Association of the two lobes of ovotransferrin is a prerequisite for receptor recognition

Studies with recombinant ovotransferrins

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Different recombinant N-lobes of chicken ovotransferrin (oTF/2N) have been isolated from the tissue-culture medium of baby hamster kidney cells transfected with the plasmid pNUT containing the relevant DNA coding sequence. Levels of up to 40, 55 and 30 mg/l oTF/2N were obtained for constructs defining residues 1–319, 1–332 and 1–337-$(Ala)_3$ respectively. In addition, a full-length non-glycosylated oTF was expressed at a maximum of 80 mg/l and a foreshortened oTF consisting of residues 1–682 was expressed at a level of 95 mg/l. These preparations were then used to produce, proteolytically, two different C-lobes (oTF/2C) comprising residues 342–686 and 342–682. The purified recombinant N-lobes (oTF/2N) are similar to the proteolytically derived half-molecule with regard to immunoreactivity and spectral properties; they show some interesting differences in thermal stability. A sequence analysis of the cDNA revealed six changes at the nucleotide level that led to six differences in the amino acid sequence compared with that reported by Jeltsch and Chambon [(1982) Eur. J. Biochem. **122**, 291–295]. Electrospray mass spectrometry gives results consistent with these six changes. Interaction between the various N- and C-lobes was measured by titration calorimetry. Studies show that only those lobes that associate in solution are able to bind to the receptors on chick embryo red blood cells. These findings do not support a previous report by Oratore et al. [(1989) Biochem. J. **257**, 301–304].

INTRODUCTION

The transferrins are glycosylated metal-binding proteins that function in the transport of iron to cells and as bacteriostatic agents in a variety of biological fluids [1–7]. The present-day 80 kDa proteins seem to have evolved by gene duplication, giving rise to two globular lobes, each containing a deep cleft capable of binding a metal ion. In all transferrins for which crystallographic data are available, each ferric ion is directly co-ordinated to the side chains of two tyrosine residues, one histidine residue, one aspartic acid residue and two oxygens from the synergistic carbonate anion [8–14].

Chicken transferrin is unique in that a single protein serves two functions. As with all serum transferrins, chicken serum transferrin is synthesized in the liver and delivers iron to cells by receptor-mediated endocytosis. Chicken ovotransferrin (oTF), which is synthesized in the oviduct and makes up approx. 15% of egg-white protein, is believed to function as a bactericidal agent. These two proteins are coded for by the same gene and differ only in their carbohydrate contents [15–18]. No role for glycosylation has been shown for human serum transferrin (hTF) in terms of receptor binding [19]. Equilibrium binding studies in our laboratory showed no significant differences between chicken serum transferrin and oTF in binding affinity or in the number of binding sites per chick reticulocyte ([20], and A. B. Mason and R. C. Woodworth, unpublished results).

Previous studies from our laboratory indicated that both the N- and C- lobes of oTF must be both present and associated to be recognized by the chick reticulocyte receptor and to allow the binding and donation of iron [21,22]. These findings were challenged in the work of Williams and co-workers [23,24], who claimed that binding takes place whether the lobes are able to pre-associate (complexable) or not (uncomplexable). Complexable oTF/2N comprises residues 1–332; uncomplexable oTF/2N comprises residues 1–319. Complexable oTF/2C is made up of residues 342–686 and uncomplexable oTF/2C residues 342–682. Recombinant technology offers an effective means of producing extremely homogeneous preparations of the putative complexable and uncomplexable lobes to test further the requirement for receptor recognition and binding. Titration calorimetry offers a sensitive and quantitative means of measuring the ability of the preparations to associate in solution. Here we describe the production, purification and characterization of the various recombinant N-lobes of chicken oTF. We have also produced and isolated non-glycosylated full-length oTF (residues 1–686) and a modified oTF that stops at residue 682. These recombinant proteins have been used to prepare two different C-lobes by proteolysis. Our studies show that association of the N- and C-lobes is an absolute prerequisite for binding to the receptor.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium/Ham F-12 nutrient mixture (DMEM-F-12) was obtained with and without Phenol Red from Sigma, as was the serum replacement LPSR-1. Fetal bovine serum was obtained from Hyclone or from Atlanta Biologicals

Abbreviations used: CERBCs, chick embryo red blood cells; DMEM-F-12, Dulbecco's modified Eagle's medium/Ham F-12 nutrient mixture; DSC, differential scanning calorimetry; ΔH_{cal}, calorimetric heat; hTF, human serum transferrin; ITC, isothermal titration calorimetry; O-ONG 1–682, recombinant ovotransferrin lacking the last four amino acids from the C-terminus; O-ONG 1–686, recombinant ovotransferrin that has a mutated inter-lobe peptide, is non-glycosylated and is full length; oTF, ovotransferrin; oTF/2C, ovotransferrin C-lobe; oTF/2N, ovotransferrin N-lobe; T_m, transition peak temperature.

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Table 1 Synthetic oligonucleotides used in cloning oTF and the various recombinant N-lobes of oTF

See the text for details of the cloning strategies.

