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Structure and thermal stability of the extracellular fragment of human transferrin receptor at extracellular and endosomal pH

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Abstract

Fourier transform infrared spectroscopy has been used to study the solution structure and thermal stability of the extracellular fragment of human transferrin receptor (tfRt) at extracellular and endosomal pH. At extracellular pH tfRt is composed of 56% α-helix, 19% β-sheet and 14% turns. Upon acidification to endosomal pH the α-helical content of the protein is reduced and the β-sheet content increased by nearly 10%. At extracellular pH, the midpoint temperature of thermal denaturation (T_m) for the loss of secondary and tertiary structure, and the formation of aggregated structures, is 71°C. At endosomal pH this temperature is reduced by ≈ 15°C. The apparent entropies of thermal denaturation indicate that the native structure of tfRt at endosomal pH is far more flexible than at extracellular pH.

Key words: Fourier transform infrared spectroscopy; Secondary structure; Tertiary structure; Thermal stability; pH-dependent conformational change

1. Introduction

The pathway of iron uptake into the vast majority of cell types involves the preferential binding of diferric transferrin with its receptor at the pH of the plasma; under these conditions the affinity of apo-transferrin for its receptor is nearly two orders of magnitude lower than that of the diferric protein [1]. The transferrin–transferrin receptor complex is then internalised via clathrin-coated pits, and the pH of the endosomes is lowered to around pH 5.6 by means of a putative proton pump [2]. At this lower pH, iron is released from one or both of the sites on transferrin and transported to cellular compartments by as yet unknown mechanisms. The resulting apo/monoferric transferrin remains bound to the receptor and is recycled back to the cell surface where, at the higher pH of plasma, it is released. At the pH of the cell surface, the receptor has been shown to retard iron release from receptor-bound transferrin [3]. However, at the pH of the endosome the receptor has the reverse effect on iron release from transferrin, accelerating the loss of iron 5-fold. Clearly the pH-dependent interaction of transferrin with transferrin receptor is of prime importance in governing the delivery of iron to cells.

Only very limited information is available on the structure of transferrin receptor. Transferrin receptor (tfR) is a transmembrane protein that is expressed by most mammalian cells. The human transferrin receptor comprises two identical, disulphide-linked, glycosylated subunits of approximately 95 kDa (for a recent review see [4]). Each subunit has an extracellular domain of 671 amino acids, a single 28-residue putative transmembrane region and a 61-residue amino-terminal cytoplasmic domain. The human transferrin receptor can be purified from term placentae as a detergent-solubilised, disulphide-linked dimer of molecular weight 185 kDa [5]. Upon digestion with trypsin a water-soluble 70 kDa monomeric fragment is generated (tfRt) which contains 95% of the external region of the transferrin receptor [5]. This soluble form of the receptor retains the ability to bind transferrin [5–7] and forms a non-covalent dimer in solution [5,6].

As yet attempts to produce large stable crystals of tfRt have been unsuccessful [7]. Biochemical data suggest that when the pH is reduced to 5.6, tfRt undergoes a conformational change which results in self association. This self association is prevented by the presence of non-ionic detergent [6]. This conformational change is poorly characterised and only very preliminary circular dichroism (CD) data are available [6].

In this study we characterise the structure of tfRt in more detail. The pH-dependent conformational transitions of tfRt, as well as the thermal stability of the protein, have been investigated by Fourier transform infrared spectroscopy (FTIR).

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Abbreviations: CD, circular dichroism; FTIR, Fourier transform infrared spectroscopy; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulp honic acid]); MES, (2-[*N*-morpholino]ethanesulfonic acid); SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; tfR, human placental transferrin receptor; tfRt, trypsin cleavable fragment of human placental transferrin receptor.

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2. Materials and methods

2.1. Purification of human transferrin receptor

Human transferrin receptor was purified from term placentae by means of previously described techniques [5]. The purity of these samples was similar to those previously reported [5]. The soluble fragment of transferrin receptor (tfRt) was generated by incubation of the intact receptor with trypsin [6]. The fragment was finally purified by HPLC using an analytical TSK G3000 SWXL gel-filtration column. Fig. 1 shows that samples of tfRt used for structural studies gave a single band when analysed by SDS-PAGE and cross-reacted with the monoclonal antibody OKT9 on Western blots.

