

$[^1\text{H}, ^{13}\text{C}]$ NMR determination of the order of lobe loading of human transferrin with iron: comparison with other metal ions

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Abstract Human serum transferrin (hTF) is a single-chain bilobal glycoprotein (80 kDa) which transports Fe^{3+} and a variety of other metal ions in blood. Only diferric transferrin, not the apo-protein, binds strongly to transferrin receptors and is taken up by cells via receptor-mediated endocytosis. We show here that 2D $[^1\text{H}, ^{13}\text{C}]$ NMR studies of recombinant ϵ - $[^{13}\text{C}]$ Met-hTF allow the order of lobe loading with various metal ions, including Fe^{3+} , to be determined. In particular, the resonance for Met-464, a residue in the hydrophobic patch of helix 5, is very sensitive to iron binding in the C-lobe. The selectivity of lobe loading with Fe^{3+} is compared to loading with Fe^{2+} (which binds as Fe^{3+}), Al^{3+} , Ga^{3+} and Bi^{3+} . Similar changes in shifts of the Met residues are observed for these metal ions, suggesting that they induce similar conformational changes in the protein.

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Key words: Iron; Metalloprotein; Metal ion; Nuclear magnetic resonance spectroscopy; Transferrin

1. Introduction

Serum transferrin is a single-chain 80 kDa glycoprotein found in the blood, consisting of two similar lobes each of 40 kDa connected by a short peptide. X-ray structures of transferrins and lactoferrins [1–7] show that each lobe contains an approximately octahedral Fe^{3+} binding site consisting of two tyrosinate oxygen atoms, one His nitrogen, one Asp carboxylate and two oxygen atoms from a bidentate carbonate anion (the synergistic anion). Metal ions cannot bind strongly without concomitant binding of a synergistic anion. Besides carbonate, other anions such as oxalate or malonate can also act as synergistic anions. While iron is the natural substrate of transferrin, many other metal ions can bind to the iron site, and this has led to the idea that transferrin acts as a 'delivery system' for both harmful and beneficial metal ions in the body.

An important feature which has emerged from X-ray studies of transferrin and lactoferrin is that iron induces a conformational change from a lobe-open (apo) to lobe-closed (holo) form. Only diferric transferrin and not the apo-form

binds strongly to the receptor protein on the surface of the cell and is taken up. Small angle X-ray scattering studies have shown that loading of transferrin with Fe^{3+} , In^{3+} , Cu^{2+} or Al^{3+} causes a decrease in the radius of gyration of the protein consistent with lobe closure [8]. Ga^{3+} also probably induces lobe closure since gallium transferrin is recognised by the transferrin receptor [9]. This process may be relevant to the anticancer activity of $\text{Ga}(\text{NO}_3)_3$ and to the delivery of the radioactive isotope ^{67}Ga to cells for diagnostic imaging. In blood plasma, transferrin is only about 30% saturated with iron, with occupies the N-lobe [10]. There is much interest in the functional differences between the two binding sites and numerous experiments in which metal ions are thought to load either the N- or C-lobes have been reported [11].

There appear to be few methods which currently allow a direct determination of the order of lobe loading of transferrin with metal ions. Methods such as EPR [12], DSC [13,14] and heteronuclear NMR spectroscopy [15–22] can often distinguish two stages of loading, but comparison with isolated individual lobes is necessary before the steps can be assigned to specific lobes. A direct determination of which lobe is loaded using gel electrophoresis is possible for some metal ions, but the procedure involves the use of 6 M urea which allows examination only of metals which bind tightly [23,24]. In the present work we show that the order of lobe loading can be determined directly under conditions of physiological relevance via 2D $[^1\text{H}, ^{13}\text{C}]$ NMR studies of recombinant (non-glycosylated) hTF labelled with ϵ - $[^{13}\text{C}]$ Met. This method is successful even for paramagnetic Fe^{3+} . The $[^1\text{H}, ^{13}\text{C}]$ NMR resonances are used to compare the conformational changes induced by binding of Fe^{3+} with those induced by Al^{3+} , Ga^{3+} and Bi^{3+} .

