[¹³C]Methionine NMR and metal-binding studies of recombinant human transferrin N-lobe and five methionine mutants: conformational changes and increased sensitivity to chloride

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The N-lobe of human serum transferrin (hTF/2N) and single point mutants in which each of the five methionine residues was individually mutated have been produced in a mammalian tissue-culture expression system. Since the five methionine residues are well distributed in the transferrin N-lobe, ¹³C NMR of the [¹³C]methionine-labelled proteins has been used to monitor conformational changes of the protein during metal binding. All five methionine residues have been assigned [Beatty, Cox, Frenkiel, Tam, Mason, MacGillivray, Sadler and Woodworth (1996) *Biochemistry* **35**, 7635–7642]. The tentative two-dimensional NMR assignment for two of the five methionine residues, namely Met²⁶ and Met¹⁰⁹, has been corrected. A series of NMR spectra for the complexes of ¹³C-Met-labelled hTF/2N with six different metal ions, Fe(III), Cu(II), Cr(III), Co(III), Ga(III) and In(III), demonstrate that the conformational change of the protein upon metal binding can be observed by means of the changes in the NMR chemical shifts associated with certain methionine residues, regardless of whether diamagnetic or para-

magnetic metals are used. Changing any of the methionine residues should have minimal effects on transferrin function, since structural analysis shows that none of these residues contacts functional amino acids or has any obvious role in iron uptake or release. In fact, UV–visible spectra show little perturbation of the electronic spectra of any of the mutants. Nevertheless, the M109L mutant (Met¹⁰⁹ → Leu) releases iron at half the rate of the wild-type N-lobe, and chloride shows a significantly greater retarding effect on the rate of iron release from all five mutants. All the methionine mutants (especially in the apo form) show a poor solubility in Hepes buffer lacking anions such as bicarbonate. These findings imply a more general effect of anion binding to surface residues than previously realized.

Key words: iron–protein, iron release, two-dimensional NMR, chloride effect.

INTRODUCTION

Human serum transferrin (hTF) (≈ 80 kDa) consists of two structurally similar halves, termed the N- and C-lobes, connected by a short peptide chain [1–3]. Each lobe can be further divided into two domains, the NI and NII domains for the N-lobe and the CI and CII domains for the C-lobe. At neutral pH, hTF binds iron and has a ‘closed’ conformation; at lower pH, the two domains of the N-lobe of hTF rotate 63° to release iron, leading to an ‘open’ conformation [4,5]. By repeating this process, hTF reversibly carries iron from plasma into cells. hTF N-lobe (hTF/2N) has four iron-binding ligands, namely Asp⁶³, Tyr⁹⁵, Tyr¹⁸⁸ and His²⁴⁹. Iron is bound octahedrally by co-ordinating to these four ligands and two oxygen atoms from the synergistic anion, carbonate. In addition to iron, other metal ions having a similar positive charge and ionic radius can occupy the binding site of hTF/2N [6–11]. A hydrogen-bond network, the so-called ‘second shell’, surrounds the binding site and stabilizes it [4–12]. The amino acid residues in the network can be said to have a functional role in hTF by providing a scaffold for stable metal binding.

In previous studies we have shown that mutations of the iron-binding ligands resulted in dramatic alterations in the metal-binding behaviour of hTF/2N [12–14]. For example, in the case of the Y188F mutant, in which the tyrosine ligand was changed

to phenylalanine, the resulting protein was completely unable to bind iron under normal conditions [14]. Dramatic effects on the iron-binding properties of hTF/2N were also found when mutations were made at the second-shell residues [12–15].

There are five methionine residues in the N-lobe of hTF, most of which are located on the surface of the protein molecule (Figure 1). These methionine residues are well distributed throughout the hTF/2N protein and therefore their behaviour could be helpful for probing protein function. As part of our effort to explore the role that a particular residue plays in the metal binding of transferrin, each of the five methionine residues of hTF/2N was mutated individually to an apolar residue, namely leucine, isoleucine or valine.