OTF-1	5′-ACA CCC GGG ATG AAG CTC ATC CTC TGC AC-3′
OTF-2	5′-ACA GCG GCC GCA GTC AGC TGA TCT TTC CGC A-3′
OTF-3	5′-ACA CCC GGG AGG GCC CTT CCC TCC CTT TA- 3′
OTF-4	5′-ACA GCG GCC GCC AGA GAA AAC AGG ATC CAA T-3′
OTF-5	5′-GGC TTG ATT GAG GAC AGA ACA GGG ACC-3′
OTF-6	5′-ACA CCC GGG TCA TTA GAA GCT GCA CAT CTG GAG-3′
OTF-7	5′-AAA ATC CGT GAT CTG CTG GA-3′
OTF-8	5′-G GCC GCC TAA TGA CCC-3′
OTF-9	5′-GGG TCA TTA GGC-3′
OTF-10	5′-ACA CCC GGG TCA TTA CCG CAT GCT CTG GAT GGC-3′
OTF-11	5′-GAG TGG CTG CTC ACG CCG TC-3′
OTF-12	5′-ACA CCC GGG TCA TTA GTA GAG CTG GCA ATC CAT-3′
OTF-13	5′-AAG TGT TTG AGG CAG GCC TT-3′

and was tested before use to ensure adequate growth of baby hamster kidney cells. Penicillin/streptomycin sulphate solution was from Gibco. Corning expanded surface roller bottles, Wheaton Omnivials and Dynatech Removawells were obtained from a local distributor. $Na^{125}I$ was from DuPont-NEN®. The chromatographic resins, DEAE-Sephacel, Sephacryl S-100 HR and Polyanion SI were from Pharmacia. The resin, Poros 50 HQ and the QE column were from PerSeptive Biosystems. Methotrexate from Cetus was purchased at a local hospital pharmacy. Centricon 10 and 30 microconcentrators, YM-10 and YM-30 ultrafiltration membranes and a spiral cartridge concentrator (CH2PRS) fitted with an S1Y10 cartridge were from Amicon. Rabbit anti-mouse IgG was purchased from Southern Biological Associates. All chemicals and reagents were analytical grade.

A monoclonal antibody designated $aOT + N_1$ was prepared in our laboratory and found to be specific for the N-terminal lobe of oTF. A complete description of this antibody is given elsewhere [25].

Isolation of chicken oTF cDNA

The plasmid pBR322-con1 containing a full-length cDNA for chicken oTF cloned into pBR322 was generously provided by Dr. G. S. McKnight (University of Washington, Seattle, WA, U.S.A.). This plasmid is the same clone used by Jeltsch and Chambon [26] to determine the cDNA sequence and the predicted amino acid sequence of chicken oTF.

The general strategy followed to create the various plasmids involved synthesizing oligonucleotides that contained the desired nucleotide changes, and then amplifying the oTF DNA sequences by PCR. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. The PCR fragments produced were gel-purified with GENECLEAN (Bio101, La Jolla, CA, U.S.A.) and ligated into Bluescript. In all cases, the amplified portion of the DNA was sequenced to ensure the absence of PCR-induced mutations. The constructs were then excised with *Sma*I, gel-purified and ligated into the *Sma*I site of the pNUT vector.

To facilitate cloning and DNA manipulations, a *Not*I site was introduced into the cDNA region coding for the bridging peptide of the oTF between the N- and C-lobes. This resulted in the alteration of the wild-type amino acid sequence from Pro-Ser-Pro at positions 338–340 to Ala-Ala-Ala. In addition, *Sma*I sites were engineered into the 5′ and 3′ ends of the cDNA to allow direct cloning into the *Sma*I site of the expression vector pNUT. The oligonucleotides used are listed in Table 1.

OTF-1 and OTF-2 were used to amplify residues −19 to 337 of oTF/2N, and OTF-3 and OTF-4 were used to amplify residues 341–686 of oTF/2C. Both the N-lobe and C-lobe fragments were ligated individually in Bluescript (BS oTF/2N *Not*I/*Sma*I and BS oTF/2C *Not*I/*Sma*I respectively) as well as being ligated together into Bluescript to give the construct BS O-O 1–686.

Chicken oTF contains a single N-linked oligosaccharide at Asn-473 corresponding to the codon AAC in the cDNA sequence [26]. To create a non-glycosylated mutant, the codon was converted to GAC (to code for an aspartate residue) by oligonucleotide directed mutagenesis with the dut^-/ung^- method [19]. The template for the mutagenesis was BS oTF/2C *Not*I/*Sma*I. Oligonucleotide OTF-5 was used for the mutagenesis. The *Bam*HI fragment containing the mutated Asp codon at position 473 was cloned back into the full-length BS O-O 1–686 to give BS O-ONG 1–686.

To produce the truncated oTF terminating at residue 682, two oligonucleotides (OTF-5 and OTF-6) were used to amplify a fragment 254 bases long corresponding to amino acids 600–682. OTF-6 introduces two stop codons and an *Sma*I at the 3′ end of the cDNA. A fragment created by digestion with *Bgl*II and *Sma*I was cloned back into the original template to give the construct designated BS O-ONG 1–682.

To obtain an N-lobe construct suitable for expression, two stop codons were introduced into BS oTF/2N *Sma*I/*Not*I. Two overlapping oligonucleotides (OTF-8 and OTF-9) were used to introduce the stop codons and an *Sma*I site at the 3′ end of the cDNA. The two adaptors were ligated together with the *Sma*I/*Not*I fragment into the *Sma*I site of Bluescript to create BS oTF/2N 1–340, which was then excised with *Sma*I and ligated into the *Sma*I site of pNUT.