2. Materials and methods

2.1. Materials

Recombinant hTF was expressed in baby hamster kidney cells using a pNUT plasmid with ϵ -L- $[^{13}\text{C}]$ methionine in the growth medium and purified as previously described [25,26]. Iron was removed from the proteins by treatment with NTA and EDTA (both 1 mM) in 0.5 M sodium acetate at pH 4.9, and followed by concentration in a Centricon 30 microconcentrator (Amicon). Potassium chloride (ACS reagent), $[\text{Al}_2(\text{SO}_4)_3]$ (A.R.), ferrous ammonium sulphate ($[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2]$, A.R.) and sodium bicarbonate (ACS reagent) were purchased from Aldrich. Stock solutions of Fe^{3+} and Ga^{3+} were prepared from atomic absorption standards (1000 ppm, in 5% HNO_3 , Johnson Matthey) by adding of 2 mol equiv of H_3NTA (Aldrich) followed by pH adjustment to 6.0–7.0, freeze-drying, and redissolution in D_2O . A $[\text{Bi}(\text{NTA})]$ stock solution was prepared by dissolving $[\text{Bi}(\text{NTA})]$ [27] in D_2O . Fresh Fe^{2+} solutions were prepared by dissolving known amounts of ferrous ammonium sulphate in D_2O .

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Abbreviations: DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; HMQC, heteronuclear multiple-quantum coherence; hTF, human serum transferrin; hTF/2N, N-lobe of hTF; NTA, nitrilotriacetate; pH*, pH meter reading in D_2O

2.2. Sample preparations

NMR samples of ϵ - ^{13}C Met-hTF (ca. 0.3 mM) were prepared in 0.1 M KCl in D_2O , and pH* values were adjusted to 7.4 ± 0.1 using NaOD or DCl. Sodium bicarbonate in D_2O (0.25 M) was added to transferrin solutions to give a concentration of 10 mM. Addition of metal complexes was made to protein samples containing bicarbonate to give metal: protein ratios of up to 2.5:1, and the pH* was readjusted to 7.4 ± 0.1 (8.8 ± 0.1 for Al^{3+}) after each addition; the samples were left 30 min for equilibration.

2.3. NMR spectroscopy

$[\text{H},^{13}\text{C}]$ HMQC [28] spectra were acquired on Varian Unity 600 and Varian Unity-Plus 500 NMR spectrometers at 600 and 500 MHz, respectively, at 310 K. The sequence was optimised for $^1\text{J}(\text{H}-^{13}\text{C})=140$ Hz, and 8–16 transients were acquired using 2 K points in f2 dimension (^1H), 32–64 increments of t_1 , ^{13}C frequency width of 3 kHz (sw1), and relaxation delay of 1.6 s giving a total ca. 30 min for the acquisition of each spectrum. The GARP-1 sequence [29] was used to decouple ^{13}C . After zero filling to 4 K \times 1 K points, unshifted-Gaussian functions were used for processing. The residual water signal was suppressed by a combination of presaturation and pulsed-field gradients. Peaks were referenced to sodium 3-(trimethylsilyl)propionate-2,2,3,3- d_4 (TSP) via the ϵ - CH_3 peak of L-methionine (external, 15.14 ppm) for ^{13}C , and via formate (8.465 ppm, a minor impurity always present in the protein) for ^1H .

3. Results

Recombinant non-glycosylated human transferrin with $>95\%$ ϵ - $^{13}\text{CH}_3$ enrichment at all nine Met residues (Fig. 1) was prepared according to published procedures [30]. 2D $[\text{H},^{13}\text{C}]$ NMR spectra of the labelled protein at low concentration (ca. 300 μM) were obtainable within a short time (ca. 0.5 h) using inverse detection techniques. Cross-peaks for eight Met residues were readily observed, and that for the ninth appeared when the temperature was raised from 310 to 318 K, or when bicarbonate was added. Reasonable assignments of these cross-peaks, as deduced previously from 2D

