Pulsed ELDOR Determination of the Intramolecular Distance Between the Metal Binding Sites in Dicupric Human Serum Transferrin and Lactoferrin

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Characterisation of recombinant unglycosylated human serum transferrin purified from *Saccharomyces cerevisiae*

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Abstract

Structural identity between a recombinant transferrin mutant (N413Q, N611Q) secreted from *Saccharomyces cerevisiae* and the native protein was shown by CD analysis and immunodiffusion assays against anti-hSTf. The ability of the recombinant protein to bind iron was confirmed by urea–PAGE and EPR analysis of the iron-saturated protein revealed the characteristic holo-transferrin spectrum, indicating conservation of both iron-binding sites. The integrity of the unglycosylated recombinant protein indicates that such protein could be a valuable tool not only for structure–function characterisation but also crystallisation assays. In addition, the recombinant transferrin was found to be as effective as native transferrin as a growth factor in cell culture medium.

Introduction

The transferrins (Tfs) constitute a family of homologous monomeric glycoproteins of molecular weight approximately 80 kDa that have an important role in iron metabolism due to their ability to bind iron (Baker *et al.* 2003). Even though transferrins are abundant in nature, the family can be divided into just four main members: the serum transferrins, which have a role in iron transport around the body, the lactoferrins, found in milk, tears and other bodily secretions of numerous mammals, the ovotransferrins, found in avian egg white, and the melanotransferrins, found on the surface of melanocytes (Bullen *et al.* 1999). These proteins are thought to originate from a common ancestor and to have evolved, through gene-duplication, into bilobal molecules, with each N- and C-lobe containing a deep cleft capable of reversibly binding one metal ion. The residues involved in the metal binding are conserved amongst all the transferrins. In each lobe, two tyrosine residues, one aspartic acid and one histidine residue have been found to be involved in iron binding, along with two oxygens from a synergistically bound carbonate anion (Baker *et al.* 2002). Although these proteins share identical iron-binding residues and high sequence homology, substantial differences in the binding affinity for iron both within and between the transferrins are found, but not well understood (Adams *et al.* 2003).

Within the transferrin family, human serum transferrin (hSTF) provides not only a means of

transporting iron from the sites of absorption and storage to the sites of utilisation, but also a protection against the damaging effects of iron-catalysed free radical cascades (Von Bonsdorff *et al*. 2001). Its role in iron homeostasis is therefore essential, but although the protein was identified more than half a century ago (Schade and Caroline 1946) its complete mechanism of iron loading and releasing is still to be elucidated. In order to study the factors affecting this mechanism, the expression of recombinant full-length transferrin, as well as the truncated protein, has proved to be an important tool (Mason *et al*. 2001). The presence of 19 disulfide bonds present in serum transferrin implies that the production of such recombinant protein might not easily be achieved in microorganisms. As a secreted protein, the presence of a leader sequence to correctly target the expressed protein out of the cell, can also be another constraint. Several expression systems have been tried in different organisms, with a number of laboratories reporting success (Evans *et al*. 1978; Mason *et al*. 2001). The most common system, which uses baby hamster kidney cells (BHK) (Mason *et al*. 2001), has been shown to be successful but due to the relatively low yield obtained, other systems have been investigated. Bacterial expression systems have also been reported in the literature, but the final yield of functional protein was unsatisfactory for in-depth structure–function studies (Ideda *et al*. 1992; DeSmit *et al*. 1995). In order to mimic the native human expression still using microorganisms, hSTf has been expressed in the methylotrophic yeast *Pichia pastoris*. Functional hSTf N-lobe was efficiently produced with a satisfactory high yield but the expression of the full-length protein in yeast was found to be unsuccessful (Steinlein *et al*. 1995). Such a result is very unfortunate since a reliable expression system in yeast has the potential to achieve a higher yield than the previously less easily sustainable mammalian systems. In addition, it would not only be important for the availability of a homogeneous stock of fully functional protein, but also making it possible to study the importance of specific residues by site-directed mutagenesis and to determine whether they contribute structurally or functionally to the transferrin integrity. The expression of non-glycosylated recombinant hSTf would lead to a more homogenous population of protein, which has been suggested to be a requirement for obtaining crystals suitable to solve the structure of the full-length hSTf (Mason *et al*. 1993).