To make the construct designated oTF/2N 1–332, an oligonucleotide (OTF-10) was synthesized to add two stop codons and an *Sma*I site after Arg-332. An internal primer (OTF-11) was used to create a fragment of 274 bp. The fragment thus produced was digested with *Ava*I and *Sma*I to yield a fragment of 250 bp. The same restriction enzymes were used to digest the cDNA for the oTF/2N 1–340 construct to give a fragment of 823 bp. After gel purification, ligation into the *Sma*I site of Bluescript and sequencing to confirm the orientation and fidelity of the changes, the construct was ligated into pNUT.

To create the construct designated oTF/2N 1–319, two additional oligonucleotides, OTF-12 and OTF-13, were synthesized and used to amplify a fragment of 700 bp that was digested with *Eco*I and with *Sma*I to yield a fragment of 665 bp. The *Eco*I/*Sma*I digest of the oTF/2N 1–340 cDNA gave a fragment of 369 bp. The two pieces were ligated into Bluescript, sequenced, cut out with *Sma*I and cloned into pNUT.

Expression vector and cell culture

Baby hamster kidney cells were grown in DMEM-F12 medium with 5% (v/v) fetal bovine serum and transfected as previously described [27]. Selection of transfected cells with 500 μM methotrexate and expansion to roller bottles have also been described in detail [19,28]. Better adhesion of the cells to the roller bottles was achieved by using DMEM-F12/5% fetal bovine serum through two or three medium changes before switching to DMEM-F12/1% LPSR-1. As described previously, the entire population of cells that survived selection was taken because clonal selection afforded no great advantage [19].

Isolation and characterization of the recombinant oTF/2N

Isolation and purification followed the general strategy used

for the N-lobe of hTF [27,28] with a few modifications: after addition of PMSF, sodium azide and a saturating amount of $Fe(NTA)_2$ (NTA is nitrilotriacetate), the harvested medium was reduced in volume and exchanged into 5 mM Tris/HCl, pH 8.0, by using a spiral cartridge. The samples were kept frozen until a total of four or five batches had accumulated. These were pooled and subjected to the series of chromatographic steps previously described [28]. For the more recent samples, a Poros 50 HQ column was substituted for the DEAE-Sephacel column in the first step of the purification. After the sample had been clarified by centrifugation at 5900 ***g*** at 4 °C for 15 min, it was applied to the Poros column (2.6 cm × 20 cm) with a Pharmacia P-1 pump at a rate of approx. 10 ml/min. Elution from the column involved a single step of 180 mM Tris/HCl, pH 8.0. In addition, at the final step, a Poros QE/M (10/100) column was substituted for the Polyanion SI column. The Poros QE/M column was run on a PerSeptive Biosystems Sprint chromatography system rather than the FPLC system used previously. The column was equilibrated and run in 50 mM Tris/1,3-bis[tris(hydroxymethyl)-methylamino]propane, pH 8.0, at a rate of 7 ml/min. A linear gradient of 0–400 mM NaCl in the same buffer over five column volumes was used to develop the column. The Sprint system allows simultaneous monitoring of absorbance, conductivity and pH. Fractions of 3 ml were collected. The homogeneity of the various protein samples was assessed by gel electrophoresis and spectral ratio analysis as described [19].

N-terminal sequence

The N-terminal sequence of the recombinant oTF/2N 1-340 was determined on an Applied Biosystems 470A protein sequencer in the Given Analytical Facility at the University of Vermont College of Medicine.

Electrospray mass spectrometry analysis

Samples were analysed on a Micromass Quattro II mass spectrometer (Micromass Ltd., Altrincham, Greater Manchester, U.K.). The details of the analysis procedure have been described [19].

Radioimmunoassay of recombinant oTF/2N

The competitive solid-phase immunoassay used to determine the concentration of the recombinant N-lobes and of the full-length constructs of oTF in the culture medium and at various stages of the purification has been discussed previously [28]. Initially holo-oTF was used as the standard.

Differential scanning calorimetry (DSC) studies

The DSC measurements were performed on a MicroCal MC-2 ultrasensitive microcalorimeter (MicroCal, Northampton, MA, U.S.A.), interfaced with an IBM-compatible personal computer. A Windows-based software package (Origin), supplied by MicroCal, was used for data analysis and plotting. The DSC scan rate was 82 °C/h for all experiments and protein concentrations were 1–2 mg/ml in 0.5 M Hepes, pH 7.5, containing 25 mM $NaHCO_3$. The re-association experiments between the two lobes of oTF were performed on a MicroCal MCS ultrasensitive isothermal calorimeter with Observer software for instrument control and data acquisition. Isothermal titration calorimetry (ITC) experiments were performed in 0.1 M Hepes, pH 7.5, containing 25 mM $NaHCO_3$. Further details of the DSC and ITC experiments have been given elsewhere [29,30].