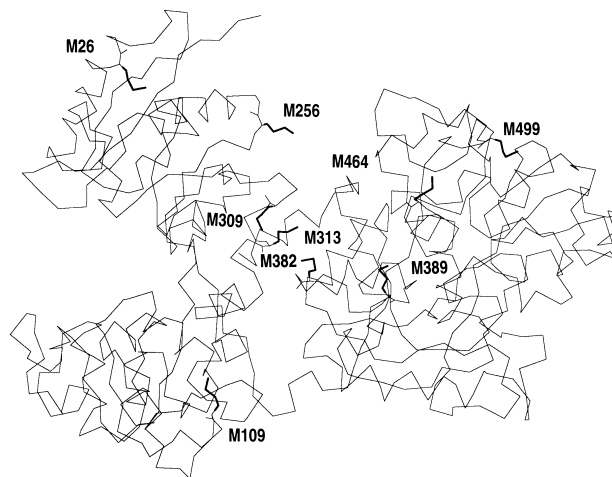


Fig. 1. Distribution of the nine Met residues in human serum transferrin, based on the X-ray crystal structure of Fe_C -hTF [1]. The N-lobe with an open interdomain cleft is on the left-hand side. The Met side-chains are shown in bold, and the residue numbers are indicated.

NMR data for hTF and hTF/2N [30], are shown in Fig. 2. The chemical shifts were constant over the range pH* 6.8–8.8.

3.1. Detection of Fe^{3+} binding to transferrin and order of lobe loading

Fe^{3+} , as $[\text{Fe}(\text{NTA})_2]$, was added to hTF at pH* 7.4 to give $\text{Fe}:\text{hTF}$ mol ratios of 0.5:1 and 2.0:1 (0.5 mol equiv steps). The addition of the first mol equiv of Fe^{3+} led to the dramatic disappearance of the cross-peak for Met-464 and was accompanied by the appearance of a new broad peak shifted by ca. 2 ppm to low field in the ^{13}C dimension and ca. 0.2 ppm to

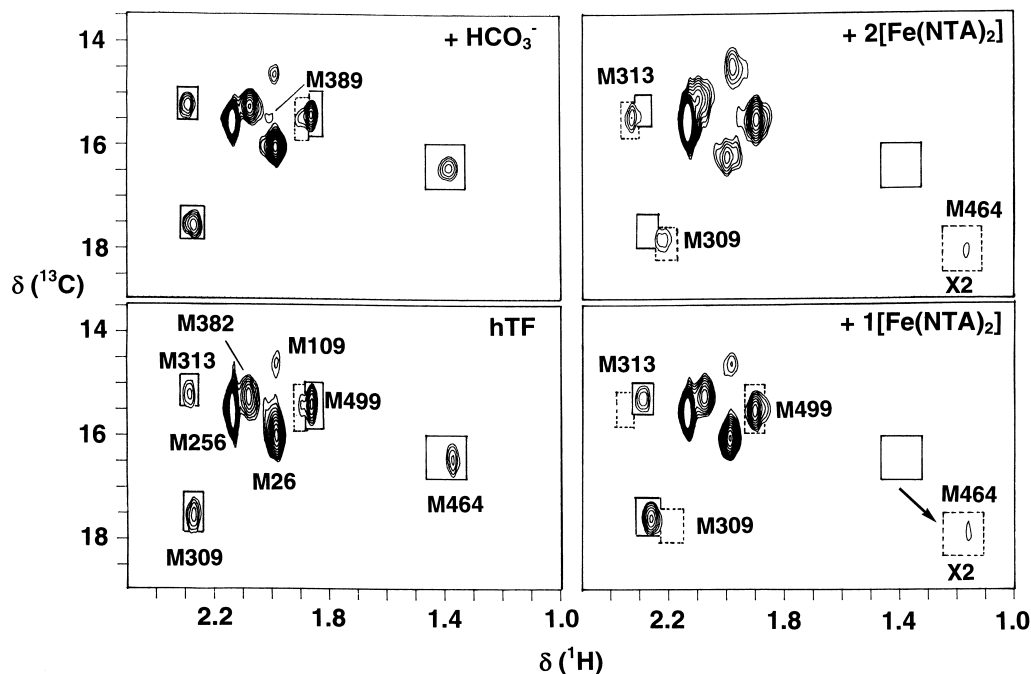


Fig. 2. 2D $[\text{H},^{13}\text{C}]$ HMQC spectra of ϵ - ^{13}C Met-apo-hTF, and after addition of 10 mM bicarbonate at pH* 7.4, and 1.0 and 2.0 mol equiv of Fe^{3+} as an NTA complex. The assignments for Met resonances are based on [30]. The specific shifts of cross-peaks for the C-lobe residue Met-464 and Met-499 on binding the first equiv of Fe^{3+} and N-lobe residues Met-309 and Met-313 on binding the second equiv are notable. The cross-peak for Met464 is broadened, see Fig. 4. The solid boxes indicate initial peaks and dotted boxes show new peaks.

