Spectral and metal-binding properties of three single-point tryptophan mutants of the human transferrin N-lobe

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Human serum transferrin N-lobe (hTF/2N) contains three conserved tryptophan residues, Trp⁸, Trp¹²⁸ and Trp²⁶⁴, located in three different environments. The present report addresses the different contributions of the three tryptophan residues to the UV-visible, fluorescence and NMR spectra of hTF/2N and the effect of the mutations at each tryptophan residue on the iron-binding properties of the protein. Trp⁸ resides in a hydrophobic box containing a cluster of three phenylalanine side chains and is H bonded through the indole N to an adjacent water cluster lying between two β -sheets containing Trp⁸ and Lys²⁹⁶ respectively. The fluorescence of Trp⁸ may be quenched by the benzene rings. The apparent increase in the rate of iron release from the Trp⁸ → Tyr mutant could be due to the interference of the mutation with the H-bond linkage resulting in an effect on the second shell network. The partial quenching in the fluorescence

of Trp¹²⁸ results from the nearby His¹¹⁹ residue. Difference-fluorescence spectra reveal that any protein containing Trp¹²⁸ shows a blue shift upon binding metal ion, and the NMR signal of Trp¹²⁸ broadens out and disappears upon the binding of paramagnetic metals to the protein. These data imply that Trp¹²⁸ is a major fluorescent and NMR reporter group for metal binding, and possibly for cleft closure in hTF/2N. Trp²⁶⁴ is located on the surface of the protein and does not connect to any functional residues. This explains the facts that Trp²⁶⁴ is the major contributor to both the absorbance and fluorescence spectra, has a strong NMR signal and the mutation at Trp²⁶⁴ has little effect on the iron-binding and release behaviours of the protein.

Key words: fluorescence, kinetics, metal protein, NMR, paramagnetic effect.

INTRODUCTION

Serum transferrins are a group of proteins responsible for iron transport to cells [1–3]. A molecule of transferrin (~80 kDa) is made up of two similar halves, the N- and C-lobes, each containing an iron-binding site defined by two domains, the NI and NII for the N-lobe and the CI and CII or the C-lobe [4–6]. A pronounced conformational change between the apo- and iron-forms of transferrin has been demonstrated by X-ray crystal-structural analysis. Interpretation of the structural data implies that, when iron is released from the N-lobe of human serum transferrin hTF(1), the NI and NII domains rotate 63° around central hinges to form an ‘open’ conformation [5,6]. The flexibility of transferrin is also demonstrated by its ability to accommodate different metal ions and anions in the iron- and anion-binding sites. Crystal structures of lactoferrin, containing copper and oxalate, showed that substitution with a metal ion larger than iron or an anion larger than carbonate did not result in significant changes in overall polypeptide folding or domain closure [7,8].

hTF N-lobe (hTF/2N) binds iron by coordination to the side chains of four amino acids, Asp⁶³, Tyr⁹⁵, Tyr¹⁸⁸ and His²⁴⁹, and two oxygens from the synergistic anion, carbonate [5]. Surrounding the binding site, there is a network of hydrogen bonds, the so-called ‘second shell’, which helps to maintain the stability of the central-binding site [6,9]. Trp⁸, Trp¹²⁸ and Trp²⁶⁴ are three highly conserved residues in transferrins [10] that reside in three different environments in the N-lobe (Figure 1). Since there are only three tryptophan residues in hTF/2N, a reasonable

strategy is to study the NMR spectra of the protein by specifically labelling these residues. Previous NMR studies from our laboratory using [5-¹⁹F]Trp-labelled W8Y and W128Y mutants (where Trp⁸ has been replaced with Tyr and Trp¹²⁸ with Tyr) and wild-type hTF/2N protein showed that the local environment of Trp¹²⁸ changes significantly upon metal binding [11]. This metal-induced structural change has also been revealed by ¹H- and ¹⁵N-NMR studies with the ¹⁵N-Tyr-labelled W128Y mutant protein [12]. To allow direct observation of the tryptophan residues and to overcome the low amount of incorporation of the fluorine label (approx. 15%), the wild-type hTF/2N and the W8Y and W128Y mutants have been labelled with 2-[¹³C]Trp for study by NMR. In addition, the fluorescence spectra and iron-release behaviours of the three mutant proteins are compared with the wild-type N-lobe. This allows determination of the contribution of each of the three individual tryptophan residues to the fluorescence spectra and provides a quantitative measurement of the effect of the mutation on metal binding. In addition, spectral measurements for a histidine mutant, H119Q (where His¹¹⁹ has been replaced with Gln), suggest a possible structural basis for the fluorescence of the Trp¹²⁸ residue.