In the present study, we report the structural and functional characterisation of a recombinant full-length hSTf from *Saccharomyces cerevisiae* with mutations (N413Q, N611Q) that knock out its two N-linked glycosylation sites (comparatively with native hSTf). In addition, native and recombinant serum transferrins are compared for their ability to be used as a growth factor in cell culture media for a source of iron for cells (Koivisto *et al*. 2004).

Materials and methods

Purification

A partially purified eluate containing unglycosylated recombinant transferrin (N413Q, N611Q) was obtained from Delta Biotechnology Ltd. The protein was further purified in a single step using immunoaffinity chromatography (Evans *et al*. 1994). The protein was eluted using 1 M ammonia solution and then desalted on a Sephadex G25 column equilibrated with 50 mM ammonium bicarbonate before being freeze-dried. Purity of proteins was determined by SDS–polyacrylamide gel (SDS–PAGE) (8%) using the discontinuous method adapted from (Laemmli 1970).

Electrospray ionization mass spectroscopy (ESI-MS)

Samples were prepared in 20 mM NH_4HCO_3 and analysed using an ESI-Quadrupole-TOF mass spectrometer (Applied Biosystems, QStar) by the Proteomic Research Group, The Babraham Institute, Cambridge.

Urea–PAGE

Polyacrylamide gel electrophoresis in 6 M urea was carried out using the method previously described (Evans *et al*. 1980, 1982).

UV/Visual spectroscopy

Absorption spectra of native and recombinant human serum transferrins were recorded over the range of 300–700 nm using a Shimadzu

UV–Visible Recording Spectrophotometer (UV-260). The protein was fully saturated with iron, using Fe(III)$(NTA)_2$ following its resuspension in 0.02 M $NaHCO_3$ at 10 mg/ml. Unbound iron was removed by ultrafiltration using Amicon Ultra-15 (300,000 MWCO) centrifugal filter units. Measurements were made in quartz cuvettes with a 10 mm path length.

Electron Paramagnetic Resonance (EPR) spectroscopy

EPR spectra were obtained using a Bruker ELEXSYS E500 spectrometer. The protein sample (60 mg/ml) in 50 mM $NaHCO_3$, was frozen in 3 mm diameter quartz EPR tubes in liquid nitrogen. The microwave frequency was 9.38 GHz and the field set at 2499G with modulation amplitude at 0.1 mT. The sample was scanned twice using a microwave power of 10 mW over 4000 G with a receiver gain of 2×10^3.

Circular dichroism analysis

Circular dichroism spectra were acquired using a Jasco J720 spectropolarimeter, scanning in the 200–260 nm regions (0.02 cm cell path length). Protein solutions were made at 0.5 mg/ml in 50 mM $NaHCO_3$. All spectra were corrected for solvent baseline and, where appropriate, normalised for concentration and path length (mean molecular weight = 113).

Immunodiffusion

Sheep antiserum raised against human serum transferrin (Micropharm Ltd, UK) was assayed against native and recombinant proteins using the precipitin reaction in 1% agar gel containing 50 mM Tris pH 7.8.

Cell proliferation assay

Cellular proliferation was determined by quantification of 5-bromo-2′-deoxy-uridine (BrdU) integrated into cellular DNA. TRVb-1 cells (McGraw *et al*. 1987) (kindly provided by Dr. H. Kawabata, Kanazawa Medical University Uchinada-machi, Ishikawa-ken, Japan) or HepG2 cells were seeded in 6-well tissue culture plates at a density of 5×10^4 cells per well and grown to 50% confluence in F12HAM (Invitrogen, Paisley, UK) or RPMI160 (Invitrogen, Paisley, UK), respectively [supplemented with 10% foetal bovine serum (FBS) 2 mM glutamine and penicillin/streptomycin]. Cells were then synchronised by cultivation in the absence of FBS for 24 h. Immediately after addition of transferrin or FBS to the cells, BrdU (Roche Applied Sciences, Lewes, UK), at a final concentration of 10 μM, was added and the cells were grown for further 16 h. Integration of BrdU into cellular DNA was then quantified immunometrically using the BrdU detection Kit III (Roche Applied Sciences, Lewes, UK) according to the manufacturer's directions.