2.2. SDS-PAGE and Western blotting

SDS-PAGE was carried out essentially according to the method of Laemmli [8]. Samples (typically 7.5 μg) were run under reducing conditions (10% separating gel) using an LKB vertical slab gel system. For Western blot analysis the protein was transferred to nitrocellulose membrane using a Bio-Rad semi-dry electroblotter. Transferred protein was visualised with the monoclonal antibody OKT9 and alkaline phosphatase-conjugated donkey anti-mouse IgG.

2.3. FTIR spectroscopic studies

FTIR spectra were recorded using a Perkin-Elmer 1750 FTIR spectrometer equipped with a fast recovery TGS detector and a Perkin-Elmer 7300 Data Station as described previously [9]. All spectra were recorded at a resolution of 4 cm^{-1}. Buffer subtraction was carried out digitally to give a straight baseline in the 2000–1800 cm^{-1} area. Further details on solvent subtraction can be found in previous publications [10].

The broad amide I bands were analysed using second-derivative and deconvolution techniques. Second-derivative spectra were calculated using a 13 data point Savitzky-Golay smoothing window (13 cm^{-1}) using the Perkin-Elmer DERIV function. Deconvolution of spectra was performed using the Perkin-Elmer ENHANCE function. Typically a band width of 16 cm^{-1} and a resolution enhancement factor of 2.25 were used.

Quantitative analysis of the thermally induced changes in protein structure as reflected in the FTIR spectra was performed using global analysis [11]. In this method, curve fitting in terms of skewed Gausians is combined with linking of the spectral shape parameters across temperature. This method has the advantage that no deconvolution or derivative of the original spectra is required. Assuming a one-step transition the estimated band amplitudes were then fitted with a two-state thermodynamic model [12,13]. This yields the midpoint of the transition (T_m) and the van't Hoff enthalpy (ΔH_{VH}).

3. Results

3.1. Structure

The absorption spectra of tfRt recorded at pH 7.4 and pH 5.6 are shown in Fig. 2A. Both spectra show an amide I maximum at 1656 cm^{-1}. However, the spectrum recorded at pH 5.6 is broader and there appears to be increased absorption in the low frequency part of the amide I region. Qualitative information on the secondary structure of a protein can be obtained from an analysis of its amide I band (1700–1600 cm^{-1}) using derivative techniques. The second-derivative spectra of tfRt at pH 7.4 and pH 5.6 are shown in Fig. 2B. Similar bands can be seen in both spectra and these can be assigned as follows: 1656 cm^{-1} α-helical and/or random coil structures [10,14–16], 1639 cm^{-1} and 1694/1692 cm^{-1} β-sheet [10,16], 1679/1678 cm^{-1} turns [10,16]. The amide II band at 1550 cm^{-1} can not be assigned unambiguously to any particular secondary structure. The components at 1516 cm^{-1} are due to the vibration of tyrosine side chains. Spectra of tfRt were also recorded in ^2H_2O at pH 7.4 and pH 5.6. Analysis of these spectra (not shown) using second-derivative techniques revealed that, like spectra in H_2O (Fig. 2B), major bands can be seen at 1656 cm^{-1} and 1639 cm^{-1}. These are consistent with the presence of α-helical and β-sheet structures, respectively.

The absorption spectra of the protein in H_2O at both pH's were quantitatively analysed for secondary structure content by means of a multivariate linear model [17], and the results are shown in Table 1. Similar results were also obtained when the spectra were analysed using factor analysis [18]. tfRt is predominantly α-helical with substantial β-sheet and turns content. There is, however, a reduction in the α-helical and an increase in β-sheet content upon lowering the pH. Although the magnitude of the difference is close to the uncertainty of the technique, these results are in agreement with the increased absorption in the β-sheet region of the absorption spectrum ($\approx$ 1635 cm^{-1}) recorded at pH 5.6 (Fig. 2A).