EXPERIMENTAL

Materials

Chemicals used were of reagent quality. Stock solutions of Hepes, Mes, and other buffers were prepared by dissolving the anhydrous salts in Milli-Q (Millipore) purified water, and

Abbreviations used: hTF, human serum transferrin; hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337; DMEM, Dulbecco's modified Eagle's medium; BHK, baby-hamster kidney cells; NTA, nitrilotriacetate; Tiron, 4,5-dihydroxy-1,3-benzenedisulphonate; HSQC, heteronuclear single-quantum coherence; W8Y etc., protein where the Trp⁸ residue has been replaced with a tyrosine residue etc.

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Figure 1 Structure of human transferrin N-lobe

The locations of three tryptophan residues W8, W128 and W264 [5] are shown.

adjusting the pH to the desired values with 1 M NaOH or 1 M HCl. Standard solutions of copper(II) (10000 µg/ml), iron(II) (1000 µg/ml), gallium(III) (10000 µg/ml) and indium(III) (10000 µg/ml) in 5% nitric acid were obtained from Johnson Matthey (Ward Hill, MA, U.S.A.). Cupric chloride was from J. T. Baker Chemical Co.; 4,5-dihydroxy-1,3-benzenedisulphonate (Tiron) was from Fisher Scientific Co.; EDTA was from Mann Research Laboratories Inc.; nitrilotriacetate (NTA) was from Sigma, and 2-[¹³C]Trp and [ϵ -¹³C]Met were from Cambridge Isotope Laboratories. Dulbecco's modified Eagle's medium (DMEM)-F12, lacking aromatic amino acids and methionine, was obtained from Gibco-BRL. Centricon 10 microconcentrators were from Amicon. Milli-Q (Millipore) purified water was used for all solutions and dilutions. Tiron stock solutions were prepared by dissolving the Tiron in the appropriate buffers and adjusting the pH to the desired values with 1 M NaOH.

Molecular biology

The tryptophan and histidine mutations were performed by using a PCR-based mutagenesis procedure [13]. The oligonucleotides corresponding to the W8Y and W128Y mutants have been reported previously [11]. The primers corresponding to the W264Y and H119Q mutants were made up as follows 5'-GACTTGATGATGAGCTTCTC-3' and 5'-AGTCCTGC CAGACGGGTCTAG-3'. A similar protocol described previously in detail for mutagenesis experiments was employed to confirm the presence of the desired mutation and to construct the appropriate plasmid [9].

Expression, purification and preparation of proteins

The N-lobe of hTF and the single-point tryptophan and histidine mutants of hTF/2N were expressed into the medium of baby-hamster kidney (BHK) cells containing the relevant cDNA in the pNUT vector, as reported previously [9, 14]. Purification of the protein required passage over an anion-exchange column (Poros 50 HQ) followed by separation from full-length transferrin using a Sephacryl-100 HR gel-filtration column [14–16]. In some cases, a Sephadex G-75 column was used to remove residual hTF. SDS/PAGE was used to monitor protein production and purity. Preparation of apo- and Fe-loaded protein samples followed the procedure described previously [9]. Samples were saturated with metal by adding Fe(III)-NTA₂, CuCl₂, Ga(III)-NTA₂, Cr(III)-NTA₂, In(III)-NTA₂ and Na₃[Co(III)(CO₃)₃] [17] into Hepes buffer (50 mM, pH 7.4) containing apo-hTF/2N and bicarbonate, then exchanging into KCl solution. Slight excess (iron, copper and cobalt) or 2-fold excess (gallium, indium and chromium) of metal reagent was used to saturate the samples. If necessary, the BioCad SPRINT system, employing a QE/M anion exchange column (10 mm × 100 mm), was used to purify the samples further before NMR analysis. Details of the buffers and elution protocol have been given previously [15]. Protein samples for NMR containing 2-[¹³C]Trp-hTF/2N were prepared by supplementing the DMEM-F-12 medium, lacking aromatic amino acids and methionine, with L-tyrosine, L-phenylalanine, L-histidine, L-methionine and 2-[¹³C]Trp. NMR samples containing both 2-[¹³C]Trp and [ϵ -¹³C]Met were prepared by replacing the corresponding amino acids with 2-[¹³C]Trp and [ϵ -¹³C]Met in the medium.

Electronic spectra and Co(III) titration

UV-visible spectra were recorded on a Cary 219 spectrophotometer with the computer program OLIS-219s (On-line Instrument Systems Inc., Bogart, GA, U.S.A.) at 25 °C. The appropriate buffer served as the reference for full-range spectra from 250–650 nm. Co(III) titrations, to determine the molar absorption coefficients at ϵ_{280} for each mutant, were conducted as described previously with sodium bicarbonate/CO₂ buffer [17]. Titration of wild-type hTF/2N was performed before titration of each mutant sample to standardize the procedure. The results reported are the average values derived from at least two titrations.