Preparation of the N- and C-lobes of oTF by proteolysis

oTF was prepared from hen egg-white as described previously [22]. The N- and C-lobes of oTF were prepared from diferric oTF by trypsin digestion by the method of Oe et al. [31]. Briefly, 245 mg of Fe_2oTF in 0.1 Tris/HCl, pH 8.0, containing 10 mM $CaCl_2$ was digested for 4 h at 37 °C with trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (Worthington Biochemical Corporation) at a 1:50 ratio of enzyme to substrate. The reaction was terminated by the addition of trypsin inhibitor (Sigma) and the sample was then dialysed against cold running water overnight. The lobes were almost totally resolved by electrofocusing for approx. 30 h on a large LKB column (500 ml) with 0.4% pH 4–6 ampholytes (Serva, Servalyt® 4-6) in a gradient of 0–50% (w/v) sucrose. After dialysis against water as above, the samples were concentrated by ultrafiltration. The individual lobes were further purified by chromatography on a Sephadex G-75 column (2.6 cm × 100 cm) in 100 mM NH_4HCO_3 and a Polyanion SI column (1 cm × 10 cm) as described [27,28] or Poros QE/M as above. The same procedure was followed to prepare oTF/2C from the two different recombinant non-glycosylated oTF samples, O-ONG 1–686 and 1–682.

Cell-binding studies

The isolation of chick embryo red blood cells (CERBCs) from 14-day embryos and the protocol for measuring the binding of oTF and the isolated lobes of oTF to the chick reticulocytes have been described [21,22,32,33]. Proteins were iodinated by the method of McFarlane [34,35]. For the studies in which the ability of the various recombinant proteins to bind to CERBCs was tested, cells were treated to remove endogenous oTF and then incubated with NH_4Cl to inhibit iron uptake. To measure binding, recombinant iron-saturated, iodinated, full-length oTF (final concentration 1 μM) or recombinant, iodinated oTF/2N in the presence or absence of unlabelled proteolytic oTF/2Cs (both at a final concentration of 3 μM) were preincubated in a volume of 40 μl for 15 min at room temperature in Omnivials. The particular concentrations were selected to duplicate those used in the experiments of Oratore et al. [24]. Then NH_4Cl-treated cells (80 μl) were pipetted into each vial and the samples were placed in a standard CO_2 incubator on an orbital shaker for 20 min. Three 35 μl aliquots of cell suspension were then pipetted into 1.5 ml polypropylene conical tubes containing a bottom layer of 300 μl of dibutyl phthalate and a top layer of 900 μl of ice-cold incubation buffer. The samples were processed and assayed for radioactivity as described previously [22].

RESULTS

Sequence of the oTF cDNA

The cDNA obtained from Dr. Stanley McKnight contained several changes in the published nucleotide sequence that led to six changes from the amino acid sequence reported by Jeltsch and Chambon [26] (Table 2). In addition, although the nucleotides at positions 472 and 1027 were incorrect in the original report, the correct amino acid was assigned. This implies that the latter two errors were probably mistypings. Six of the changes in the nucleotide sequence led to changes in the amino acids, resulting in significant differences in the mass of the expressed protein. Nucleotide changes compared with the published sequence [26] were confirmed by determining the corresponding sequence in the plasmid pBR322-con-1 DNA, thereby eliminating the possibility that PCR errors were introduced during the amplification of oTF cDNA fragments. The validity of the

Table 2 Differences found in the sequence of oTF in the present work compared with previous work [26]

Nucleotide sequence number	Codon change: Previous work		Codon change: Present work	Amino acid residue	Amino acid change: Previous work		Amino acid change: Present work	Net change in molecular mass (Da)
374	ATT	→	GTT	81	Ile	→	Val	−14.03
472	AAC*	→	ACC	113	Thr	→	Thr	–
530	CTC	→	ATC	133	Leu	→	Ile	–
536	TGG	→	CGG	135	Trp	→	Arg	−30.03
792	CTG	→	CAG	220	Leu	→	Gln	+14.97
796	AAT	→	AAG	221	Asn	→	Lys	+14.07
803	TAT	→	TAC	224	Tyr	→	Tyr	–
1027	TTC*	→	TTG	298	Leu	→	Leu	–
2133	AAC	→	AGC	667	Asn	→	Ser	−27.03

* In these two changes the nucleotide appears to have been incorrectly typed but the amino acid was correctly translated.

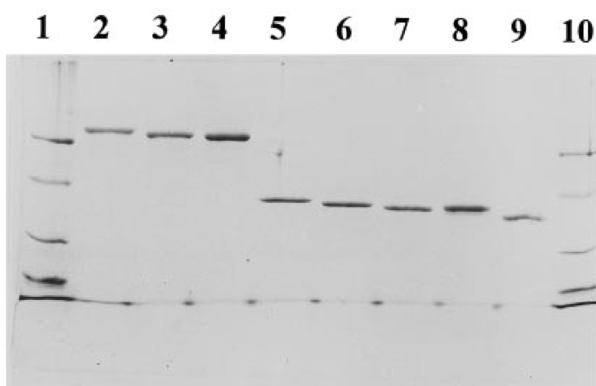

Figure 1 SDS/PAGE of various oTF samples run under reducing conditions and revealed with Coomassie Blue

Lanes 1 and 10, Bio-Rad low-molecular-mass standards of (top to bottom) 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa; lane 2, holo-oTF; lane 3, O-ONG 1–686; lane 4, recombinant O-ONG 1–682; Lane 5, recombinant oTF/2N 1–340; lane 6, recombinant oTF/2N 1–332; lane 7, recombinant oTF/2N 1–319; lane 8, proteolytically produced oTF/2C 342–686 (glycosylated); lane 9, proteolytically produced oTF/2C 342–682 (non-glycosylated). Approx. 1 μg of each sample was run for better detection of the molecular mass differences.

