Articles

Interlobe Communication in ^{13}C-Methionine-Labeled Human Transferrin†

Emma J. Beatty,‡ Mark C. Cox,‡ Tom A. Frenkiel,§ Beatrice M. Tam,|| Anne B. Mason,⊥ Ross T. A. MacGillivray,|| Peter J. Sadler,*,‡ and Robert C. Woodworth⊥

Department of Chemistry, Birkbeck College, University of London, Gordon House and Christopher Ingold Laboratories, 29 Gordon Square, London WC1H 0PP, U.K., MRC Biomedical NMR Centre, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada, and Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405

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ABSTRACT: [^{1}H, ^{13}C] NMR investigations of metal-induced conformational changes in the blood serum protein transferrin (80 kDa) are reported. These are thought to play an important role in the recognition of this protein by its cellular receptors. [^{1}H, ^{13}C] NMR resonance assignments are presented for all nine methionine $^{13}CH_3$ groups of recombinant deglycosylated human transferrin on the basis of studies of recombinant N-lobe (40 kDa, five Met residues), NOESY-relayed [^{1}H, ^{13}C] HMQC spectra,[1] and structural considerations. The first specific assignments for C-lobe resonances of transferrin are presented. Using methionine $^{13}CH_3$ resonances as probes, it is shown that, with oxalate as the synergistic anion, Ga^{3+} binds preferentially to the C-lobe and subsequently to the N-lobe. The NMR shifts of Met464, which is in the Trp460-centered hydrophobic patch of helix 5 in the C-lobe in contact with the anion and metal binding site, show that Ga^{3+} binding causes movement of side chains within this helix, as is also the case in the N-lobe. The C-lobe residue Met382, which contacts the N-lobe hinge region, is perturbed when Ga^{3+} binds to the N-lobe, indicative of interlobe communication, a feature which may control the recognition of fully-metallated transferrin by its receptor. These results demonstrate that selective ^{13}C labeling is a powerful method for probing the structure and dynamics of high-molecular-mass proteins.

Binding events in proteins often produce local conformational changes and also transmit effects to distant sites via connecting α-helices, *β*-strands, and networks of H-bonds (Mathews & van Holde, 1990). Such allosteric interactions are the basis of, for example, the cooperative uptake of dioxygen molecules by hemoglobin. The blood serum protein transferrin has two Fe^{3+} binding sites, and occupation of these sites increases the strength of binding of transferrin to its cellular receptors by about 2 orders of magnitude (Brock, 1985; Klausner et al., 1983; Young et al., 1984). Therefore, the conformational changes which are induced in the protein by metal binding are central to understanding the biological function of this protein.

Transferrin is a single-chain 80 kDa glycoprotein consisting of two similar lobes each of 40 kDa connected by a short peptide (Baker, 1994). The X-ray crystal structures of diferric rabbit serum transferrin (Bailey et al., 1988), the related protein diferric lactoferrin (Anderson et al., 1987, 1989), and monoferric human transferrin (Zuccola, 1993) 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).

An intriguing aspect of the chemistry of transferrins is that metal ions cannot bind without concomitant binding of a synergistic anion. Synergistic anions have the common features of a carboxylate donor and a second (proximal) electron donor one or two carbon atoms removed (Schlabach & Bates, 1975). Although carbonate is thought to be the natural synergistic anion and has a higher affinity than most other anions, oxalate is also efficient in promoting metal ion binding. The crystal structure of diferric lactoferrin with oxalate in both lobes shows that oxalate is bidentate to Fe^{3+} in both lobes (Baker, 1994), and solution NMR on Tl^{3+} transferrin using [^{13}C]oxalate has confirmed this mode of binding (Bertini et al., 1983, 1988; Aramini et al., 1994a).

Each lobe of transferrin is itself composed of two domains which have alternating α-helical and *β*-sheet segments, a feature common to binding proteins of the "Venus fly-trap" family (Louie, 1993). X-ray crystallographic studies of Fe_2-lactoferrin and Fe_C-lactoferrin (Fe^{3+} in the C-lobe only) have shown that the binding of Fe^{3+} and carbonate causes the N-lobe to change conformation from wide open to closed

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* To whom correspondence should be addressed.
‡ University of London.
§ National Institute for Medical Research.
|| University of British Columbia.
⊥ University of Vermont.

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[1] Abbreviations: DQF-COSY, double-quantum-filtered correlation spectroscopy; NTA, nitrilotriacetate; EDTA, ethylenediaminetetraacetate; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; hTF, human transferrin; hTF/2N, the N-lobe of human transferrin (residues 1−337); NOESY, nuclear Overhauser effect spectroscopy; PCR, polymerase chain reaction.