Results and discussion

Of the transferrin family, hSTf is now one of the best-characterised proteins, but its mechanism of iron loading and release is still poorly understood. hSTf is not only one of the most important proteins of iron homeostasis as the main iron transporter in the body, but it is also used extensively as an animal cell culture medium additive. In order to produce a homogenous population of deglycosylated proteins, which has been suggested to improve the quality of crystallisation (Pace *et al*. 1990; Mason *et al*. 2001), the double mutant (N413Q, N611Q) was used. Insertion of both mutations has been shown to compromise neither the structural integrity of the protein nor its ability to bind iron and the transferrin receptor (Mason *et al*. 1993).

A partially purified eluate containing recombinant transferrin (N413Q, N611Q) was further purified using affinity chromatography (Figure 1), and after purification, the final yield was estimated to be of 1.5 mg/ml of original culture fluid. The purity and molecular weight of the purified recombinant hSTf were checked using ESI-MS (Figure 2). As shown in Figure 2, several peaks were obtained, which correspond to a heterogeneous population of the four different forms of iron-bound transferrin, and also to possibly post-translational modifications, such as varying number of O-linked mannoses present due to glycosylation. Since the presence of one Fe^{3+} and therefore one CO_3^{2-} ion, or one molecule of mannose on the protein increases its MW by 118 Da or by 162 Da respectively, the maximum

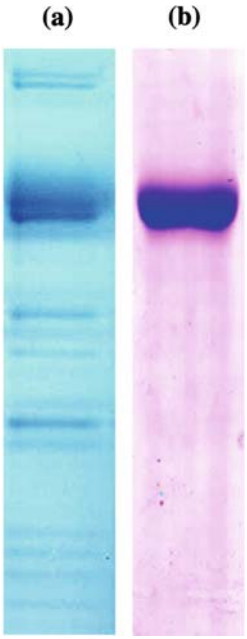

Figure 1. 8% SDS–PAGE gel of research grade recombinant hSTf purification: (a) partially purified eluate and (b) affinity chromatography eluate.

number of mannoses attached to hSTf was deduced to be three.

The CD spectra of native and recombinant proteins were found to be superimposable, which implies that both secondary structure content were identical and suggests that the recombinant hSTf had conserved its correct fold (Figure 3). In immunodiffusion experiments, a polyclonal sheep antiserum raised against native hSTf reacted identically with both native and recombinant proteins, which indicates the same antigenicity and again is consistent with an identical structure for both (Figure 4)

The integrity of the iron-binding site was verified by electron paramagnetic resonance spectroscopy (EPR). The transferrin iron-binding site has a very characteristic double peak spectrum (Hoefkens *et al.* 1996) and its line shape is a sensitive indication of conformation of the iron-binding site. The spectra obtained for both the recombinant and the native hSTf are identical and contain typical spectrum of transferrin, which is a strong indication that the integrity of