Table 3 Electrospray mass spectrometric analytical results for recombinant and proteolytically derived N-terminal lobes of chicken oTF and recombinant full-length oTF

Ovotransferrin	Calculated molecular mass* (Da)	Experimental molecular mass† (Da)	Difference in molecular mass (Da)
Recombinant			
1–340	37268.2	37270.5	+2.3
1–332	36469.3	36470.9	+1.6
1–319	34922.6	34923.0	+0.4
1–686	75759.0	75762.5	+3.5
1–682	75331.2	75334.6	+3.2
Native oTF	77531.7	77529.3	+2.4
Proteolytic			
1–332	36469.3	36466.4	−2.9
		(30429.3)‡	−40.0
1–332§	36469.3	36468.3	−1.0
		(36428.6)	−40.7

* The calculated molecular masses assume the mass changes listed in Table 1 and the presence of six disulphide bonds (−12.09 Da).

† The experimental molecular masses listed are the means for three to five repeat determinations with S.D. = 2.4 Da.

‡ The molecular masses in parentheses are those for the other major component present in the spectrum. The absence of a second component for the recombinant protein and the near constancy of the molecular mass difference observed for the proteolytic samples suggest that the second component is a variant.

§ Sample provided by Dr. John Williams (University of Bristol).

corrected sequence also seems to be supported by the mass spectrometry data (see below). Five of the six changes are in oTF/2N and result in an overall change in molecular mass of −15.01 Da. The one change in oTF/2C at position 667 results in a change in molecular mass of −27.03.

Protein expression, isolation and characterization

The amount of recombinant oTF/2N that was secreted into the medium by the baby hamster kidney cells was measured with a solid-phase radioimmunoassay and purified as decribed in the Materials and methods section. The oTF/2N 1–340 construct was produced at a maximum concentration of approx. 30 μg/ml. The constructs oTF/2N 1–319 and oTF/2N 1–332 were produced at 40 and 55 μg/ml. The full-length non-glycosylated oTf had a maximum concentration of 80 μg/ml; the same construct lacking the last four amino acids was produced at a maximum concentration of 95 μg/ml. Recombinant oTF and the recombinant oTF/2Ns are shown in comparison with the proteolytically prepared oTF/2Cs on SDS/PAGE (Figure 1). The recombinant proteins are clearly homogeneous. The spectral ratios for the iron-saturated recombinant oTF/2Ns were typically $A_{280}/A_{465} = 26.9$ and $A_{465}/A_{410} = 1.39$. Typical ratios for transferrin are $A_{280}/A_{465} \cong 20$ and $A_{465}/A_{410} = 1.4$.

The sequence of the first seven residues of the recombinant oTF/2N at the N-terminus of oTF/2N 1–340 was Ala-Pro-Pro-Lys-Ser-Val-Ile. This sequence is identical with that reported for holo-oTF [26] and shows that the signal peptide was properly cleaved. Because the other two N-lobes were derived from this construct, N-terminal sequencing was not performed on them. The mass spectrometry results are consistent with proper cleavage of the signal peptide from both oTF/2N 1–319 and oTF/2N 1–332.

Electrospray mass spectrometry analyses of the proteolytic and recombinant oTF/2N are presented in Table 3. The calcu-

Table 4 Thermodynamic parameters obtained from DSC for the apo- and holo-forms of recombinant and proteolytic oTF/2N and recombinant hTF/2N and its weakest binding single point mutant in which the Asp (D) residue at position 63 was mutated to a Ser (S) residue

Protein*	Apo-form		Iron-bound form	
	ΔH_{cal} (kJ/mol)	T_m (°C)	ΔH_{cal} (kJ/mol)	T_m (°C)
Recombinant oTF/2N 1–340	561	55.2	1075	77.7
Recombinant oTF/2N 1–332	527	55.2	1083	74.7
Recombinant oTF/2N 1–319	510	55.2	795	70.7
Proteolytic oTF/2N	598	50.9	962	76.9
Recombinant hTF/2N†	929	66.4	1477	86.0
Recombinant hTF/2N D63S†	690	65.6	1038	74.3

* The data for oTF/2N should be considered semi-quantitative owing to aggregation of the protein at high-temperature transition curves. The thermal reversibility for these runs was less than 5% as judged by second scans. Estimated uncertainties: $\pm$10% for ΔH_{cal} and $\pm$0.5 °C for T_m.

† From Lin et al. [37].

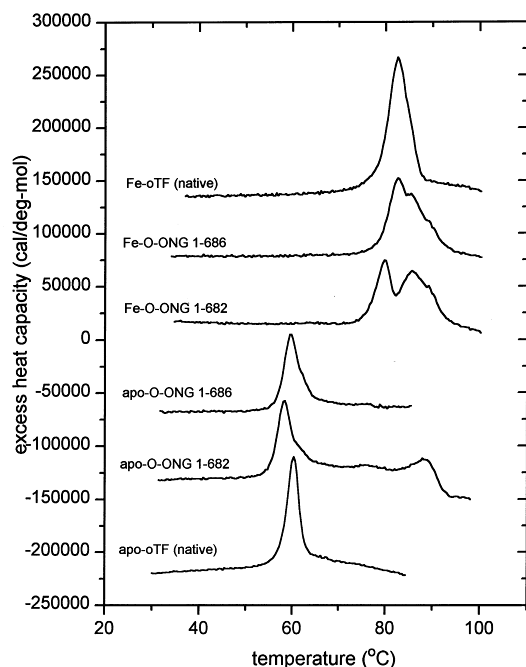

Figure 2 DSC of apo- and iron-containing recombinant and native oTF

The data have been normalized with respect to concentration. The scan rate was 82 °C/h. For clarity, DSC traces have been arbitrarily shifted on the ordinate scale. (1 cal ≈ 4.2 J.)