Experiments by our collaborators using [¹³C]methionine-labelled hTF/2N led to a definite assignment for two of the methionine residues, namely Met³⁰⁹ and Met³¹³ [16]. On the basis of nuclear-Overhauser-effect (NOE) connectivity analysis, a tentative assignment for two other methionine residues, Met²⁶ and Met¹⁰⁹, was also made, and by elimination the assignment of the remaining residue, Met²⁵⁶, was suggested [16]. The present study provides a definite assignment for the five methionine residues and indicates that the NMR resonances of Met²⁶ and Met¹⁰⁹ must be switched. The chemistry and function of the five methionine residues were determined by means of UV–visible and NMR spectroscopies and iron-release kinetics. Different

Abbreviations used: hTF, human serum transferrin; hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337; mutants of hTF/2N are designated by the wild-type amino acid residue, the sequence number and the amino acid to which the residue was mutated (e.g. M109L is Met¹⁰⁹ → Leu); NTA, nitrilotriacetate; Tiron, 4,5-dihydroxy-1,3-benzenedisulphonate; Ac[−], acetate; DMEM, Dulbecco’s modified Eagle’s medium; 2D, two-dimensional; HSQC, heteronuclear single-quantum coherence.

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Figure 1 Structure of human transferrin N-lobe [4] showing the locations of five methionine residues

functional behaviours shown by the mutants lead to the conclusion that mutation, even at a so-called 'inert' residue, may result in a more general effect on the protein.

EXPERIMENTAL

Materials

Chemical reagents used were of commercial quality. Stock solutions of Hepes, Mes and other buffers were prepared by dissolving the anhydrous salts in Milli-Q (Millipore)-purified water, and adjusting the pH to desired values with 1 M NaOH or HCl. Standard solutions of copper(II) (10 mg/ml), iron(II) (1 mg/ml), gallium(III) (10 mg/ml) and indium(III) (10 mg/ml) in 5% HNO_3 were obtained from Johnson Matthey, Ward Hill, MA, U.S.A. CuCl_2 came from J. T. Baker Chemical Co., 4,5-dihydroxy-1,3-benzenedisulphonate (Tiron) from Fisher Scientific Co., EDTA from Mann Research Laboratories, ethylenediamine- N,N' -diacetate from Aldrich Chem. Co., nitrilotriacetate (NTA) from Sigma, and [^{13}C]methionine from Cambridge Isotope Laboratories. Dulbecco's modified Eagle's medium (DMEM)/F12 medium 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.

Methods

Molecular Biology

Mutating each of the five methionine residues to alternative amino acids was carried out by using a PCR-based mutagenesis procedure [17]. Synthetic oligonucleotides were used to introduce the mutations; a similar protocol to that described previously in

detail was employed to confirm the presence of the desired mutation and to place the mutagenized hTF/2N into the PNUT vector [13–18]. Two of the mutants, M309I and M313I, have been described previously [16].

Expression, purification and preparation of proteins

The N-lobe of hTF and the single-point methionine mutants of hTF/2N were expressed into the medium of baby-hamster kidney cells containing the relevant cDNA in the pNUT vector and were purified as reported previously [19,20]. The preparation of apo- and Fe-loaded protein samples followed the procedure described previously [13]. The NMR protein sample containing [^{13}C]methionine-labelled hTF/2N was prepared by substituting [^{13}C]methionine for L-methionine in the custom DMEM/F12 medium and addition of L-tyrosine, L-phenylalanine, L-tryptophan and L-histidine. Metal-saturated samples were prepared by adding Fe(III)-NTA, CuCl_2 , Ga(III)-NTA, Cr(III)-NTA, In(III)-NTA and $\text{Na}_3[\text{Co}(\text{CO}_3)_3]$ [21] 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 (Ga, In and Cr) of metal was required to saturate the samples. The BioCad (Framingham, MA, U.S.A.) SPRINT system employing a QE/M (quaternized polyethyleneimine) anion-exchange column (10 mm $\times$ 100 mm) was used to further purify the samples before NMR if necessary. Details of the buffers and elution protocol have been given previously [20]. Samples of hTF/2N used for NMR were at a final concentration of 0.5–1.0 mM in 0.1 M KCl (0.6 ml), pH 7.4, with 20% $^2\text{H}_2\text{O}$, unless otherwise stated.