3.2. Thermal stability

In order to study the conformational stability of transferrin receptor, spectra of tfRt at pH 7.4 and pH 5.6 in ^2H_2O were recorded over a linear temperature gradient from 20 to 80°C. The intensity of specific bands in the deconvolved spectra are plotted against temperature in Fig. 3. Several observations can be made. At a given pH the bands attributed to the main secondary structural elements (α-helix 1655 cm^{-1}, β-sheet 1638 cm^{-1}) reduce

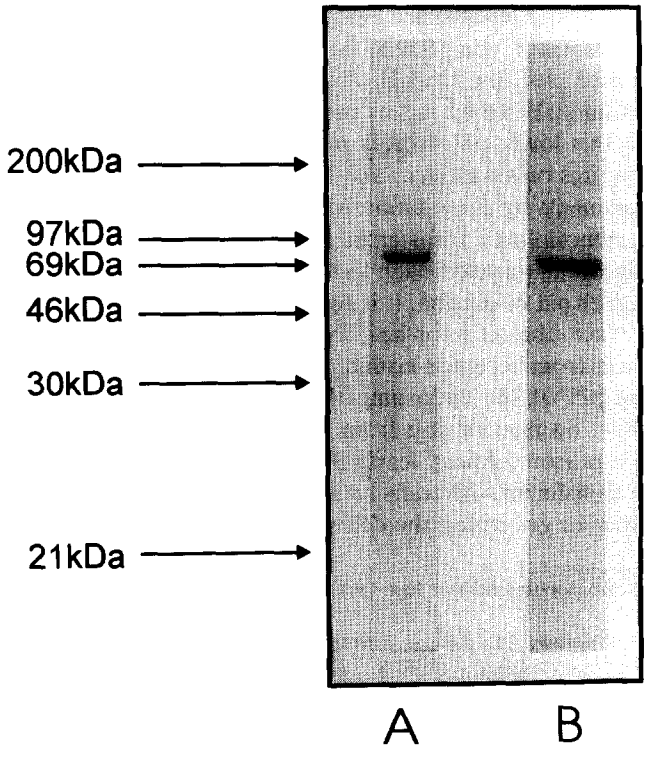


Fig. 1. SDS-PAGE (A) and Western blot (B) analysis of purified tfRt.