lated molecular mass for the recombinant proteins takes into account the amino acid changes in the sequence that result in changes in mass. For oTF/2N 1–340 the addition of three Ala residues at the C-terminus is included. The proteolytic oTF/2N is assumed to comprise residues 1–332 and also to contain the five changes shown in Table 2. As indicated in Table 3, two major components were found in the proteolytically derived samples prepared in our laboratory and in the laboratory of Dr. John Williams (University of Bristol). One component corresponds within experimental error to the expected molecular mass. The other component, with a molecular mass 40 Da lower,

Table 5 Thermodynamic parameters obtained from DSC for the apo- and holo-forms of native and recombinant oTF

Estimated uncertainties: $\pm$10% for ΔH_{cal} and $\pm$0.5 °C for T_m.

Protein	Apo-form		Iron-bound form		
	ΔH_{cal} (kJ/mol)	T_m (°C)	ΔH_{cal} (kJ/mol)	T_m1 (°C)	T_m2 (°C)
Native oTF	1314	60.2	2795	82.5	–
Recombinant O-ONG 1–686	1498	59.6	2510	82.6	85.4
Recombinant O-ONG 1–682	1469	58.3	2335	79.7	85.6

Table 6 Binding of native and recombinant holo-oTF and various combinations of N- and C-lobes of oTF to CERBC receptors

As detailed in the Materials and methods section, protein samples were incubated for 20 min with a constant amount of CERBCs. The results shown are the means $\pm$ S.D. for triplicate samples.

Sample	TF molecules/cell
Fe_2^{125}I-oTF	39732 $\pm$ 2413
Fe_2^{125}I-O-ONG 1–686	46200 $\pm$ 1302
Fe_2^{125}I-O-ONG 1–682	48492 $\pm$ 1768
Fe^{125}I-oTF/2N 1–340	5755 $\pm$ 831
+ Fe-oTF/2C 342–686	30824 $\pm$ 3553
+ Fe-oTF/2C 342–682	7237 $\pm$ 398
Fe^{125}I-oTF/2N 1–332	3444 $\pm$ 595
+ Fe-oTF/2C 342–686	41619 $\pm$ 1600
+ Fe-oTF/2C 342–682	12246 $\pm$ 1859
Fe^{125}I-oTF/2N 1–319	4428 $\pm$ 25
+ Fe-oTF/2C 342–686	4902 $\pm$ 585
+ Fe-oTF/2C 342–682	5130 $\pm$ 1016

might be the result of polymorphism. To calculate the molecular mass of O-ONG 1–686 a number of corrections were made, including replacement of the residues at positions 338–340 (Pro-Ser-Pro) with three Ala residues, replacement of Asn-473 by Asp and of Asn-667 by Ser. As shown in Table 3 the experimental values are within 3.5 Da for these two recombinant proteins.

Thermodynamic parameters from DSC of apo- and iron-containing recombinant and proteolytically derived oTF/2Ns are shown in Table 4. The calorimetric heat, ΔH_{cal}, was determined as the total area between the experimental data and the progress baseline as described previously in detail for apo-hTF/2N [30]. The ΔH_{cal} values for oTF/2N thus obtained are subject to a large error (estimated to be 10%) owing to the distortion of the post-transition baseline caused by protein aggregation, which occurred in the post-transition region. The thermal reversibility for all the oTF/2Ns, as judged by the DSC traces of the second upscan, was found to be less than 5% (it was 60–80% for the human N-lobe). The T_m values presented in Table 4 are the apparent transition temperature (i.e. transition peak temperature). The results are compared with the previously reported parameters found for recombinant human N-lobe and the single point mutant D63S of hTF/2N, which was found to be the most thermally unstable of the five human N-lobe mutants tested [30].

DSC of the native holo-oTF against the recombinant O-ONG samples showed that the changes made, i.e. substitution of the three Ala residues in the bridging peptide, elimination of the carbohydrate and the absence of four amino acids from the C-

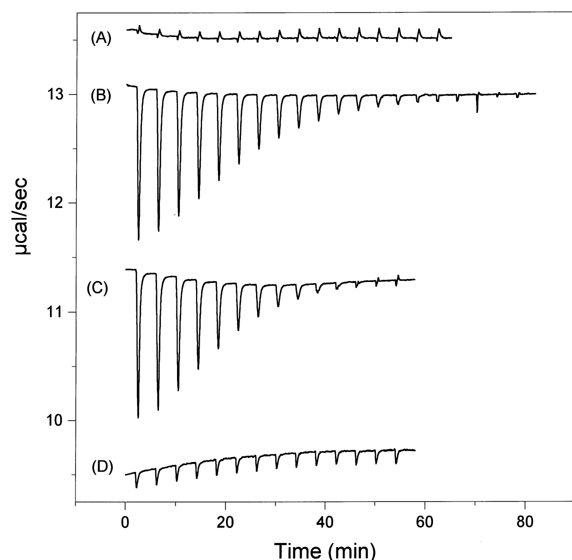

Figure 3 Raw data for ITC of various ferric oTF/2Ns with ferric oTF/2Cs used to determine the binding parameters in Table 6