Fluorescence spectroscopy

Fluorescence spectra for each Trp mutant and for the wild-type hTF/2N were obtained using an OLIS RSM-1000 spectrophotometer with an excitation wavelength of 280 nm, entrance slit of 1.24 mm and exit slit of 3.16 mm. A 720-W xenon light source was used to excite 4 µM protein samples. A total of 312 scans over a range of 345 ± 78 nm were averaged to obtain the final spectrum. The background (Hepes solution) showed only a small amount of Rayleigh scattering at 280 nm.

NMR spectroscopy

¹H-¹³C heteronuclear single-quantum coherence (HSQC) two-dimensional spectra were recorded on a Varian INOVA 500 MHz NMR spectrometer. Experiments were carried out at 298 K, with 112–640 transients per t_1 increment, acquired using 2048 complex points in the proton dimension and 96–160 increments in the carbon dimension. The spectral-sweep widths were 6 kHz for ¹H and 6 or 40 kHz for ¹³C, depending on which sample was used.

Protein samples containing both 2- ^{13}C Trp and $[\epsilon\text{-}^{13}\text{C}]$ Met were prepared initially for the purpose of observing the chemical shifts associated with both tryptophan and methionine residues simultaneously. For these samples, a large sweep width of 40 kHz was used to cover the entire ^{13}C chemical-shift range to eliminate possible alias effects from $[\epsilon\text{-}^{13}\text{C}]$ Met. Water (4.77 p.p.m.) and 3-(trimethylsilyl)propionic acid (0.0 p.p.m.) were used as references for ^1H and ^{13}C respectively. The final concentration of hTF/2N for each of the NMR experiments was 0.5–1.0 mM in 0.1 M KCl (0.6 ml), pH 7.4, with 10 % $^2\text{H}_2\text{O}$.

Kinetics of metal removal

The kinetics of iron release from transferrin ($\sim 40 \mu\text{M}$) by Tiron (12 mM) were measured in a Hepes buffer (50 mM, pH 7.4) at 25 °C by monitoring the absorbance spectral change at 480 nm for a period of at least four half-lives [18,19]. Rate constants were obtained by fitting the absorbance-versus-time data to a single-exponential function, giving R^2 values (coefficients of determination) greater than 0.99 in all cases.

RESULTS

Absorbance spectra

The spectral characterization of the iron-containing mutant proteins was carried by scanning the range 250–650 nm. The intrinsic parameters, λ_{max} , λ_{min} , $A_{\text{max}}/A_{\text{min}}$ and A_{280}/A_{max} , derived from the spectra, are listed in Table 1. Compared with wild-type hTF/2N, the W128Y mutant has similar spectral features, whereas the W8Y and W264Y mutant proteins show slight shifts in λ_{max} and lower A_{280}/A_{max} ratios. These results suggest that the point mutations of Trp to Tyr do not significantly alter the iron-binding site. In marked contrast, the H119Q mutant protein reveals a pronounced enhancement of the A_{280}/A_{max} ratio.

Co(III) titration

A method to determine the molar absorption coefficient at ϵ_{280} of apo-hTF/2N by titration with $\text{Co}(\text{CO}_3)_3^{3-}$ has been reported previously [17]. Obtaining accurate absorption coefficients for the Trp mutants is critical, as Trp residues are major contributors to the electronic spectra of most proteins [20]. The ϵ_{280} values derived for the three Trp mutants and the H119Q mutant from titration with Co(III) are listed in Table 1. Compared with wild-type hTF/2N, the W8Y and W128Y mutants gave slightly lower values, whereas the W264Y mutant had a value that was lower by approx. 10 %. This suggests that Trp²⁶⁴ makes a major contribution to the ϵ_{280} of the wild-type protein. The 40 % increase in ϵ_{280} for the H119Q mutant is of particular interest, and the structural basis for this increase will be considered in the Discussion section. The theoretically calculated ϵ_{280} for each of

Figure 2 Fluorescence spectra for the Trp and His mutants and wild-type hTF/2N

the Trp $\rightarrow$ Tyr mutants was 34.4, and that for the wild-type protein was 38.4 (Table 1 and [20]).