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(Anderson et al., 1987, 1990; Gerstein et al., 1993). This involves a 54° rotation of the N2 domain relative to domain N1 and flexing of the two antiparallel extended polypeptide strands which run behind the iron binding site connecting domains N1 and N2. One of the Fe^{3+} ligands (Asp63 in human transferrin, hTF)[1] is located on domain 1, one is on domain 2 (Tyr188), and two are on the interdomain strands (Tyr95 and His249) which form the hinge. Binding by the synergistic anion is further stabilized by H-bonding to the N-terminal end of helix 5 (Baker, 1994).

Small-angle X-ray scattering studies have shown (Grossmann et al., 1992, 1993; Castellano et al., 1994) that loading of transferrin with Fe^{3+}, Cu^{2+}, and In^{3+} causes a decrease in the radius of gyration of the protein consistent with lobe closure. Because transferrin has a high molecular mass, ^{1}H NMR structure determinations in solution are not feasible, but a few resonances can be used to "fingerprint" the binding of metal ions to the N- and C-lobes (Kubal et al., 1992, 1993; Battistuzzi et al., 1995). In particular, the high-field-shifted resonances of Leu122 and Ile132, residues which form part of a hydrophobic patch around Trp128 in helix 5 of the N-lobe, can be used to monitor the movements of these residues on metal loading (E. J. Beatty, M. C. Cox, A. B. Mason, P. J. Sadler, A. Tucker, and R. C. Woodworth, to be submitted).

The question of possible cooperativity between the two metal binding sites of transferrin is an interesting one (Brock, 1985). Calorimetric studies have suggested the presence of interlobe interactions in transferrins (Lin et al., 1991, 1994), and X-ray studies show that there is an area of contact between the two lobes involving hydrophobic interactions, salt bridges, and H-bonds (Baker, 1994; Kurokawa et al., 1995).

As summarized recently by Aramini et al. (1994a), three general NMR methods have been used to monitor metal binding to transferrins: (1) direct detection of bound diamagnetic metal ions, (2) observation of bound ^{13}C-enriched anions, and (3) use of ^{1}H NMR to investigate protein conformational changes. In this work we introduce a new approach, involving the labeling of specific amino acid residues of transferrin with ^{13}C. In order to avoid the paramagnetic effects of high-spin Fe^{3+}, we use diamagnetic Ga^{3+} as a replacement. Ga^{3+} has a similar six-coordinate ionic radius (62 pm) to Fe^{3+} (65 pm) (Shannon & Prewitt, 1969) and is known to bind strongly to both lobes (Harris & Pecoraro, 1983). In addition, gallium transferrin has been shown to be recognized by transferrin receptors and block uptake of iron into cells. For this reason there is considerable interest in the role of gallium transferrin in the anticancer activity of $Ga(NO_3)_3$ and in the delivery of the radioactive isotope ^{67}Ga to cells for diagnostic imaging purposes (Ward & Taylor, 1988).

Most previous work on ^{13}C-labeled proteins has been confined to those of molecular mass <30 kDa (Kuboniwa et al., 1995; Slupsky & Sykes, 1995; Wagner, 1993), although some early work on [^{13}C]His-labeled alkaline phosphatase (a dimer of 2 × 47 kDa) demonstrated the advantages of ^{13}C labeling for the study of larger proteins (Otvos & Browne, 1980). Chemical ^{13}C labeling of Met residues of small proteins at ϵ-CH_3 has been widely used [e.g., Jones et al. (1976), Deber et al. (1978), and Harina et al. (1978)], but such labeling is possible only for chemically accessible Met residues, unless the protein is unfolded before treatment, and a lower percentage of enrichment is obtained than is possible by recombinant technology. In the present work, we have labeled all nine Met residues of human transferrin (Figure 1) with [ϵ-^{13}C]Met using recombinant DNA methods. Using the same method, we have also labeled the five Met residues in the N-terminal half-molecule hTF/2N. The work illustrates that specific ^{13}C amino acid labeling can be advantageous even for proteins as large as 80 kDa. Using [ϵ-^{13}C]Met transferrin, the order of loading the lobes with gallium can be simply determined, and our data suggest that metal-induced compression of helix 5, which backs onto the metal and anion binding site, is a feature not only of the N-lobe but also of the C-lobe. In addition, we show that a mechanism for interlobe communication (which may be important for receptor recognition) exists in the protein.