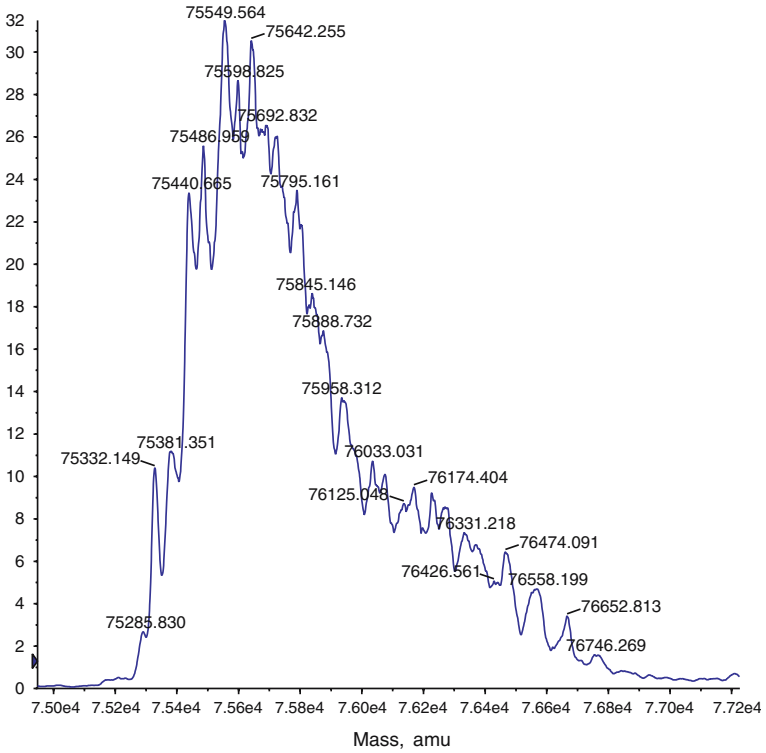

Figure 2. ESI-MS of partially Fe^{3+}-loaded recombinant hSTf. The molecular weight (Da) corresponding to each protein is indicated for each peak.

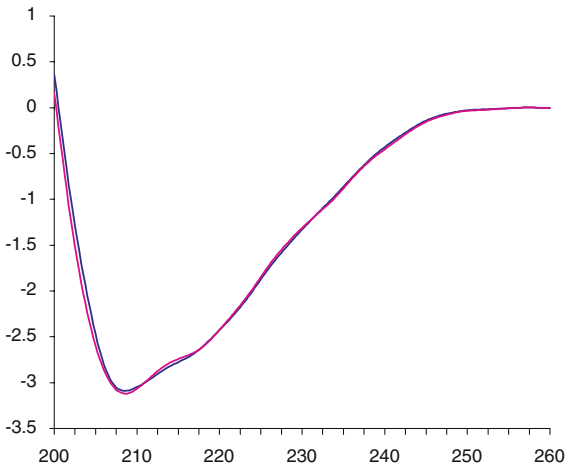

Figure 3. CD Spectra of the apo-native (—) and the apo-recombinant (...) hSTf between 200 and 260 nm.

the iron-binding site has been conserved (Figure 5) and that the iron atoms are bound identically in both proteins.

The ability to bind and retain iron for recombinant protein was checked by urea–PAGE analysis as described by Evans *et al*. 1980. For both the native and recombinant proteins, the two holo- and apo-forms can clearly be distinguished (Figure 6). The isoform that migrates the furthest

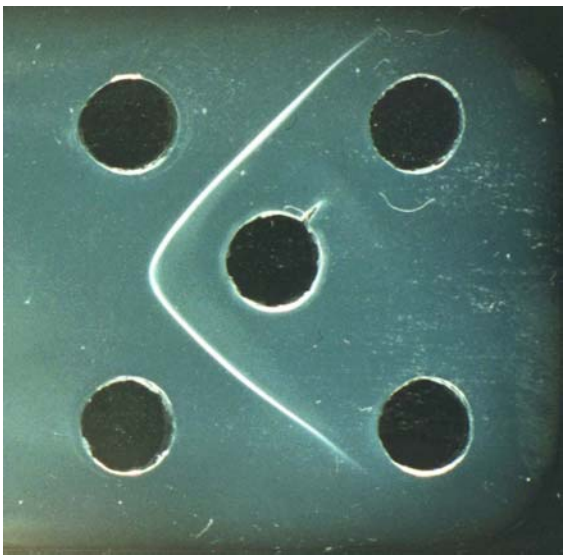

Figure 4. Precipitin reaction of recombinant human serum transferrin with native human serum transferrin antiserum. Centre well contains10 μl sheep anti-transferrin antiserum; the outer wells (clockwise from top right) contain yeast culture medium in the first two wells, followed by native human serum transferrin and finally recombinant human serum transferrin.