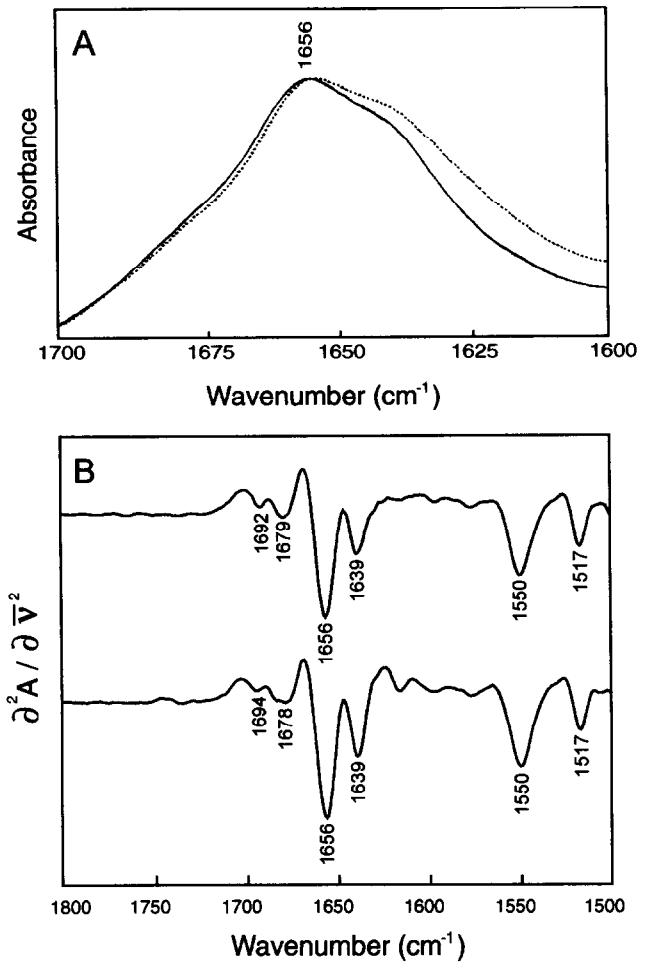


Fig. 2. (A) FTIR absorption spectra of tfRt at pH 5.6 (....) and pH 7.4 (—) in H_2O, 20°C. (B) Second-derivative FTIR spectra of tfRt at pH 5.6 (top trace) and pH 7.4 (bottom trace) in H_2O. Spectra were recorded at a protein concentration of approximately 35 mg/ml in 20 mM HEPES, 0.154 M NaCl (pH 7.4) or 20 mM MES, 0.154 M NaCl (pH 5.6).

in intensity simultaneously. Concomitant with this, bands attributed to aggregated structures that result upon thermal denaturation of proteins (1617 cm^{-1} and 1683 cm^{-1}) appear in the spectra. The onset of these transitions occur at a much lower temperature at pH 5.6 (≈ C38°C) than at pH 7.4 (≈ 62°C). In addition, the rates of the thermal transitions are different at the two pH's studied. It is interesting to note that a sudden loss in intensity of the residual amide II band at 1550 cm^{-1} coincides with the loss of secondary structure and the appearance of aggregated structures.

Table 1
Secondary structure of the tfRt estimated from FTIR

Protein	α-Helix	β-Sheet	Turns	Other
tfRt, pH 7.4	56 (2)	19 (3)	14 (2)	10 (2)
tfRt, pH 5.6	48 (3)	25 (4)	15 (3)	10 (2)
Transition				
pH 7.4 → 5.6	− 8	+ 6	+ 1	0

Values in brackets indicate estimated errors (S.D.).

Once the samples had been heated to 80°C they were cooled back to 20°C. No spectral changes were observed upon cooling, indicating that at both pH values studied thermal denaturation of tfRt was irreversible.

The variation in the denaturation properties of tfRt at pH 7.4 and pH 5.6 was quantified from the infrared spectra using global analysis [11]. The results are summarised in Table 2 and, where applicable, are in agreement with those shown in Fig. 3.

4. Discussion

This is the first study to estimate the secondary structural content and thermal stability of tfRt. At pH 7.4 tfRt