Fluorescence spectra

Based on the ϵ_{280} values in Table 1, fluorescence spectra were collected on identical concentrations of the apo-proteins (Figure 2A). The maximum fluorescence intensity of the W8Y mutant was very close to that of the wild-type protein, whereas that of the W128Y mutant was approx. 25 % lower, and that of the W264Y mutant was some 51 % lower with a slight blue shift. Addition of $\text{Fe}(\text{III})(\text{NTA})_2$ to the proteins resulted in significant quenching of the fluorescence intensity (Figure 2B): 65 % for hTF/2N, 60 % for the W8Y mutant, 38 % for the W128Y mutant, 71 % for the W264Y mutant and 51 % for the H119Q mutant proteins.

Formation of the ternary $\text{In}(\text{III})\text{-hTF/2N-CO}_3$ complexes with the proteins under study led to apparent blue shifts in the spectra in certain cases (Figure 3A). In order to enhance characterization of this effect, difference spectra were constructed by subtracting the spectra of the apo-protein from those of the respective $\text{In}(\text{III})\text{-hTF/2N-CO}_3$ complexes (Figure 3B). It was apparent that any protein containing Trp¹²⁸ showed this blue shift, whereas the W128Y mutant did not. This means that Trp¹²⁸ may be a fluorescent, as well as an NMR (see below), reporter group for cleft closing.

Table 1 Summary of spectral characteristics and molar absorption coefficients (ϵ_{280} , $\text{mM}^{-1}\cdot\text{cm}^{-1}$) for the mutants and wild-type (WT) hTF/2N

hTF/2N protein	λ_{max} (nm)	λ_{min} (nm)	$A_{\text{max}}/A_{\text{min}}$	A_{280}/A_{max}	Experimental (ϵ_{280})	Calculated* (ϵ_{280})
hTF/2N (WT)	472	410	1.37	23.5	38.5	38.4
W8Y	470	410	1.33	20.3	36.2	34.4
W128Y	473	410	1.35	22.7	37.8	34.4
W264Y	476	410	1.38	21.7	33.4	34.4
H119Q	470	410	1.31	36.2	53.8	38.4

* The absorption coefficients were calculated from the equation in [20].

Figure 3 Fluorescence spectra for In-hTF/2N-CO₃ complexes and the difference spectra between In-hTF/2N-CO₃ and apo-proteins

NMR spectra

HSQC two-dimensional NMR spectra for apo-hTF/2N, -W8Y and -W128Y mutant proteins are shown in Figure 4, and the chemical shifts are summarized in Table 2. The presence of three major spectral cross-peaks for the wild-type and only two for the W8Y and W128Y mutants allow assignment of proton resonances to Trp¹²⁸, Trp²⁶⁴ and Trp⁸ from low to high field. The two minor cross-peaks at lower and higher field in the ¹³C dimension most likely result from metabolic degradation of the

Figure 4 Two-dimensional [¹H, ¹³C] HSQC NMR spectra of [¹³C]Trp-labelled wild-type hTF/2N and mutants W8Y and W128Y in apo-form

Three cross-peaks from low to high field in the proton dimension are assigned to Trp¹²⁸, Trp²⁶⁴ and Trp⁸ respectively. The spectra were recorded at 25 °C and the spectral widths were 6 kHz and 40 kHz for ¹H and ¹³C respectively.

Table 2 ¹H- and ¹³C-NMR chemical shifts (p.p.m.) for wild-type apo- and metal-loaded hTF/2N

Protein	Peak W8		Peak W128		Peak W264	
	δ(¹ H)	δ(¹³ C)	δ(¹ H)	δ(¹³ C)	δ(¹ H)	δ(¹³ C)
Apo-hTF/2N	6.99	126.53	7.98	127.75	7.44	127.75
Apo-W8Y	—	—	7.97	127.27	7.44	127.38
Apo-W128Y	7.00	126.49	—	—	7.44	127.77
Fe-hTF/2N	6.79	126.33	—	—	7.45	127.74
Cu(II)-hTF/2N	6.79	126.31	—	—	7.42	127.74
Cr(III)-hTF/2N	6.78	126.27	—	—	7.45	127.72
Co(III)-hTF/2N	6.77	126.51	7.74	127.16	7.44	127.75
Ga(III)-hTF/2N	6.76	126.27	7.74	127.18	7.43	127.70
In(III)-hTF/2N	6.78	126.36	7.75	127.11	7.43	127.77

Figure 5 NMR spectra for the titration of apo-hTF/2N with the paramagnetic Fe(III) ion

The apparent movement of the Trp⁸ peak and the disappearance of the Trp¹²⁸ peak are shown. The spectra were recorded at 25 °C and the spectral widths were 6 kHz for both ¹H and ¹³C.

labelled Trp by the BHK cells, entry of the 2-¹³C into the C-1 pool and incorporation of this label into other amino acids, such as glycine and serine.