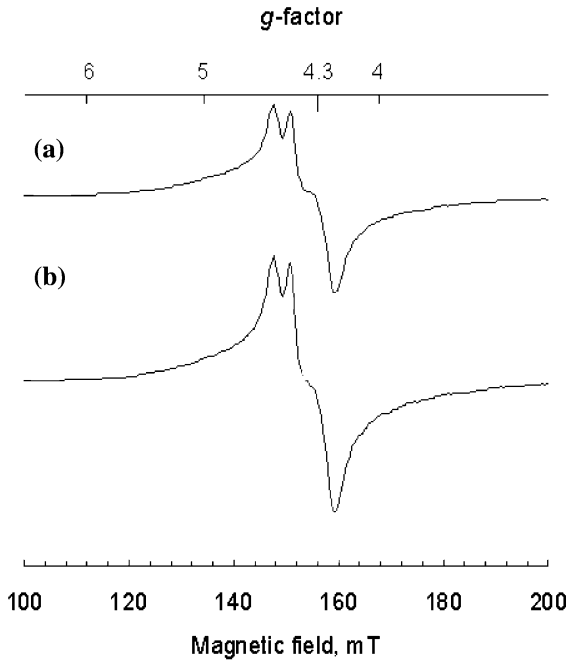

Figure 5. EPR spectra of diferric native (a) and diferric recombinant (b) hSTf (60 mg/ml in 50 mM $NaHCO_3$).

corresponds to the diferric form of hSTf, whereas the slower moving isoform corresponds to the apo-hSTf. The relative lower mobility of both

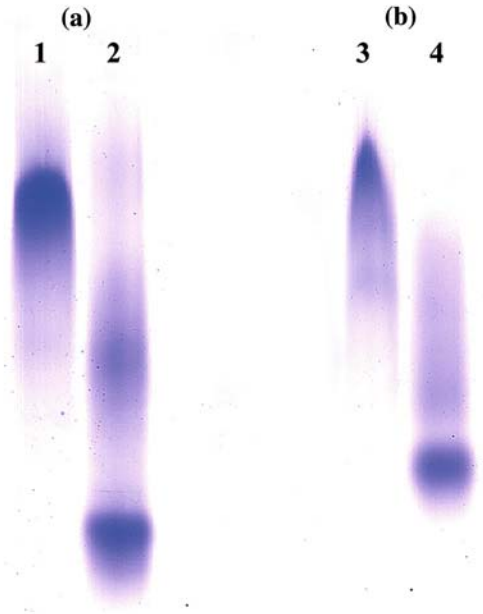

Figure 6. Electrophoresis of native (a) and recombinant (b) on a 6 M urea–PAGE isoforms 1 and 3 correspond to apo hSTf whereas, 2 and 4 correspond to diferric hSTf.

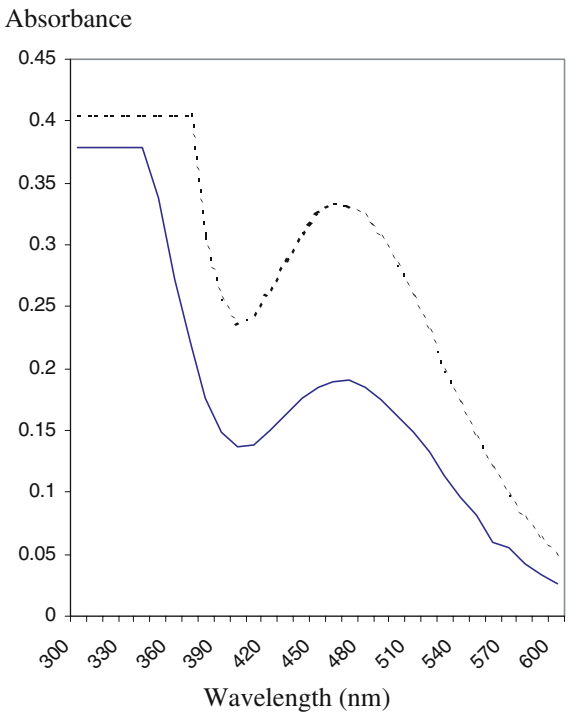

Figure 7. Absorbance spectra of iron-saturated samples of native (—) and recombinant (...) hSTf from 300 to 700 nm at concentrations of approximately 1 mg/ml.