(**A**) Fe-oTF/2N 1–319 titrated with oTF/2C; (**B**) Fe-oTF/2N 1–332 titrated with oTF/2C; (**C**) Fe-oTF/2N 1–340 titrated with oTF/2C. The concentration of Fe-oTF/2C 342-686 in the syringe was 0.396 mM for all three titrations and each peak represents a 10 μl injection. (**D**) Titration of Fe-oTF/2C 342–682 (0.015 mM) with Fe-oTF/2N 1–332 (0.21 mM) in the syringe. Each peak represents a 5 μl injection. All of the ITC experiments were performed at 28 °C in 0.1 M Hepes buffer, pH 7.5, containing 25 mM $NaHCO_3$. (1 μcal $\approx$ 4.2 μJ.)

terminus, had rather large effects on the thermal stability. The scans are shown in Figure 2 and the thermodynamic parameters are presented in Table 5.

To test the requirements for binding to the receptors on CEBRCs, an experiment was set up as decribed in the Materials and methods section in which the various samples were incubated for 20 min to reach equilibrium with cells that were treated to prevent the removal of iron. A representative experiment is shown in Table 6. ^{125}I-labelled diferric oTF, O-ONG 1–686 and O-ONG 1–682 all bound to the cells to approximately the same extent. In the same experiment the ability of ^{125}I-labelled Fe-oTF/2N 1–340, Fe-oTF/2N 1–332 and Fe-oTF/2N 1–319 to bind to chicken receptors was tested in the presence of Fe-oTF/2C 342–686, of Fe-oTf/2C 342–682 and by itself. The results show that: (1) both lobes are required to realize maximal binding, (2) the absence of the last four amino acids at the C-terminus of oTF/2C severely compromises binding, and (3) the oTF/2N lacking 14 amino acids from the C-terminus (oTF/2N 1–319) is unable to bind under any conditions. The entire experimental protocol was performed three times with differing concentrations of protein. The results were qualitatively identical in terms of the ability to bind or not.

Previous studies [29] showed that the association of proteolytically derived Fe-oTF/2N and Fe-oTF/2C could be measured by titration calorimetry. This approach was used in the present work to measure the ability of the various preparations to interact. The raw data are presented in Figure 3 and the thermodynamic parameters are given in Table 7. The only pairs for which association in solution could be measured were the recombinant oTF/2N 1–332 and oTF/2N 1–340 in the presence of oTF/2C 342–686. There was no indication of association with the oTF/2C lacking the final four amino acids or with the oTF/2N that stopped at residue 319.

Table 7 Best values for fitting parameters for binding of proteolytic or recombinant ferric oTF/2Ns to proteolytic Fe-oTF/2C 342–686 at 28 °C

No significant interaction between the oTF/2Ns and oTF/2C 342–682 could be detected from the ITC experiment under identical conditions. In addition, oTF/2N 1–319 showed no interaction with either oTF/2C under the same conditions; see Figure 3. Estimated uncertainties are ±10% for all three parameters.

	n	ΔH (kJ)	$10^{-5} \times K$ (M^{-1})
Proteolytic oTF/2N* 1–332	0.98	−83.6	3.0
Recombinant oTF/2N 1–332	0.94	−69.0	2.5
Recombinant oTF/2N 1–340	1.00	−71.1	3.4

* From Lin et al. [29].

DISCUSSION

Use of recombinant-DNA technology has led to the successful production of N-lobes of chicken transferrin of three different lengths. The recombinant proteins are homogeneous in size, show immunoreactivity with a domain-specific monoclonal antibody to the N-lobe, have the correct N-terminal sequence and spectral ratios (indicative of iron binding, which in turn is indicative of correct folding). The modified full-length recombinant oTFs (O-ONG 1–686 and O-ONG 1–682) were likewise expressed and purified to homogeneity.

Data from electrospray mass spectrometry confirm that there seem to be errors in the original report of Jelsch and Chambon [26]. Two of the changes in amino acid residues shown in Table 2 have been confirmed by peptide sequence data: the Leu residue at position 298 and the Ser residue at position 667 [36]. As mentioned by Williams et al. [36], polymorphisms in oTF seem to be common. Nevertheless it seems that the changes found are real differences in the published sequence that are not attributable to polymorphism.

DSC shows that all three recombinant oTF/2Ns are considerably more thermally stable than the proteolytic oTF/2N when the proteins are in the apo-form. The T_m values are 55.2 and 52.3 °C for the recombinant and proteolytic oTF/2Ns respectively. The relative thermal instability of the trypsin-derived oTF/2N is consistent with our experience over a number of years in which it has been found that the apo-N-lobe is much less robust than the trypsin-derived oTF/2C. With oTF/2N, iron removal is readily achieved but the apo-protein has often been difficult to reload with iron to yield the same spectral ratios as were found in the original sample. This might be the result of proteolytic nicking of oTF/2N produced by trypsinization. DSC of the iron-saturated oTF/2Ns indicates that the proteolytic oTF/2N has approximately the same T_m as the recombinant proteins oTF/2N 1–332 (75.0 compared with 74.7 °C). The T_m of the recombinant oTF/2N, oTF/2N 1–340, is higher (77.7 °C) but that of oTF/2N 1–319 is considerably lower (70.7 °C). These results seem to be consistent with the recently published crystal structure of diferric oTF [14] at 2.4 Å resolution, which indicates that helix 10 (residues 315–321) and helix 11 (residues 321–332) play a role in stabilizing the N-lobe of Fe_2oTF (see below). As shown in Table 3, recombinant human N-lobe is much more thermally stable than the recombinant chicken N-lobes. The presence of six disulphide bonds in the chicken N-lobe as opposed to eight in the human N-lobe undoubtedly contributes to the difference in structural stability.