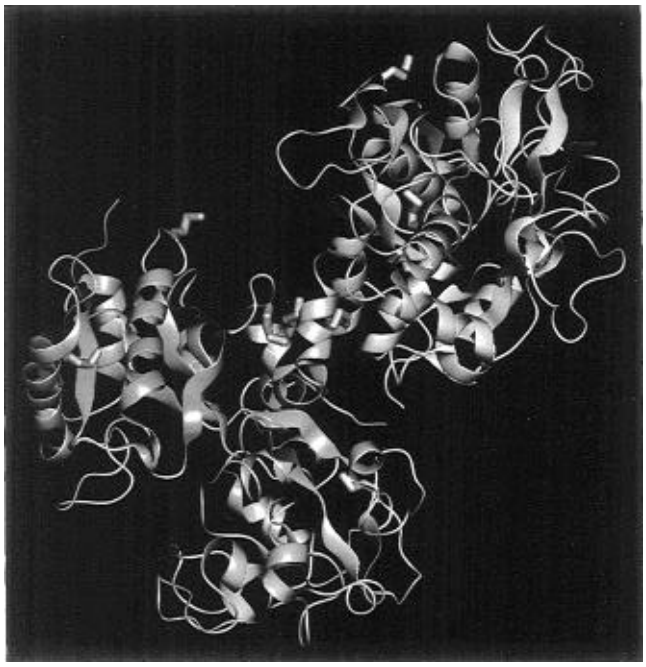

FIGURE 1: Locations of the nine Met residues of hTF as in the X-ray crystal structure of Fe_C-hTF (Zuccola, 1993). The protein backbone is shown in blue and Met side chains are in orange. The N-lobe with an open interdomain cleft is at the bottom left, and from left to right the Met residue numbers are 26, 256, 309, 313, 382 (underline = hinge region), 109 (bottom of picture), 499 (top of picture), 389, and 464.

MATERIALS AND METHODS

Materials. Recombinant hTF/2N, hTF/2N mutants, and hTF were expressed in baby hamster kidney cells using a pNUT plasmid with [ϵ-^{13}C]-L-methionine in the growth medium and purified as previously described (Mason et al., 1991, 1993).

For the mutants M309I and M313I of hTF/2N, the ATG codons for Met309 and Met313 were changed to ATC (Ile) by using *in vitro* mutagenesis. The mutagenic oligonucleotides, 5′-CCCCCAGGATCGATGCCAAGA-3′ and 5′-ATGCCAAGATCTACCTGGGCT-3′ (mutated codons underlined), were synthesized on an Applied Biosystems 391 DNA synthesizer, and were purified by reverse-phase chromatography using a SEP-PAK (Waters) column (Atkinson & Smith, 1984).

For use in the mutagenesis experiments, the plasmid BS-hTF/2N contained the hTF/2N cDNA as an *Xba*I−*Hin*dIII

fragment (Funk et al., 1990) cloned into Bluescript. This cDNA includes the signal peptide and coding region for the N-lobe of transferrin in addition to two stop codons and a *Hin*dIII site inserted after the codon for Asp337. A 180 bp *Eco*RI−*Hin*dIII fragment (containing the region for amino acid residues 281−337 plus the stop codons) was isolated from BS-hTF/2N and was subcloned into the *Eco*RI and *Hin*dIII of Bluescript to give the plasmid BS-hTF-EH. Each of the two mutagenic oligonucleotides was used separately to introduce the desired mutation into BS-hTF-EH by using a polymerase chain reaction (PCR) based mutagenesis method (Nelson & Long, 1989). For the PCR, a hot start of 5 min at 367 K was followed by 25 cycles of denaturation at 367 K for 15 s, annealing at 323 K for 30 s, and extension at 345 K for 45 s. At the end of the last PCR cycle, the extension was continued for 10 min at 345 K. Subsequent PCR steps utilized the Bluescript-specific oligonucleotides, 5′-GGAGTACTAGTAACCCTGGCCCCAGTCAC-GACGTTGTAAA-3′, 5′-CAGGAAACAGCTATGACCAT-3′, and 5′-GGAGTACTAGTAACCCTGGC-3′, and used the same conditions as the first PCR step except that the annealing temperature was 328 K and 30 cycles were used. After the PCR-directed mutagenesis steps, the fragments were cleaved with *Eco*RI and *Hin*dIII, purified by agarose gel electrophoresis using GENECLEAN (Bio101, La Jolla, CA), and religated into BS-hTF/2N in place of the wild-type fragment. The presence of only the desired mutations was confirmed by DNA sequence analysis (Sanger et al., 1977). Each plasmid was then cleaved with *Xba*I and *Hin*dIII to release the mutated hTF/2N cDNA inserts which were isolated by agarose gel electrophoresis, made blunt ended by treatment with the Klenow fragment of DNA polymerase I in the presence of dNTPs, and ligated directly into the *Sma*I site of pNUT (Palmiter et al., 1987). The correct orientation of the cDNA in the pNUT-hTF/2N plasmids was confirmed by restriction endonuclease mapping. The maximum yields of the mutant hTF/2N proteins were M309I, 42 μg mL^{-1}, and M313I, 18 μg mL^{-1}, as determined by solid-phase radioimmunoassay (Mason et al., 1991). Their purities were checked by SDS gel electrophoresis.