Electronic spectra

UV-visible spectra were recorded on a Cary 219 spectrophotometer under the control of the computer program Olis-219s (On-line Instrument Systems, Inc., Bogart, GA, U.S.A.). The appropriate buffer served as the reference for full-range spectra from 250 to 650 nm. Difference spectra were generated by storing the spectrum of the apoprotein as the baseline and subtracting it from the sample spectra.

NMR spectroscopy

$^1\text{H}/^{13}\text{C}$ heteronuclear single-quantum coherence (HSQC) two-dimensional (2D) spectra were recorded on a Varian Unity INOVA 500 MHz NMR spectrometer. Experiments were carried out at 298 K unless otherwise stated, with 48–80 transients acquired using 2000 points in the proton dimension and 32–80 increments of t_1 . The spectral widths were 6 and 1 kHz for ^1H and ^{13}C respectively. The relaxation delay was 0.5 s. Water (4.8 p.p.m.) and 3-(trimethylsilyl)propionic acid (0.0 p.p.m.) were used as references for ^1H and ^{13}C respectively.

Kinetics of iron release

Experiments to measure the kinetics of iron release from transferrin by Tiron and EDTA and data processing were carried out as described in detail elsewhere [13–22]. 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

UV-visible spectra

When bi- or ter-valent metal ions bind to transferrin, the UV-visible spectra of the resulting complexes show characteristic

Table 1 Summary of the spectral characteristics and absorption coefficients for recombinant wild-type (WT) and mutants of hTF/2N

Protein	Fe-hTF/2N-CO ₃ complex				Cu-hTF/2N-CO ₃ complex			
	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	$A_{\max}/A_{\min}$	$A_{280}/A_{\max}$	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	$A_{\max}/A_{\min}$	$A_{280}/A_{\max}$
WT*	472	410	1.37 ± 0.02	23.5 ± 0.1	425	365	1.82 ± 0.01	31.1 ± 0.2
M26I	475	410	1.41 ± 0.01	23.9 ± 0.1	425	365	2.06 ± 0.02	25.7 ± 0.2
M109L	472	410	1.40 ± 0.02	23.7 ± 0.1	425	365	1.85 ± 0.03	31.9 ± 0.3
M256V	472	409	1.42 ± 0.01	23.4 ± 0.1	420	375	1.83 ± 0.02	32.1 ± 0.4
M309I	470	410	1.35 ± 0.01	24.4 ± 0.1	422	370	1.60 ± 0.01	25.8 ± 0.1
M313I	468	410	1.26 ± 0.02	24.6 ± 0.2	415	380	1.53 ± 0.02	26.3 ± 0.2

* From He et al. [14].

Figure 2 2D (^1H , ^{13}C) HSQC NMR spectra of [^{13}C]methionine-labelled apo-hTF/2N and the M26I and M256V mutants

The assignments of the cross-peaks for the methionine residues Met³⁰⁹ and Met³¹³ were established by Beatty et al [16]. In Figures 2, 3 and 4 the values on the ordinates and abscissae are chemical shifts in p.p.m.

absorption bands in the UV, and often the visible, region. For this reason, UV-visible spectroscopy has proved to be a simple and useful technique to study the metal-binding behaviour of TF. UV-visible spectra from 250 to 600 nm were recorded for the iron and copper complexes of the methionine mutants of hTF/2N. Table 1 lists the intrinsic spectral parameters for the mutants and wild-type hTF/2N. For the iron complexes, the five methionine mutants had $\lambda_{\max}$, $\lambda_{\min}$, $A_{\max}/A_{\min}$ and $A_{280}/A_{\max}$ parameters that differ only slightly from those found for the wild-type hTF/2N, indicating that the iron-binding behaviour of hTF/2N was not affected by mutation of any of the five

methionine residues. Similar spectral characteristics were also observed for the copper-containing mutants and the wild-type hTF/2N. Small differences were found in the spectra of the M26I, M309I and M313I mutants, compared with that of the wild-type hTF/2N.