Titration of 2-[¹³C]Trp-labelled apo-hTF/2N with iron was monitored by NMR as shown in Figure 5. Omission of [¹³C]Met labelling from this sample allowed a narrower sweep width of 6 kHz and improved spectral resolution. With increasing iron load, the up-field shift of the Trp⁸ resonance and the disappearance of the Trp¹²⁸ resonance were apparent, whereas the Trp²⁶⁴ resonance remained fixed. Thus the shift in the Trp⁸ resonance may reflect cleft closure in the protein, whereas the Trp¹²⁸ resonance is most likely broadened paramagnetically.

Confirmation of the paramagnetic broadening was found in complexes with two other paramagnetic metal ions, Cu(II) and Cr(III), and three diamagnetic metal ions, Co(III), Ga(III) and In(III) (Figure 6). Again the Trp¹²⁸ resonance disappeared on addition of the two other paramagnetic metal ions; however, the three diamagnetic ions caused up-field shifts of the resonances for Trp⁸ and Trp¹²⁸ in both dimensions. It is clear that even at a

Figure 6 Comparison of the NMR spectra for the complexes of hTF/2N with six different metal ions

Upper panels; binding with paramagnetic metal ions. Lower panels; binding with diamagnetic metal ions. The spectra were recorded at 25 °C and the spectral widths were 6 kHz and 40 kHz for ^1H and ^{13}C respectively.

Figure 7 Comparison of the iron release rates for the Trp mutants and wild-type hTF/2N

See the Experimental section for details. Experiments were performed in the absence (open bars) or presence (hatched bars) of 0.5 M KCl.

2:1 ratio there was insufficient Ga(III) to completely saturate the protein.

Iron-release kinetics

Iron-release kinetics were used to assess the effect of the mutations on the rates of iron release from the ternary complexes. Rate constants for the W8Y and W128Y mutant proteins were approx. 3 to 5 times faster than that of the wild-type protein in the absence of KCl (Figure 7). Thus even in the absence of significant changes in the electronic-spectra of these mutants, the stability of the iron complexes was moderately decreased. The W264Y mutant protein appeared to be about as stable as the wild type. KCl at 0.5 M retarded the iron release rate to about one-third of that in its absence for the W8Y and W264Y mutant proteins and halved it for the wild-type and W128Y proteins (Figure 7).

DISCUSSION

Spectral contribution of the three tryptophan residues to hTF/2N

Tryptophan residues are major contributors to the electronic spectral absorbance in the UV region and also to the intrinsic fluorescence of proteins [20]. However, the extent of their contribution depends on the local environment of the specific tryptophan residue. In the case of the three tryptophan residues in hTF/2N, Trp²⁶⁴ is the major contributor to both the absorbance and fluorescence spectra of the protein, as revealed by the lower absorption coefficient ϵ_{280} (Table 1), and weaker fluorescence intensity (Figure 2A) of the apo-W264Y mutant protein. In contrast to the other two tryptophan residues, Trp²⁶⁴ is located on the surface of the protein (Figure 1) and does not appear to interact with either the metal centre or any other functional residues.

The concomitant quenching of the intrinsic protein fluorescence of transferrins upon binding iron was first reported by Lehrer [21] and further investigated by Evans and Holbrook [22]. This is the basis for the iron release assay used by many researchers [23–25]. Figure 2(B) shows that the binding of iron to hTF/2N severely quenches the fluorescence of both the wild-type and mutant proteins. Unexpectedly, the different degrees of quenching of the point mutants show that the W8Y and W128Y proteins have unusually high fluorescence intensities and the W264Y protein has an unusually low intensity relative to wild-type hTF/2N. Clearly the three tryptophan residues are unequal in fluorescence emission because of the different environment of each.

The essential lack of fluorescence by Trp⁸ may be explained in the light of its containment in a hydrophobic box containing three phenylalanine residues. Benzene is known to quench the fluorescence of indole and phenylalanine quenches the fluorescence of tryptophan [26]. In the latter case the quenching phenylalanine ring was shown by X-ray crystallography to be orthogonal to the indole ring of the nearby tryptophan residue, and the conclusion drawn was that a hydrogen bond exists between the indole N-H and the π -electron cloud of the benzene ring. In the case of hTF/2N, this geometrical relationship does not exist; rather, the indole ring lies parallel to a plane roughly defined by three nearby phenylalanine rings and the benzene ring of Phe²⁹⁵ appears to be orthogonal to the indole ring of Trp⁸ (Figure 8). This arrangement suggests that π -electron overlap among these aromatic rings may account for the observed fluorescence quenching.