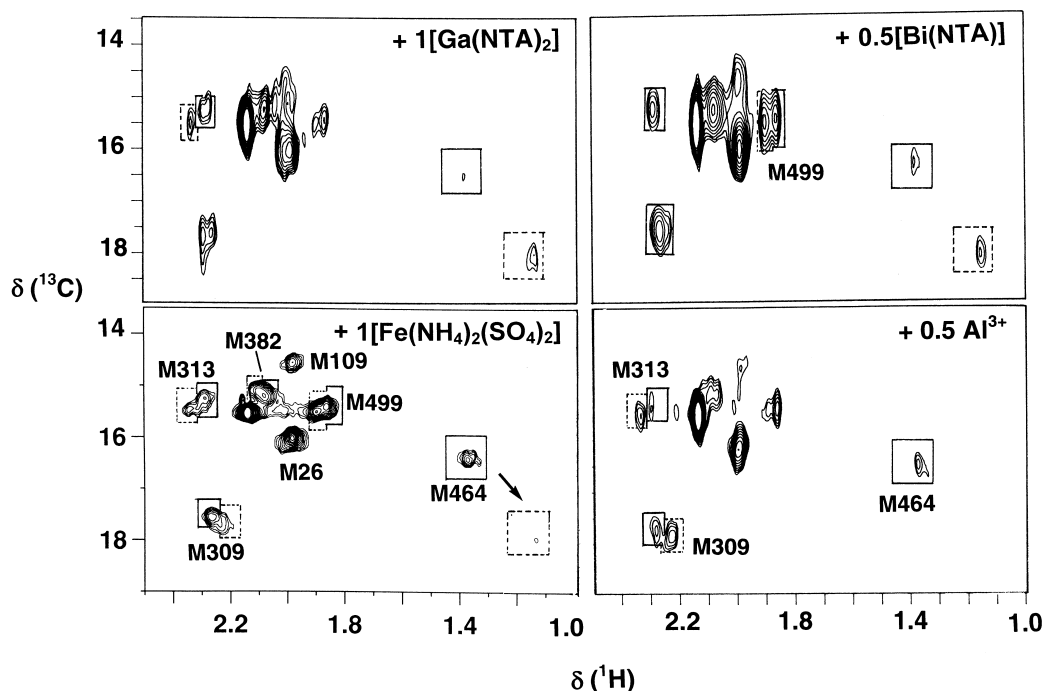


Fig. 3. 2D $[^1\text{H}, ^{13}\text{C}]$ HMQC spectra of ϵ - $[^{13}\text{C}]$ Met-apo-hTF in the presence of 10 mM bicarbonate, after addition of 1.0 mol equiv of ferrous ammonium sulphate ($\text{pH}^* 7.4$), 1.0 mol equiv. of $[\text{Ga}(\text{NTA})_2]$, 0.5 mol equiv of Al^{3+} or 0.5 mol equiv Bi^{3+} . The solid boxes indicate initial peaks and dotted boxes show new peaks.

high field in the ^1H dimension (Fig. 2). The cross-peak for Met-499 also disappeared and a new peak appeared which is shifted slightly to low field (Fig. 2). When the second mol equiv of Fe^{3+} was added, there was no further change to these peaks, but shifts were observed for the cross-peaks of N-lobe residues Met-26, Met-109, Met-309 and Met-313. In addition, a shift of the peak for Met-382, which is in the C-lobe, was observed. The chemical shift changes are listed in Table 1, and coordination shifts are calculated on the assumption that the peaks for the iron-loaded protein are close to those for the apo-protein. Although the peak for Met-464 is markedly broadened by Fe^{3+} binding, a notable sharpening of the cross-peak for Met-109 was observed.