NMR spectra

Figure 2 shows the HSQC NMR spectra for apo-(wild-type) hTF/2N and two mutants, M26I and M256V. For the wild-type hTF/2N, there were only four resonance peaks at 25 °C;

Figure 3 NMR spectra for the titration of apo-hTF/2N with the naturally bound Fe(III) at 25 °C clearly demonstrate the conformational change of the protein upon metal binding, with the apparent movement of peak M109 and the appearance of peak M26

Table 2 Summary of ¹H- and ¹³C-NMR chemical shifts for the [*ε*-¹³C]methionine-labelled residues in hTF/2N at 25 °C (except as noted)

Peak ...	Chemical shift (p.p.m.)									
	M26		M109		M256		M309		M313	
	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C
Apo-hTF/2N			1.97	17.47	2.15	17.03	2.18	17.67	2.23	17.26
Apo-hTF/2N, 37 °C	1.99	16.13	1.97	17.46	2.15	17.04	2.17	17.63	2.22	17.25
Ga-hTF/2N	1.98	16.04	2.00	17.66	2.15	17.06	2.16	17.65	2.23	17.30
Co(III)-hTF/2N	1.98	16.02	2.01	17.67	2.16	17.03	2.18	17.66	2.24	17.28
In-hTF/2N	1.99	16.03	2.01	17.66	2.16	17.04	2.17	17.63	2.23	17.29
Fe-hTF/2N	1.97	16.03	1.99	17.63	2.15	17.04	2.17	17.66	2.23	17.30
Cu-hTF/2N			1.96	17.64	2.16	17.03	2.15	17.73	2.23	17.27
Cr-hTF/2N	1.99	16.03	2.02	17.68	2.16	17.05	2.18	17.67	2.24	17.30

when the temperature was increased to 37 °C, a fifth weak peak (M26, see below) was observed. The assignment for the two cross-peaks M309 and M313 was established in the previous work by Beatty et al. [16]. The other three resonances are assigned as follows: peak M26 is missing in the M26I mutant, peak M256 is missing in the M256V mutant and the remaining peak therefore must be M109 (Figure 2). The assignment for the peaks M26 and M109 is opposite to that in the previous report [16].

NMR spectra of [¹³C]methionine-labelled hTF have been used successfully to study the metal-induced conformational change

in the protein [16]. In this work, the diamagnetic metal gallium was used because of concerns about line broadening by paramagnetic iron(III). In the present report, [¹³C]methionine 2D NMR spectra for hTF/2N complexes with five other metal ions [Fe(III), Cu(II), Cr(III), In(III) and Co(III)], were collected in order to observe the chemical shifts of the five methionine residues during the binding of hTF/2N with different metal centres. The NMR spectra at 25 °C for the titration of apo-hTF/2N with iron clearly shows the movement of some of the methionine residues upon metal binding (Figure 3): the peaks for

Figure 4 A comparison of the NMR spectra for the complexes of hTF/2N with six different metal ions

Upper panel, binding with diamagnetic metal ions; lower panel, binding with paramagnetic metal ions.

Table 3 Iron-release rate constants for the mutants and WT

Conditions were as follows: [Tiron], 12 mM; [Hepes], 50 mM; pH, 7.4; temperature, 25 °C; assay wavelength, 480 nm.

Protein	[KCl](M) ...	100 × Iron-release constant (k_{obs}) (min ⁻¹)	
		0	0.5
hTF/2N (WT)*		2.24 ± 0.04	1.26 ± 0.03
M26I		2.43 ± 0.06	0.84 ± 0.04
M109L		1.30 ± 0.06	0.45 ± 0.02
M256V		2.80 ± 0.05	0.76 ± 0.04
M309I		2.15 ± 0.07	0.79 ± 0.04
M313I		2.86 ± 0.04	0.66 ± 0.03

* From He et al. [13].

M256 and M309 essentially remain unchanged, the peak for M313 has a small shift, while the peak for M109 moves downfield in both the ¹H and ¹³C dimensions and the peak for M26 appears (Table 2).