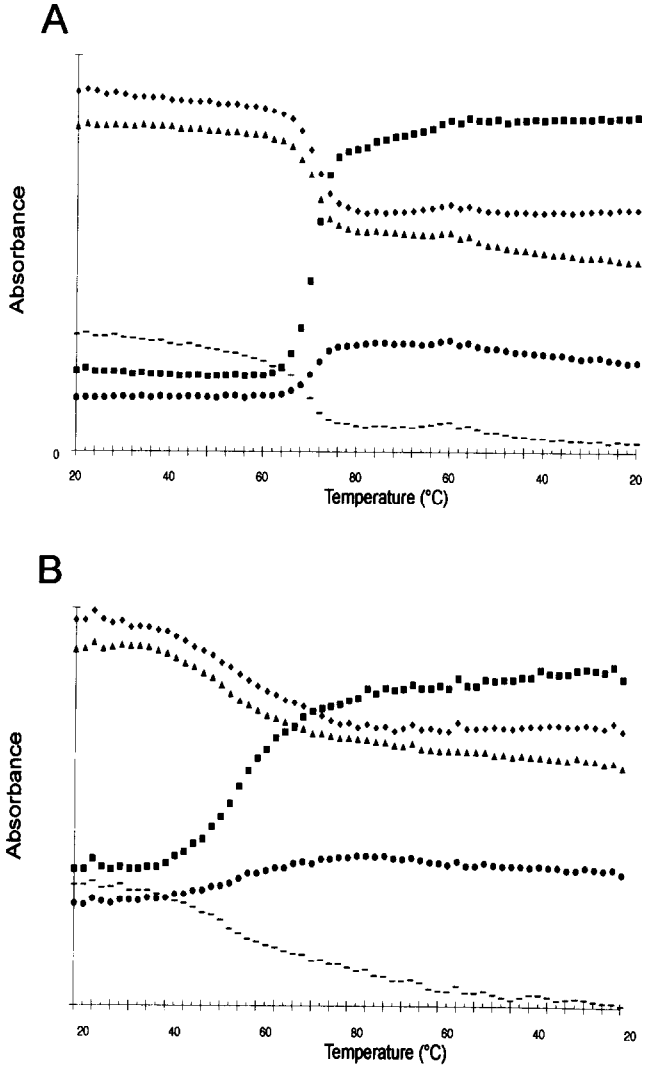


Fig. 3. Temperature-dependent intensity of selected bands in the deconvolved spectra of tfRt at pH 7.4 (A) and pH 5.6 (B) in ^2H_2O. (■) Aggregated structures, 1617 cm^{-1}; (▲) α-helix; (♦) β-sheet, (–) amide II; (●) aggregated structures, 1683 cm^{-1}. Spectra were recorded at a protein concentration of approximately 50 mg/ml in 20 mM HEPES, 0.154 M NaCl (pH 7.4) or 20 mM MES, 0.154 M NaCl (pH 5.6).

is estimated to comprise 56 ± 2% α-helix, 19 ± 3% β-sheet, 14 ± 2% turns and 10 ± 2% undefined structures. Upon acidification of tfRt to endosomal pH, the α-helical content of the protein decreases and the β-sheet content increases by nearly 10% (Table 1), suggesting a limited conformational change. Turkewitz et al. [6], using CD, also found a conformational change in tfRt upon acidification but no quantification was provided.

To further characterise the effect of pH on the structure of transferrin receptor, temperature-dependent conformational changes at pH 5.6 and pH 7.4 were investigated using FTIR combined with global analysis. At both pH 5.6 and pH 7.4 loss of secondary structure coincides with enhanced proton exchange as seen by concomitant loss of amide I and amide II band intensity (Fig. 3, Table 2). This could be due to loss of secondary and tertiary structure occurring simultaneously. Alternatively high solvent accessibility of tfRt may lead to exchange of all protons not involved in secondary structure at room temperature [19]. Consequently the reduction of amide II absorption at elevated temperature would also reflect loss of secondary structure. At pH 5.6 tfRt is thermally less stable than at pH 7.4, the midpoint of denaturation being reduced from 71°C to 55°C. In both cases the transitions are irreversible and result in protein aggregation.

Although a two state thermodynamic model implicitly assumes that unfolding of a protein is fully reversible, it has been noted [20] that acceptable Van 't Hoff plots are observed even in the case of apparently irreversible protein denaturation. Therefore the apparent entropy of unfolding at the midpoint temperature (ΔS_m) can be roughly estimated from the equation $\Delta S_m = \Delta H_{VH}/T_m$ [21]. From the values presented in Table 2 for α-helical and β-sheet structures we calculate ΔS_m to be 85 and 280 cal·mol^{-1}·K^{-1} at pH 5.6 and pH 7.4, respectively. This difference in ΔS_m suggests that the native structure of tfRt at low pH is considerably more flexible than that found at high pH. This increase in flexibility at low pH may also explain the lower thermal stability of the protein at endosomal pH.

Table 2
Transition temperatures and Van 't Hoff enthalpy of tfRt determined by FTIR

Band	pH	T_m (°C)	ΔH_{VH} (kcal/mol)	Assignment
1620 cm^{-1} and 1678 cm^{-1}	5.6	55 (< 0.5)	30 (2)	aggregated structures
1656 cm^{-1} and 1637 cm^{-1}	5.6	55 (1)	28 (2)	α-helix/β-sheet
1545 cm^{-1} and 1447 cm^{-1}	5.6	55 (2)	23 (4)	amide II/amide II′
1619 cm^{-1} and 1678 cm^{-1}	7.4	70 (< 0.5)	109 (6)	aggregated structures
1661 cm^{-1} and 1642 cm^{-1}	7.4	71 (< 0.5)	96 (6)	α-helix/β-sheet
1545 cm^{-1} and 1446 cm^{-1}	7.4	71 (3)	40 (7)	amide II/amide II′

Values in brackets indicate calculated errors. Amplitude variations of combined bands were fitted together. The bands assigned to the amide II′ vibration and aggregated structures increased in intensity during thermal denaturation while other bands decreased in intensity. Additional bands identified during the fit that did not change significantly during heating were located at 1516 cm^{-1} (tyrosine) and 1588/1590 cm^{-1} (carboxylate).