The reaction of apo-hTF with Fe^{2+} (added as a solution of ferrous ammonium sulphate) was also studied under similar conditions. In this case, after addition of 1 mol equiv of Fe^{2+}

(in the presence of 10 mM bicarbonate and after equilibration for ca. 30 min), cross-peaks for both N- and C-lobe Met residues decreased in intensity and new peaks appeared with the same chemical shifts as those observed for $\text{Fe}(\text{III})_2$ -hTF (Fig. 3 and Table 1). The disappearance of peaks for apo-hTF and the appearance of these new peaks for Fe_2 -hTF was complete after addition of 2 mol equiv of Fe^{2+} . Similar spectra were obtained after leaving the same mixture overnight (> 12 h) at 310 K showing that equilibrium had indeed been reached. An additional experiment was also carried out in which 0.8 mol equiv of Fe^{2+} (ferrous ammonium sulphate) was added to a solution of apo-hTF (80 μM , 10 mM HCO_3^- , $\text{pH}^* 7.4$) without added KCl. The 2D $[^1\text{H}, ^{13}\text{C}]$ spectra recorded after average reaction times of ca. 1 h and 6 h (2 h accumulations) were both similar to that shown in Fig. 3 for reaction in the presence of KCl.

Table 1

^1H and ^{13}C NMR chemical shifts of apo-hTF, iron(III)-induced coordination shifts ($\Delta\delta = \delta_{\text{Fe}_2\text{-hTF}} - \delta_{\text{apo-hTF}}$) for the Met-(ϵ)- $^{13}\text{CH}_3$ residues of hTF at 310 K, their locations, and distances between Met- ϵ - $^{13}\text{CH}_3$ and Fe^{3+}

Residue (lobe)	Location	ϵ - $^{13}\text{CH}_3$ distance from Fe^{3+} (\AA)		δ ($^1\text{H}/^{13}\text{C}$) (ppm)	$\Delta\delta$ ($^1\text{H}/^{13}\text{C}$) ^d (ppm)
		N-lobe ^a	C-lobe ^b		
Met-26 (N)	buried	23.1		1.98/16.02	0.03/0.13
Met-109 (N)	partially buried	16.5		1.99/14.61	-0.01/-0.10
Met-256 (N)	completely exposed	30.0		2.13/15.54	0.00/0.00
Met-309 (N)	mostly buried	17.1		2.27/17.52	-0.05/0.28
Met-313 (N)	partially exposed	21.4		2.29/15.22	0.05/0.24
Met-382 (C)	partially exposed	22.1	21.5	2.07/15.23	0.03/-0.20
Met-389 (C)	completely buried		12.2	2.01/15.48 ^c	
Met-464 (C)	Trp460 hydrophobic patch of helix 5		16.1	1.38/16.30	-0.20/1.90 ^d
Met-499 (C)	mostly exposed		21.8	1.86/15.40	0.04/0.11

^aData from model of hTF/2N [36] based on the X-ray structure of N-lobe of diferric rabbit serum transferrin [7].

^bData from the X-ray structure of Fe_2 -hTF [1].

^cPeak appears at 318 K or after addition of bicarbonate at 310 K.

^d $\Delta\delta = \delta_{\text{Fe}_2\text{-hTF}} - \delta_{\text{apo-hTF}}$.