Binding of the other metals to the protein showed similar changes in the NMR spectra (Figure 4). Copper(II) binding leads to some unique features in the NMR spectrum of the protein: the M309 peak slightly shifts upfield in the proton dimension and the M26 peak is completely absent (Figure 4 and Table 2). The spectrum of Cu(II)-hTF/2N was taken at pH 9.2 (adjusted using 1 M Na₂CO₃ solution) at 25 °C since there was no detectable NMR signal for the protein at pH 7.4. When the temperature

was increased to 37 °C, the resonance signal for the Met²⁶ was observed in the spectrum of Cu(II)-hTF/2N (results not shown). Identical NMR experiments were also carried out for Co(III)-hTF/2N complex and resulted in similar phenomena: the M26 peak was absent at pH 9.2 and 25 °C, but appeared when the temperature was raised to 37 °C.

Iron-release kinetics with Tiron

Iron release from each of the methionine mutants by Tiron was carried out at KCl concentrations of both 0 and 0.5 M. The resulting rate constants, k_{obs} , are listed in Table 3. Compared with the wild-type hTF/2N, all of the mutants except M109L had similar, or slightly faster, iron-release rates in the absence of chloride; in contrast, the M109L mutant had an iron-release rate that was approximately half that of the wild-type and the other mutant proteins. Chloride showed a retarding effect on the iron release in all cases, with larger chloride effects observed in the iron release from the mutants.

Further experiments were performed to address the chloride effect in detail. Along with that for wild-type hTF/2N, the chloride-dependent iron release from the M26I, M109L and M309I mutants is shown in Figure 5. These three mutants were selected because: (1) both residues Met²⁶ and Met¹⁰⁹ have significant NMR chemical-shift changes upon metal binding; (2) the M109L mutant has the slowest iron release; and (3) the M309I mutant is representative of a mutant with an iron-binding behaviour similar to that of wild-type hTF/2N. Over the KCl concentration range 0–2.0 M, the chloride effect on iron release from these mutants displayed a similar pattern to that for wild-type hTF/2N: the rate constant had a sharp decrease at low

Figure 5 Chloride effects on iron release from wild-type and the three mutants

The conditions used were: [Tiron], 12 mM; [Hepes], 50 mM; pH, 7.4; temperature, 25 °C.

chloride concentrations, reached a minimum point and then with increasing KCl concentration increased slightly. The minimum point for the M109L mutant and wild-type hTF/2N is at a KCl concentration of 0.5 M; it is higher ([KCl] = 1.0–1.5 M) for the M26I and M309I mutants. The negative effect of chloride is more pronounced in the mutant proteins than in wild-type hTF/2N.

DISCUSSION

Electronic spectra and iron-release kinetics

The side chains of the methionine residues in hTF/2N do not have a known specific function in the protein, nor do they contact, directly or indirectly, functional residues. It was, therefore, anticipated that the mutation of any of the methionine residues would not produce a large disturbance in the metal-binding site of hTF/2N. The similarity of the UV–visible spectra for the metal-containing mutants and wild-type hTF/2N supports this contention. The small variation in the iron-release kinetics in the absence of chloride between four of the mutants and wild-type proteins (Table 3) provides clear evidence that mutations of these methionine side chains led to minor effects on the iron-binding stability. The exception is the M109L mutant, in which slow iron release (approximately half) compared with that with hTF/2N is evident. In hTF/2N, Met¹⁰⁹ is buried in the interior of the NII domain, surrounded by a number of apolar residues, including Ala⁹⁹, Leu¹¹², Leu²²⁶, Leu²²⁸ and Leu²⁴³. Introducing another apolar amino acid, leucine, to replace methionine at position 109 might allow a better fit in this hydrophobic environment and contribute to an overall stabilizing effect on the iron–protein.

Interestingly, in iron release by Tiron, chloride has a greater retarding effect with all the mutants than with the wild-type hTF/2N (Table 3). According to the proposal of Aisen and co-workers, chloride retards iron release by competing with the chelator for the anion-binding site(s) where iron release is initiated

[23]. The larger chloride effect found in iron release with the mutants implies that the anion-binding site(s) were modified to some extent due to the mutations at the methionine residues. Although no detailed evidence from structural analysis supports this idea directly, it is clear that the overall chloride effect from the individual substitution of the methionine side chain with an apolar residue leads to an alteration of the anion effect on iron release from Fe–hTF/2N. The poor solubility of the methionine mutants in Hepes buffer, pH 7.4, lacking anions such as bicarbonate (results not shown) appears to be a further indication that this is the case. However, this influence from the mutations is relatively small, as demonstrated by the similar profile of the chloride effect shown in the iron release for both the mutants and the wild-type hTF/2N (Figure 5).

