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/oTF/2N were obtained for constructs defining residues 1–319, 1–332 and 1–337-(Ala)4, 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 these 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 studies have been made, the two lobes are an absolute prerequisite for binding to the receptor. 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°m 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 interlobe peptide, is non-glycosylated and is full length; oTF, ovotransferrin; oTF/2C, ovotransferrin C-lobe; oTF/2N, ovotransferrin N-lobe; Tm, transition peak temperature.

† To whom correspondence should be addressed.
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 into Bluescript (BS oTF/2N NotI/SmaI and BS oTF/2C NotI/SmaI respectively) as well as being ligated together into Bluescript to give the construct BS O-1 1–868.

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 NotI/SmaI. Oligonucleotide OTF-5 was used for the mutagenesis. The BamHI 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-O-1 1–868.

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 Smal at the 3’ end of the cDNA. A fragment created by digestion with BglII and Smal was cloned back into the original template to give the construct designated BS O-O-1 1–868.

To obtain an N-lobe construct suitable for expression, two stop codons were introduced into BS oTF/2N NotI/Smal. Two overlapping oligonucleotides (OTF-8 and OTF-9) were used to introduce the stop codons and an Smal at the 3’ end of the cDNA. The two adaptors were ligated together with the Smal/NotI fragment into the Smal site of Bluescript to create BS oTF/2N 1–340, which was then excised with Smal and ligated into the Smal 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 Smal at the 3’ end of the cDNA. The two adaptors were ligated together with the Smal/NotI fragment into the Smal site of Bluescript to create BS oTF/2N 1–340, which was then excised with Smal and ligated into the Smal site of 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 EcoRI and with Smal 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 Smal 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 EcoRI and with Smal 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 Smal site of Bluescript and sequencing to confirm the orientation and fidelity of the changes, the construct was ligated 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

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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.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTF-1</td>
<td>5′-ACA GCC GGG ATG AAG CTC ATC TGC AC-3'</td>
</tr>
<tr>
<td>OTF-2</td>
<td>5′-ACA GCG GCC GTA AGC TGA TCT TCC CCC A-3'</td>
</tr>
<tr>
<td>OTF-3</td>
<td>5′-ACA CCC GGG AGG GCC TCT CCC TCC TTA TA-3'</td>
</tr>
<tr>
<td>OTF-4</td>
<td>5′-ACA ACC GGG GGC CGA AGA AAC AGG ATC CTA T-3'</td>
</tr>
<tr>
<td>OTF-5</td>
<td>5′-GGC TGG ATT GAG GAC AGA ACA GGC ACC-3'</td>
</tr>
<tr>
<td>OTF-6</td>
<td>5′-ACA CCC GGG TCA TTA GAA GCT GCA CAT GAG-3'</td>
</tr>
<tr>
<td>OTF-7</td>
<td>5′-AAA ATC CTC GAT CTC CTA GAG-3'</td>
</tr>
<tr>
<td>OTF-8</td>
<td>5′-GCA GCC CTA TAA TGA CCC-3'</td>
</tr>
<tr>
<td>OTF-9</td>
<td>5′-GGC TCA TTA GCC-3'</td>
</tr>
<tr>
<td>OTF-10</td>
<td>5′-ACA CCC GGG TCA TTA CCG CAT GCT GTG GGC-3'</td>
</tr>
<tr>
<td>OTF-11</td>
<td>5′-GAG TGG CTC CTC ACC CGG TC-3'</td>
</tr>
<tr>
<td>OTF-12</td>
<td>5′-ACA CCC GGG TCA TTA GTA GAG CTG GCA ATC CAT-3'</td>
</tr>
<tr>
<td>OTF-13</td>
<td>5′-AAG TGT TGG AGG CAG GCG TT-3'</td>
</tr>
</tbody>
</table>

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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-Sephael 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 Polyion 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 loaded with 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 Hapes, 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 Hapes, 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$_2$-oTF 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-toluene-p-sulphonamidobutan-2-one (Warthington 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 amphotelys (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$_4$ HCO$_3$ and a Polyion 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 I–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$_4$Cl 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/2C (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$_4$Cl-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 polypyrrole 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]