Although spontaneous release of iron from transferrin at low pH has been proposed as a mechanism of iron release from the receptor–transferrin complex, Bali et al. [22] have shown that the interaction of transferrin with transferrin receptor can promote or retard iron release depending on pH. On the basis of these results it was suggested that receptor binding to diferric transferrin at low pH may force the latter into a more open or labile conformation, thereby facilitating iron release. Direct evidence to support this suggestion requires structural studies of the transferrin–transferrin receptor complex. So far it has been shown that in the absence of transferrin receptor the secondary structure of diferric transferrin is very similar at extracellular and endosomal pH [23]. The limited change in secondary structure and enhanced flexibility seen in tfRt at low pH reported in this study may induce a conformational change in bound transferrin, and thus facilitate iron release.

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References

[1] Young, S.P., Bomford, A. and Williams, R. (1984) Biochem. J. 219, 505–510.
[2] de Jong, G., van Dijk, J.P. and van Eijk, H.G. (1990) Clin. Chim. Acta 190, 1–46.
[3] Bali, P.K. and Aisen, P. (1991) Biochemistry 30, 9947–9952.
[4] Testa, U., Pelosi, E. and Peschle, C. (1994) Crit. Rev. Oncogenesis 4, 241–276.
[5] Turkewitz, A.P., Amatruda, J.F., Borhani, D.W., Harrison, S.C. and Schwartz, A.L. (1988) J. Biol. Chem 263, 8318–8325.
[6] Turkewitz, A.P., Schwartz, A.L. and Harrison, S.C. (1988) J. Biol. Chem 263, 16309–16315.
[7] Borhani, D.W. and Harrison, S.C. (1991) J. Mol. Biol. 218, 685–689.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Hadden, J.M., Bloemendal, M., Haris, P.I., Srai, S.K.S. and Chapman, D. (1994) Biochim. Biophys. Acta 1205, 59–67.
[10] Haris, P.I., Lee, D.C. and Chapman, D. (1986) Biochim. Biophys. Acta 874, 255–265.
[11] Van Stokkum, I.H.M., Scherer, T., Brouwer, A.M. and Verhoeven, J.W. (1994) J. Phys. Chem. 98, 852–866.
[12] Elwell, M.L. and Schellman, J.A. (1977) Biochim. Biophys. Acta 494, 367–383.

[13] Pace, C.N., Shirley, B.A. and Thompson, J.A. (1989) in: Protein Structure: A Practical Approach (Creighton, T.E., ed.) pp. 311–330, IRL Press, Oxford.
[14] Haris, P.I. and Chapman, D. (1992) Trends. Biochem. Sci. 17, 328–333.
[15] Susi, H. and Byler, D.M. (1986) Methods Enzymol. 130, 290–311.
[16] Olinger, J.M., Hill, D.M., Jakobsen, R.J. and Brody, R.S. (1986) Biochim. Biophys. Acta 869, 89–98.
[17] Pribić, R., Van Stokkum, I.H.M., Chapman, D., Haris, P.I. and Bloemendal, M. (1993) Anal. Biochem. 214, 366–378.
[18] Lee, D.C., Haris, P.I., Chapman, D. and Mitchell, R.C. (1990) Biochemistry 29, 9185–9193.
[19] Pedersen, T.G., Sigurskjold, B.W., Andersen, K.V., Kjaer, M., Poulsen, F.M., Dobson, C.M. and Redfield, C. (1991) J. Mol. Biol. 218, 413–426.
[20] Sturtevant, J.M. (1987) Annu. Rev. Phys. Chem. 38, 463–488.
[21] Hilser, V.J., Worosila, G.D. and Freire, E. (1994) Anal. Biochem. 208, 125–131.
[22] Bali, P.K., Zak, O and Aisen, P (1991) Biochemistry 30, 324–328.
[23] Shen, Z.M., Yang, J.T., Feng, Y.-O. and Wu, C.-S.C (1992) Prot. Sci. 1, 1477–1484.