In contrast, Trp¹²⁸ appears to be partially quenched relative to Trp²⁶⁴. This may result from the nearby His¹¹⁹ residue, imidazole being a known quencher of tryptophan fluorescence [26]. This hypothesis was tested by observing the fluorescence spectrum of the H119Q mutant protein (Figure 2). The 60% enhancement of the fluorescent intensity of the H119Q mutant relative to wild-type hTF/2N reveals the role that His¹¹⁹ plays in quenching the fluorescence of Trp¹²⁸ in the wild-type protein. The greater quenching by iron of wild-type hTF/2N relative to the H119Q protein might result from a change in the degree of protonation of His¹¹⁹ on formation of the iron–protein complex. We believe that the significant increase in the molar absorptivity and the absorption coefficient at 280 nm for the H119Q mutant can also be explained by the absence of interaction of histidine with Trp¹²⁸. A detailed study of effect of His¹¹⁹ on the spectral properties of the protein is ongoing.

Formation of a colourless, non-quenched metal complex of hTF/2N resulted in a blue shift of the fluorescence spectrum (Figure 3A). This is best shown by constructing difference spectra by subtracting the spectrum of the apo-protein from that of

Figure 8 Hydrophobic box location of Trp⁸ (W8)

Trp⁸ side chain is located near to a cluster of three phenylalanine residues (F22, F295 and F302) and adjacent to a 'water pool' [5].

the metal–protein complex (Figure 3B). In the present study, the metal used was In(III) because it forms a tight complex with transferrin, unlike Ga(III), which, even at two-fold excess, failed to completely saturate hTF/2N (Figure 6). Comparison of the difference spectra of the wild-type with the three Trp → Tyr mutants showed that Trp¹²⁸ was the main fluorescent reporter group for metal binding, and possibly for cleft closure in hTF/2N. This finding agrees with the results of NMR with 5-F-Trp-labelled [11] and 2-[¹³C]Trp-labelled hTF/2N (see below) showing the importance of Trp¹²⁸ as a primary reporter group for metal binding and possibly cleft closure. The blue shift of the λ_{em} for Trp¹²⁸ on metal binding suggests that, on closure of the binding site cleft, Trp¹²⁸ undergoes a change to a more hydrophobic environment [26]. Indeed the structure of apo-hTF/2N shows a water hydrogen bonded to the indole N, whereas this water is absent from the Fe-hTF/2N structure [5,6].

Metal-binding effect revealed through ¹³C-Trp NMR spectra

The NMR cross-peaks for the three tryptophan residues were assigned by means of ¹³C-labelled and site-directed mutations (Figure 4). The low and high intensity of NMR signals for the Trp¹²⁸ and Trp²⁶⁴ residues respectively probably corresponds to the stable, buried structure of Trp¹²⁸ and the mobile surface structure of Trp²⁶⁴.

The effect of metal binding on the NMR spectra was clearly demonstrated when iron was added to apo-hTF/2N (Figure 5). Two interesting and diagnostic changes occur as iron is titrated into the protein: (1) movement of the Trp⁸ peak and (2) broadening and disappearance of the Trp¹²⁸ peak as a result of

the paramagnetic effect. The paramagnetic metal effect on the Trp¹²⁸ peak was confirmed in other metal-binding experiments (Figure 6). Whereas binding of three diamagnetic metals caused the Trp¹²⁸ peak to move up field, the other two paramagnetic metals caused it to disappear completely.

Two-dimensional NMR spectra with paramagnetic metallo-proteins have been extensively studied and reviewed [27–30]. Paramagnetic centres exert their effects on NMR spectra through two pathways. The first one is the Fermi or contact contribution, in which the unpaired spin electron(s) delocalize the resonating nucleus. This contribution is transmitted through bonds and only works with chemical bonds which are very close to the metal. The second effect is the pseudocontact or dipolar contribution, in which the unpaired electron(s) interact with the nuclear-spin magnetic moments through dipole–dipole interaction or through space. The second contribution usually functions within 7.5 Å (1 Å = 0.1 nm) [27]. These two pathways simultaneously affect the NMR spectra, leading to short relaxation times, line broadening and shifts of NMR signals. Trp¹²⁸ does not engage in any direct contact with the metal binding site, and it is about 10 Å away from the metal centre [5]. Obviously, the first type of paramagnetic effect described could not apply in the case of Trp¹²⁸. Therefore the second effect must be evoked and extended to a distance of 10 Å.

