell and showed consistently lower non-specific binding and higher incorporation of labelled iodine, it is probably the procedure of choice for iodinating Fe₂OTF and the half-molecule domains of OTF.

Although the present study raises almost as many questions as it answers, it indicates the adverse result which iodination can have on binding particularly of the C-terminal half-molecule domain to the specific TF receptor. It is hoped that this iodination effect can be used to probe the molecular basis for the transferrin-receptor interaction.

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