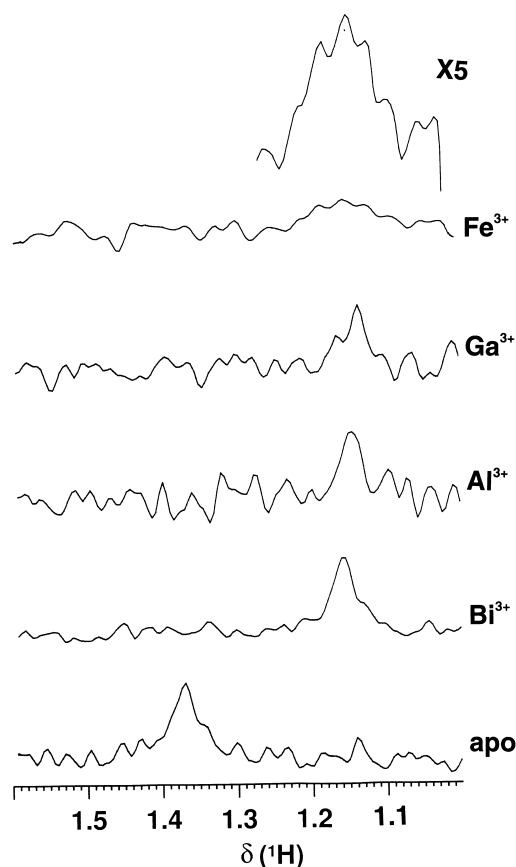


Fig. 4. Slices through 2D [^1H , ^{13}C] HMQC spectra of ϵ - ^{13}C Met-apo-hTF, and after addition of one mol equiv of Bi^{3+} , two mol equiv of Al^{3+} , two mol equiv of Ga^{3+} , or one mol equiv of Fe^{3+} (as $\text{Fe}(\text{NTA})_2$), showing the similar shifts of Met-464 on occupation of the C-lobe site. The ^1H linewidth of Met-464 is ca. 30 Hz for apo-hTF, Bi_1 -hTF, Al_2 -hTF and Ga_2 -hTF, and ca. 80 Hz for Fe_C -hTF.

3.2. Uptake Ga^{3+} , Al^{3+} and Bi^{3+} by transferrin

The binding of these metal ions to apo-hTF was also studied by 2D [^1H , ^{13}C] NMR under similar conditions. On addition of $[\text{Ga}(\text{NTA})_2]$, the behaviour of the cross-peaks was similar to that observed for Fe^{2+} , with addition of 1 mol equiv of Ga^{3+} causing a decrease in intensity of the initial peaks for apo-hTF and appearance of a new set of peaks

for both N- and C-lobe Met residues (Fig. 3). However, there was less broadening of peaks for Met-464 than in the case of the reaction with Fe^{2+} (or Fe^{3+}). Addition of the second mol equiv of Ga^{3+} led to a further increase in intensity of the peaks for Ga_2 hTF and almost complete disappearance of peaks for apo-hTF. The chemical shifts changes observed were identical to those observed for binding of Fe^{3+} .

No changes to the 2D [^1H , ^{13}C] NMR spectrum were observed on reaction of apo-hTF with Al^{3+} (added as $\text{Al}_2(\text{SO}_4)_3$) at pH* 7.4 in the presence of 10 mM bicarbonate. However, on increasing the pH* to 8.8, peaks for the N-lobe resonances Met-109, Met-309, Met-313 and interlobe Met-382 decreased in intensity and new cross-peaks appeared (Fig. 3). On addition of the second equiv of Al^{3+} , peaks for Met-464, Met-389 and Met-499 were perturbed. The new cross-peak for Met-464 appeared at 1.17/18.20 ppm, a similar shift change to that observed for Fe^{3+} binding to the protein. The shift changes for the other Met peaks in the spectrum were also similar to those observed with Fe^{3+} .

Addition of 0.5 mol equiv of Bi^{3+} (as $[\text{Bi}(\text{NTA})]$) led to a decrease in intensity of peaks for C-lobe residues and appearance of new peaks characteristic of Bi -hTF (Fig. 3). Again the large shift of the peak for Met-464 to 1.17/18.20 ppm was notable, with no changes in shift for the N-lobe peaks. These changes were complete in the presence of 1 mol equiv of Bi^{3+} . Addition of a second mol equiv of Bi^{3+} caused a decrease intensity of peaks for N-lobe residues and appearance of new peaks with shifts similar to those of Fe_2 hTF, but peaks for apo-hTF still remained in the spectrum. Interestingly, when 1 mol equiv of Fe^{3+} (as $\text{Fe}(\text{NTA})_2$) was added to Bi_1 -hTF, Fe^{3+} did not occupy the vacant N-lobe, but instead displaced Bi^{3+} from the C-lobe, as judged from the 2D NMR spectra.