<table>
<thead>
<tr>
<th>Nucleotide sequence number</th>
<th>Previous work</th>
<th>Present work</th>
<th>Amino acid change</th>
<th>Net change in molecular mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>374</td>
<td>ATT → GTT</td>
<td>Ile → Val</td>
<td>14.03</td>
<td></td>
</tr>
<tr>
<td>472</td>
<td>TAC → AGC</td>
<td>Thr → Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>530</td>
<td>CTC → GTC</td>
<td>Leu → Ile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>536</td>
<td>TGG → TGG</td>
<td>Trp → Arg</td>
<td>30.03</td>
<td></td>
</tr>
<tr>
<td>792</td>
<td>CTC → TCT</td>
<td>Leu → Gin</td>
<td>14.97</td>
<td></td>
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<tr>
<td>796</td>
<td>ATC → AAT</td>
<td>Asn → Lys</td>
<td>14.07</td>
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<tr>
<td>803</td>
<td>TAT → TAC</td>
<td>Tyr → Tyr</td>
<td></td>
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</tr>
<tr>
<td>1027</td>
<td>TTG → TAC</td>
<td>Leu → Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2133</td>
<td>AAC → AGC</td>
<td>Asn → Ser</td>
<td>27.03</td>
<td></td>
</tr>
</tbody>
</table>

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

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

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

**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-IONG 1–686; lane 4, recombinant O-IONG 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.

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 described 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 $A_{365}/A_{402} = 26.9$ and $A_{365}/A_{410} = 1.39$. Typical ratios for transferrin are $A_{365}/A_{402} \approx 20$ and $A_{365}/A_{410} = 1.4$.

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

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Apo-form</th>
<th>Iron-bound form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta H_{\text{cal}}$ (kJ/mol)</td>
<td>$T_m$ (°C)</td>
</tr>
<tr>
<td>Recombinant oTF/2N 1–340</td>
<td>561</td>
<td>55.2</td>
</tr>
<tr>
<td>Recombinant oTF/2N 1–332</td>
<td>527</td>
<td>55.2</td>
</tr>
<tr>
<td>Recombinant oTF/2N 1–319</td>
<td>510</td>
<td>55.2</td>
</tr>
<tr>
<td>Proteolytic oTF/2N</td>
<td>598</td>
<td>50.9</td>
</tr>
<tr>
<td>Recombinant hTF/2N†</td>
<td>929</td>
<td>66.4</td>
</tr>
<tr>
<td>Recombinant hTF/2N D63S†</td>
<td>690</td>
<td>65.6</td>
</tr>
</tbody>
</table>

* 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: ±10% for $\Delta H_{\text{cal}}$ and ±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/n. For clarity, DSC traces have been arbitrarily shifted on the ordinate scale. (1 cal = 4.2 J.)

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-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, $\Delta H_{\text{cal}}$, 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 $\Delta H_{\text{cal}}$ 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-
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 described 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. $^{131}$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 Fe-oTF 2N and Fe-oTF 2C 342–686 to bind to chicken receptors was tested in the presence of Fe-oTF 2N 1–332 and Fe-oTF 2N 1–319 to binding proteins oTF 2N severe 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 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**

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>$\Delta H$ (kJ)</th>
<th>$10^{-3} \times K$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic oTF 2N* 1–332</td>
<td>0.98</td>
<td>−83.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Recombinant oTF 2N 1–332</td>
<td>0.94</td>
<td>−69.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Recombinant oTF 2N 1–340</td>
<td>1.00</td>
<td>−71.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* 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 Chambron [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-oTF (see below). As shown in Table 3, recombinant human N-lobe is much more thermally stable than the proteolytic oTF 2Ns are con-
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 oTF/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 \(^{59}\text{Fe}\) in the work of Williams and co-workers and \(^{125}\text{I}\) 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 \(^{59}\text{Fe}\)-labelled samples are difficult to do. Counting efficiency 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 \(^{59}\text{Fe}\)-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 \(^{59}\text{Fe}\)-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 an 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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REFERENCES


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