Mutations at tryptophan residues result in effects on iron release of hTF/2N

In the iron-loaded protein, Trp⁸ is located in a hydrophobic box adjacent to a 'water pool' between two β -sheets containing Trp⁸ and Lys²⁹⁶ respectively [5]. The Trp⁸ side chain has no direct or indirect contact with the iron-binding ligands or any of the 'second shell' residues, although the indole N-H is evidently hydrogen bonded to one of these 'bound' water molecules [5]. This observation explains why the mutation Trp⁸ → Tyr does not disturb the iron binding of hTF/2N, as demonstrated by its electronic spectrum (Table 1). However, there is a hydrogen-bond network among a number of water molecules and residues in the vicinity of both Trp⁸ and Lys²⁹⁶. The mutation at Trp⁸ could interfere with the H-bond linkage, which could result in an effect on the di-lysine trigger (K206–K296) [9], leading to a four-fold decrease in the kinetic stability of iron binding in the mutant protein.

Trp¹²⁸ is a residue within helix 5, from which a number of side chains, including Thr¹²⁰, Arg¹²⁴, Ala¹²⁶ and Gly¹²⁷, have extensive hydrogen bonding with the synergistic anion carbonate [5]. It is therefore reasonable to postulate that the mutation of Trp¹²⁸ → Tyr could have some effect on iron release from the W128Y mutant; iron removal from W128Y is three times faster than from the parent protein. Almost identical UV–visible spectra for both the W128Y mutant and the wild-type protein suggest that they both have a stable iron-binding structure, a fact consistent with the results from the CD spectra and energy calculations [12]. As mentioned above, Trp²⁶⁴ is a surface residue that lies far from the metal-binding centre. Mutation at the Trp²⁶⁴ position therefore does not have any effect on the iron release properties of the protein.

Summary

In hTF/2N, Trp²⁶⁴ is the largest contributor to both absorbance at 280 nm and to fluorescence, but mutation at Trp²⁶⁴ does not affect the kinetic stability of the protein. Trp¹²⁸ is the residue closest to the metal-centre, consequently a diagnostic paramagnetic effect broadens out the NMR signal of this residue correlated to metal binding and the conformational change with

small effects on the iron release from the W128Y mutant. The effect of mutation at Trp⁸ is demonstrated by the faster iron release of the W8Y mutant with a rate 4–5 times faster than that for wild-type hTF/2N. These spectral and metal-binding properties of the Trp mutants are closely related to the unique locations of these three highly conserved tryptophan residues.