4. Discussion

Transferrin is a major transport protein in blood, and binds not only to the natural metal ion Fe^{3+} , but also to other metal ions of toxic (e.g. Al^{3+} [9]), therapeutic (e.g. Bi^{3+} [31]) and diagnostic (e.g. $^{67}\text{Ga}^{3+}$ [9]) interest. The protein possesses two similar metal binding sites in the N- and C-lobes which differ slightly in their affinities, but the factors which direct metal ions to specific lobes of hTF are poorly understood. The strength of metal binding to hTF correlates with metal ion acidity: the most acidic metal ions (Fe^{3+} , Bi^{3+}) binding the

Table 2
Order of lobe loading of serum apo-transferrin with various metal ions using different techniques

Metal	Loading complex	Technique	pH or pH*	Anion	Lobe preference	
					N-	C-
Al	$\text{Al}^{\text{III}}_2(\text{SO}_4)_3$	^1H , [^1H , ^{13}C] NMR	8.8	HCO_3^-	+	
Sc	$\text{Sc}^{\text{III}}(\text{NTA})_2$	^1H NMR	7.4	HCO_3^-		+
Fe	$\text{Fe}^{\text{III}}(\text{NTA})_2$	[^1H , ^{13}C] NMR	7.4	HCO_3^-		+
		electrophoresis	6–8.5	HCO_3^-		
		calorimetry				+
	$\text{Fe}^{\text{II}}(\text{NH}_4)_2(\text{SO}_4)_2$	[^1H , ^{13}C] NMR	7.4	HCO_3^-	+	+
		electrophoresis	7.4–8.5	HCO_3^-	+	
Ga	$\text{Ga}^{\text{III}}(\text{NTA})_2$	[^1H , ^{13}C] NMR	7.4	HCO_3^-	+	+
		^1H , [^1H , ^{13}C] NMR	7.2	oxalate		+
Tl	$\text{Tl}^{\text{III}}\text{Cl}_3$	^{205}Tl NMR	7.3–7.8	HCO_3^-		+
Bi	$\text{Bi}^{\text{III}}(\text{NTA})$	^1H , [^1H , ^{13}C] NMR	7.4	HCO_3^-		+

The results of the present studies as well as data from previous reports, for which the references are given in [11] and [12], are included.

most strongly [32]. Only when hTF is loaded with metal ions in both lobes does it bind strongly to the receptor and so the conformational changes which accompany metal binding are of direct relevance to its recognition by cells. The 2D [^1H , ^{13}C] NMR method reported here allows the direct determination of the order of loading of the lobes of intact hTF with metal ions, and, moreover, for the first time using NMR, it is possible to compare directly structural changes induced by Fe^{3+} in comparison to other metal ions.

Using recombinant hTF labelled specifically with ϵ - ^{13}C Met, it is possible to detect peaks for eight (and sometimes all) of the nine Met residues in hTF at micromolar concentrations, and although the protein is expensive to produce, it can be demetallated and recycled for studies under different conditions. The recombinant hTF is non-glycosylated, but there is no evidence that glycosylation affects metal uptake [26]. The SCH_3 peaks are convenient for NMR studies since they are singlets, and moreover the Met residues are well spread throughout the protein and occupy several types of environment (Table 1 and Fig. 1). Since the (carbon atoms of the) SCH_3 groups are all over 12 Å from Fe^{3+} , it is a reasonable assumption that the SCH_3 cross-peaks are little affected by the paramagnetism of Fe^{3+} if the effects are predominantly dipolar (high-spin $S = 5/2$ [12], dipolar shifts $\propto 1/r^3$, dipolar broadening $\propto 1/r^6$, where r is the distance between Fe^{3+} and nucleus of interest [33,34]).

Previously we have described the use of ^1H NMR to determine the order of lobe loading of hTF with Al^{3+} and Ga^{3+} [35–37]. This required studies on the isolated N-lobe alone and the use of specific resonances as fingerprints. Although His ^1H NMR resonances were useful for fingerprinting, they were difficult to follow on account of their sensitivities to pH near pH 7. This is not the case with ϵ - ^{13}C Met resonances, and, moreover, the resonance for Met-464 is a specific and sensitive indicator for C-lobe binding. It is also possible to determine successfully the order of loading using, e.g. heteronuclear NMR, DSC and EPR [12,13], but again comparison with isolated N-lobe is necessary. Although apo-hTF can sometimes be separated from mono- (Fe_C or Fe_N) and di-metal transferrin by electrophoresis, the method requires harsh conditions (6 M urea) and is successful only for very tightly bound metal ions [23,24,38].