The DSC results for the full-length constructs show that the change of amino acids in the bridging peptide, the elimination of the carbohydrate and deletion of the final four amino acids at the

C-terminus have only a small effect on the thermal stability of the apo-proteins. Apo-forms of O-ONG 1–686 and 1–682 showed a single thermal transition, with T_m values 0.6 and 1.9 °C lower than found for the native oTF. However, the DSC traces in Figure 2 show that the coupling of the two transitions for native apo-oTf is stronger than that observed for the two recombinant proteins. In the iron-bound form, the recombinant proteins show two thermal transitions as opposed to a single transition reported previously for native oTF, where the two transitions are strongly coupled [37]. Remarkably, the higher transition temperature for the two recombinant proteins is larger than the single transition measured for the oTF by 3 °C. This might be due to the change in the amino acid residues in the bridging peptide. As pointed out by Kurokawa et al. [14], the Pro-Ser-Pro sequence in the bridge residues only allows an extended conformation. Elimination of the final four residues at the C-terminus seems to weaken the lobe–lobe interaction considerably. This finding is consistent with a role for these residues in interacting with the N-lobe and stabilizing the overall structure [14].

Our previous work showed that both the N- and C-lobes of oTF must be present and associated to be recognized by the chick reticulocyte receptor and to result in binding and iron donation [21,22]. Studies by Williams and co-workers [23,24] challenged this work, claiming that prior association in solution is not a prerequisite for binding. In these studies the 'complexable' and 'uncomplexable' lobes were made by proteolysis. The complexable N-lobe was assumed to be oTF2/N 1–332 and the C-lobe oTF/2C 342–686; the uncomplexable N-lobe was assumed to be oTF/2N 1–319 and the C-lobe oTF/2C 342–682. The results with the recombinant proteins reported here agree with the previous work in terms of the ability of the various N- and C-lobes to complex or associate with each other in solution. Titration calorimetry provides a more elegant and quantitative approach than either the gel filtration or fluorescent binding assays described in the earlier studies.

The cell-binding studies presented in Table 6 clearly show that if the N- and C-lobes are unable to associate in solution they are unable to bind to receptors on CERBCs. Thus the oTF/2N that comprises residues 1–319 has no ability to bind alone or in the presence of either complete or truncated oTF/2C. This finding is not predicted by the crystallographic structure in which 'the major interactions between the two lobes involve hydrophobic residues Val310, Pro311, Leu313, Met314, Leu318 and Tyr319 in the N-terminal lobe and Ile382, Met385, Ile676, Met679, Phe682 and Leu683 in the C-terminal lobe' [14]. All of these residues are present in oTF/2N 1–319 and yet there is no detectable interaction between this N-lobe and either C-lobe. The interruption of helix 10 (315–321) and the absence of helix 11 are apparently strong factors in inhibiting or promoting the interaction between the two lobes. The oTF/2Ns comprising residues 1–332 and 1–340 are able to bind to about the same extent as holo-oTF in the presence of the complexable oTF/2C (residues 342–686), but show very low binding in the presence of uncomplexable oTF/2C (residues 342–682). These results seem to confirm the important observation made by Williams and co-workers [23,24] (and confirmed in the X-ray studies) that the amino acid residues that allow the two lobes to bind non-covalently to each other in solution reside in the C-terminal portion of each lobe.

There are a number of possible explanations for the discrepancy between the present work and the earlier results. In general, proteins produced by recombinant technology lead to more homogeneous products than proteins prepared by proteolysis. In particular, both SDS/PAGE and mass spectrometric analyses confirm the identity and the purity of the samples of recombinant proteins. The major difference between the two studies, however, is the use of ^{59}Fe in the work of Williams and co-workers and ^{125}I in our work to label the different lobes. To allow a direct comparison, our binding studies used about the same concentration of protein used in the earlier work at the plateau. In our experience, equilibrium binding studies with ^{59}Fe-labelled samples are difficult to do. Counting efficency is low (approx. 10–15 %). High specific radioactivities are necessary to ensure a reasonable radioactive count at the lower concentrations. These higher specific radioactivities can result in radiological damage to the protein, as indicated by high 'non-specific' binding when measuring ^{59}Fe-labelled N- or C-lobes alone. In the binding experiments presented by Oratore et al. [24], the non-specific binding of the lobes alone at the higher concentrations was greater than or equal to that of the combined lobes. This indicates a large amount of non-specific binding, which casts serious doubt on the validity of the results presented there. With the ^{59}Fe-labelled N-lobe there is the additional problem, mentioned above, of successfully resaturating this site with iron once the iron has been removed. An essential control in all binding experiments is the measurement of the binding of diferric oTF with the same cells under the same conditions to allow a assessment of the significance of the findings.

In conclusion it seems that preassociation of the N- and C-lobes is required for full binding to the chicken transferrin receptor.

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