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REFERENCES

- Evans, R. W., Crawley, J. B., Joannou, C. L. and Sharma, N. D. (1999) Iron proteins. In *Iron and Infection: Molecular, Physiological and Clinical Aspects* (Bullen, J. J. and Griffiths, E., eds.), pp. 27–86, John Wiley and Sons, Chichester.
- Aisen, P. (1998) Transferrin, the transferrin receptor, and the uptake of iron by cells. In *Iron Transport and Storage in Microorganisms, Plants and Animals* (Sigel, A. and Sigel, H., eds.) vol. 35, pp. 585–631, Marcel Dekker, New York.
- Wessling-Resnick, M. (1999) Biochemistry of iron uptake. *Crit. Rev. Biochem. Mol. Biol.* **34**, 285–314.
- Baker, E. N. (1994) Structure and reactivity of transferrins. *Adv. Inorg. Chem.* **41**, 389–463.
- MacGillivray, R. T. A., Moore, S. A., Chen, J., Anderson, B. F., Baker, H., Luo, Y. G., Bewley, M., Smith, C. A., Murphy, M. E. and Wang, Y. et al. (1998) Two high-resolution crystal structures of the recombinant N-lobe of human transferrin reveal a structural change implicated in iron release. *Biochemistry* **37**, 7919–7928.
- Jeffrey, P. D., Bewley, M. C., MacGillivray, R. T. A., Mason, A. B., Woodworth, R. C. and Baker, E. N. (1998) Ligand-induced conformational change in transferrins: crystal structure of the open form of the N-terminal half-molecule of human transferrin. *Biochemistry* **37**, 13978–13986.
- Smith, C. A., Anderson, B. F., Baker, H. M. and Baker, E. N. (1992) Metal substitution in transferrins: The crystal structure of human copper-lactoferrin at 2.1-Å resolution. *Biochemistry* **31**, 4527–4533.
- Baker, H. M., Anderson, B. F., Brodie, A. M., Shongwe, M. S., Smith, C. A. and Baker, E. N. (1996) Anion binding by transferrins: Importance of second-shell effects revealed by the crystal structure of oxalate-substituted diferric lactoferrin. *Biochemistry* **35**, 9007–9013.
- He, Q.-Y., Mason, A. B., Tam, B. M., MacGillivray, R. T. A. and Woodworth, R. C. (1999) Dual role of Lys206-Lys296 interaction in human transferrin N-lobe: Iron-release trigger and anion-binding site. *Biochemistry* **38**, 9704–9711.
- Baldwin, G. S. (1993) Comparison of transferrin sequences from different species. *Comp. Biochem. Physiol. [B]* **106B**, 203–218.
- Luck, L. A., Mason, A. B., Savage, K. J., MacGillivray, R. R. A. and Woodworth, R. C. (1997) ¹⁹F NMR studies of the recombinant human transferrin N-lobe and three single point mutants. *Magnetic Resonance in Chemistry* **35**, 477–481.
- Beatty, E. J., Cox, M. C., Frenkiel, T. A., He, Q.-Y., Mason, A. B., Sadler, P. J., Tucker, A. and Woodworth, R. C. (1997) Trp¹²⁸Tyr mutation in the N-lobe of recombinant human serum transferrin: ¹H- and ¹⁵N-NMR and metal binding studies. *Protein Eng.* **10**, 583–591.
- Nelson, R. M. and Long, G. L. (1989) A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal. Biochem.* **180**, 147–151.
- Woodworth, R. C., Mason, A. B., Funk, W. D. and MacGillivray, R. T. A. (1991) Expression and initial characterization of five site-directed mutants of the N-terminal half-molecule of human transferrin. *Biochemistry* **30**, 10824–10829.
- Mason, A. B., Tam, B. M., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Savage, K. J., Lineback, J. A. and MacGillivray, R. T. A. (1997) Receptor recognition sites reside in both lobes of human serum transferrin. *Biochem. J.* **326**, 77–85.
- Mason, A. B., Funk, W. D., MacGillivray, R. T. A. and Woodworth, R. C. (1991) Efficient production and isolation of recombinant amino-terminal half-molecule of human serum transferrin from baby hamster kidney cells. *Protein Exp. Purif.* **2**, 214–220.
- He, Q.-Y., Mason, A. B. and Woodworth, R. C. (1996) Spectrophotometric titration with cobalt (III) for the determination of accurate absorption coefficients of transferrins. *Biochem. J.* **318**, 145–148.
- He, Q.-Y., Mason, A. B., Woodworth, R. C., Tam, B. M., Wadsworth, T. and MacGillivray, R. T. A. (1997) Effects of mutations of aspartic acid 63 on the metal-binding properties of the recombinant N-lobe of human serum transferrin. *Biochemistry* **36**, 5522–5528.
- He, Q.-Y., Mason, A. B. and Woodworth, R. C. (1997) Iron release from recombinant N-lobe and single point Asp 63 mutants of human transferrin by EDTA. *Biochem. J.* **328**, 439–445.
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411–2423.
- Lehrer, S. S. (1969) Fluorescence and absorption studies of the binding of copper and iron to transferrin. *J. Biol. Chem.* **244**, 3613–3617.
- Evans, R. W. and Holbrook, J. J. (1975) Differences in the protein fluorescence of the two iron(III)-binding sites of ovotransferrin. *Biochem. J.* **145**, 201–207.
- Egan, T. J., Zak, O. and Aisen, P. (1993) The anion requirement for iron release from transferrin is preserved in the receptor-transferrin complex. *Biochemistry* **32**, 8162–8167.
- Zak, O., Aisen, P., Crawley, J. B., Joannou, C. L., Patel, K. J., Rafiq, M. and Evans, R. W. (1995) Iron release from recombinant N-lobe and mutants of human transferrin. *Biochemistry* **34**, 14428–14434.
- Muralidhara, B. K. and Hirose, M. (2000) Anion-mediated iron release from transferrins – The kinetic and mechanistic model for N-lobe of ovotransferrin. *J. Biol. Chem.* **275**, 12463–12469.
- Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York.
- Xavier, A. V., Tumer, D. L. and Santos, H. (1993) Two-dimensional nuclear magnetic resonance of paramagnetic metalloproteins. *Methods Enzymol.* **227**, 1–16.
- Canter, G. W., Hilbers, C. W., van de Kamp, M. and Wijmenga, S. S. (1993) Multidimensional nuclear magnetic resonance methods to probe metal environments in proteins. *Methods Enzymol.* **227**, 244–290.
- Banci, L., Bertini, I. and Luchinat, C. (1994) Two-dimensional nuclear magnetic resonance spectra of paramagnetic systems. *Methods Enzymol.* **239**, 485–514.
- Bertini, I., Luchinat, C. and Rosato, A. (1996) The solution structure of paramagnetic metalloproteins. *Prog. Biophys. Mol. Biol.* **66**, 43–80.

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