forms of the recombinant hSTf is to be expected, as it lacks the four negatively charged sialic acid residues that are present at the ends of the two branched N-linked glycans of the native protein (Evans *et al*. 1982). The integrity of iron binding was further checked by measuring the absorbance, for both proteins, between 300 and 600 nm, in the presence of iron since native diferric transferrin has a broad absorbance maximum between 465 and 470 nm. As expected, the maximum of absorbance was obtained in the same region for both proteins, between 460 and 470 nm (Figure 7), with a slight variation due to differences in protein concentration.

To further compare the recombinant with the native protein for its biological application, a cellular proliferation assay was performed using both proteins as growth factors. The results showed that the recombinant transferrin was as potent as serum transferrin from other sources in its ability to support cellular proliferation (Figure 8).

Based on the biophysical results described in the present study, not only was the secondary structure content of the recombinant protein was shown to be identical to the native protein by CD analysis, but also its reactivity against antiserum

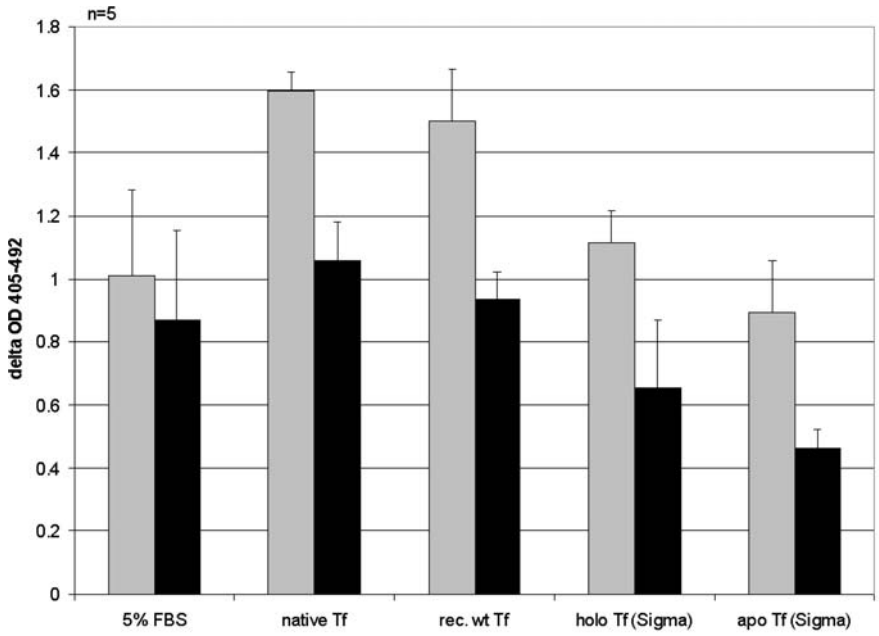

Figure 8. Cell proliferation assay. The effect of using recombinant transferrin (rec. wt. Tf) on the cellular proliferation of TRVb-1 (grey) and HepG2 (black), was compared to the effect of different sources of serum transferrin: serum purified native transferrin (native Tf), native holo-transferrin from Sigma (holo Tf) and native apo-transferrin from Sigma (apo Tf).

raised against native hSTf were identical further confirming structural integrity and correct folding of the recombinant protein. When conservation of the iron-binding site was investigated, EPR results revealed a characteristic spectrum characteristic of the native hSTf, identical to the native protein. In addition, electrophoresis of both proteins on urea denaturing PAGE showed identical behaviour with the presence of both forms, apo- and holo-proteins, confirming the ability of the recombinant protein to bind iron reversibly.

With the substantial amount of protein now available, crystallisation trials are in progress in an attempt to solve the 3D structure of the full-length protein in its iron-free and iron-loaded forms.

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