Importantly the present study has allowed determination of the order of lobe loading of hTF with Fe^{3+} (as an NTA complex in the presence of HCO_3^- as synergistic anion), and, in agreement with previous reports, the C-lobe is occupied preferentially. Met-464 is situated in the hydrophobic patch (V454-W460-M464, the analogue of L122-W128-I132 in the N-lobe) of helix 5, which backs onto the metal binding sites and H-bonds to the synergistic anion [1,7]. This result indicates that the cross peak for Met-464 is a sensitive indicator of metal binding to the C-lobe. We found that reaction of one mol equiv of Fe^{2+} with apo-hTF led to the occupation of both lobes with iron, in contrast to previous reports in which it was concluded that this method of loading with iron leads to selective loading of the N-lobe [23,24]. Although Cl^- ions have been reported to affect the distribution of iron between the two lobes [39], this did not appear to be the case under the conditions used here.

Since the shifts and broadening of [^1H , ^{13}C] NMR resonances upon loading apo-hTF with 2 mol equiv of Fe^{2+} were the same as that with Fe^{3+} , it can be concluded that Fe^{2+} is

oxidised as it is taken up, and this was confirmed by the appearance of the characteristic tyrosinate-to- Fe^{3+} charge-transfer band at 465 nm [12]. The detailed mechanism of this process appears to be unknown, but probably involves O_2 as oxidant.

The results obtained here for the order of lobe loading with Ga^{3+} , Bi^{3+} and Al^{3+} agree with previous reports. The behaviour of Bi^{3+} resembles that of Fe^{3+} (as NTA complexes) with preferential binding to the C-lobe, whereas Ga^{3+} shows little preference for either lobe. Under the conditions of our experiments, Al^{3+} does not load either the N-lobe or the C-lobe at pH* 7.4, but at pH* 8.8, preferentially loads the N-lobe, in contrast to Fe^{3+} and Bi^{3+} at pH* 7 (Table 2). The difference may be related both to the presence of NTA as a bound ligand on the loading Fe^{3+} and Bi^{3+} complexes, and to the change in charge of the protein (and especially the charge in the interdomain cleft) at high pH.

Although the shift changes for Met resonances induced by Fe^{3+} are similar to those induced by Ga^{3+} , Al^{3+} and Bi^{3+} , implying that these metals all induce similar conformational changes on binding, Fe^{3+} markedly broadens the cross-peak for Met-464 (Fig. 4). The contribution of paramagnetic effects to peak broadening remains to be determined, but may be very small at such a large distance ($> \text{ca. } 12 \text{ Å}$) from Fe^{3+} if dipolar in origin. It is therefore possible that iron causes a reduction in mobility of this group, or a change in its dynamic exchange behaviour, which is different from that induced by the other metals studied. Further work is required to investigate this.

5. Conclusion

2D [^1H , ^{13}C] NMR provides a rapid method of determining the order of lobe loading of transferrins with metal ions via the use of ϵ - ^{13}C Met-labelled recombinant transferrin. Under the conditions we have used (10 mM bicarbonate, 0.1 M KCl at pH* 7.4), NTA complexes of Fe^{3+} and Bi^{3+} preferentially load the C-lobe, whereas Ga^{3+} shows little preference, and, in the absence of NTA, Al^{3+} does not bind at pH* 7.4 but preferentially loads the N-lobe at pH* 8.8. Reaction of hTF with Fe^{2+} gave rise to both N- and C-lobe loading (with Fe^{3+}) under the conditions used, in contrast to previous reports [24,38]. The Met residues of hTF are well distributed throughout the protein, and the changes in shift of their [^1H , ^{13}C] NMR resonances can be used as finger-prints of conformational changes induced by various metal ions. A significant result from this work is that Fe^{3+} , Ga^{3+} , Al^{3+} and Bi^{3+} all induce similar conformational changes as judged from the changes in shifts of resonances.

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