NMR spectra and conformational change

Consistent with the results by Beatty and co-workers [16], the 2D HSQC spectra for [¹³C]methionine-labelled hTF/2N gave five clear cross-peaks for the five methionine residues. The assignment of these peaks is unambiguously established by assistance from the NMR spectra for recombinant single-point mutant proteins. The tentative assignment for the residues Met²⁶ and Met¹⁰⁹ in the earlier work [16] is reversed. It appears that the assignment based on the NOE effects is not reliable in this case. Methionine residues at positions 256, 309 and 313 are three very accessible residues on the surface of hTF/2N that do not have any contact with functional residues [4]. The isolated locations of these methionine residues in hTF/2N make their NMR signals quite sharp (Figure 2). Met²⁶ has a broad NMR signal and is absent in the apo form at 25 °C because it is surrounded by a number of apolar residues, including Phe²², Val²⁹, Ile³⁰, Pro³⁵, Leu²⁶⁶, Ala²⁷⁰ and Phe²⁷⁴. Increasing the temperature to 37 °C enhances the motional relaxation of the Met²⁶ residue, and therefore its resonance becomes observable.

As stated above, since the five methionine residues are well distributed in the N-lobe, it was hoped that the NMR spectra associated with the [¹³C]methionine residues would provide a diagnostic pattern indicating the dynamics of the protein when binding or releasing metal. For example, diamagnetic Ga(III) was used to demonstrate the metal-induced conformational change by NMR spectroscopy and to avoid the paramagnetic effects of high-spin Fe(III) [16]. In the present study, titration of hTF/2N with the naturally bound Fe(III) ion at 25 °C allowed observation by NMR of the conformational change of the protein during metal binding, with the apparent movement of peak M109 and the appearance of peak M26 (Figure 3). The latter phenomenon may be due to the fact that iron-containing hTF/2N is more globular and thus has higher rotational diffusion than the apo form.

Similar effects were observed for other metals binding the [¹³C]methionine labelled protein, no matter whether diamagnetic or paramagnetic metals are used. A comparison of the NMR spectra for six different metal complexes (Figure 4) shows that the resonance associated with M109 becomes broader after Fe(III) and Cr(III) binding. This change could be due to the pseudo-contact paramagnetic effect of the high-spin Fe(III) and Cr(III) which penetrates to the Met¹⁰⁹ residue and shortens its relaxation time since the distance between the Met¹⁰⁹ residue and the metal centre is within 2 nm [20 Å (≈ 17.4 Å)] [4]. No NMR signals for the methionine residues in Cu(II)–hTF/2N can be observed at pH 7.4. This may be due to the weakness of the interaction of copper(II) with the protein in which reversible binding of the paramagnetic copper ion broadens the signals and makes them impossible to see. Increasing the pH for Cu(II)–

hTF/2N leads to tighter copper binding and results in observable NMR peaks. The absence of the M26 peak in the spectrum of Cu(II)-hTF/2N can be accounted for by the pH effect rather than the paramagnetic effect of Cu(II), since the same phenomenon is found in the NMR spectrum of the protein complex with diamagnetic Co(III) ion at pH 9.2. Raising the pH somehow slows down the motion of the Met²⁶ residue, leading to increased relaxation time. This situation can be improved by increasing the temperature, as shown by the spectra for both Cu(II)- and Co(III)-hTF/2N at 37 °C, where the M26 peak can be observed even at pH 9.2 (results not shown).

In conclusion, the present work corrects the NMR assignment for the Met²⁶ and Met¹⁰⁹ residues and thereby establishes the unambiguous assignment for all five methionine residues in hTF/2N. Metal-induced conformational change of hTF/2N can be observed by NMR spectroscopy upon binding of the protein with both diamagnetic and paramagnetic metal ions. Minor functional effects are found in the mutations at the methionine residues of hTF/2N, although most of these residues are located on the surface. These effects are demonstrated by different metal removal rates and differences in the chloride effect in the iron-release kinetics. This finding shows that a more general impact resulted from mutations made, even at so-called inert residues.

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