Iron was removed from the proteins by treatment with NTA and EDTA (both 1 mM) in 0.5 M sodium acetate, pH 4.9, followed by concentration in a Centricon 10 microconcentrator (Amicon). Potassium oxalate (ACS reagent) was purchased from Aldrich. Stock solutions of Ga^{3+} [47 mM; referred to as $Ga(NTA)_2$] were prepared from gallium atomic absorption standards (1000 ppm, in 5% HNO_3, Johnson Matthey) by addition of 2 mol equiv of H_3NTA (Aldrich) and pH adjustment to 6.5, followed by freeze-drying and redissolution in D_2O.

NMR samples of [ϵ-^{13}C]Met-hTF/2N (ca. 2 mM) and [ϵ-^{13}C]Met-hTF (ca. 0.2 mM) were prepared in 0.1 M KCl in D_2O, and the pH* values (meter reading in D_2O solution) were adjusted to pH* 7.1 ± 0.1 using NaOD and DCl. Five mole equivalents of the synergistic anion oxalate (microliter aliquots of 0.1 M $K_2C_2O_4$ in D_2O) per lobe was added. Additions of $Ga(NTA)_2$ were made to protein samples containing oxalate to give Ga:protein ratios of up to 1.5:1 for hTF/2N and 3:1 for hTF, and the pH* was readjusted to pH* 7.1 ± 0.1 after each addition.

NMR Spectroscopy. [^{1}H, ^{13}C] HMQC (Bax et al., 1983) and HSQC (Wider & Wüthrich, 1993) spectra were acquired on Varian Unity 600 or Bruker AMX600 and Varian Unity-Plus 500 NMR spectrometers at 600 and 500 MHz for ^{1}H and at 150 and 125 MHz for ^{13}C, respectively, at 310 K unless otherwise stated. Essentially identical data were obtained from HMQC and HSQC experiments. The sequences were optimized for $^1J(^1H-^{13}C) = 140$ Hz and 16−128 transients were acquired using 4K points in the ^{1}H dimension and 32 −80 increments of t_1, with spectral widths of 5 and 1 kHz for ^{1}H and ^{13}C, respectively, and a relaxation delay of 1.2−1.6 s. For the HMQC-NOESY (Gronenborn et al., 1989) spectra, a mixing time of 100 ms was used and spectral widths of 8 and 1 kHz for ^{1}H and ^{13}C, respectively. The ^{13}C spins were decoupled using the GARP-1 sequence. After zero filling to 4K × 256 points, shifted Gaussian functions were used for processing. The residual HDO peak was suppressed via a combination of presaturation and pulsed field gradients. Peaks were referenced to TSP via the ϵ-CH_3 peak of L-methionine (15.14 ppm) for ^{13}C and via formate (8.465 ppm, always present as a minor impurity) for ^{1}H.

pH Measurements. These were made directly in NMR tubes using a Corning 145 meter and an Aldrich ultrathin combination electrode standardized with Aldrich buffers at pH 4, 7, and 10.

Table 1: ^{1}H and ^{13}C NMR Chemical Shifts for the [ϵ-$^{13}CH_3$]Met Residues of hTF/2N and hTF at 310 K

		hTF/2N		hTF	
residue	location[b]	^{1}H	^{13}C	^{1}H	^{13}C
Met26 (5)[a]	N1, helix 1, buried	1.95	15.96	1.98	16.02
Met109 (4)	N2, partially buried	1.97	14.62	1.98	14.55
Met256 (3)	N1, completely exposed	2.13	15.54	2.13	15.53
Met309 (2)	N1, hinge region, mostly buried	2.16	16.12	2.27	17.52
Met313 (1)	N1, hinge region, partially exposed	2.21	15.73	2.29	15.18
Met382	C1, hinge region, partially exposed			2.08	15.22
Met389	C1, completely buried			2.02[c]	15.49[c]
Met464	C2, Trp460 hydrophobic patch of helix 5			1.38	16.30
Met499	C2, mostly exposed			1.86	15.41

[a] Numbers in parentheses refer to peak labels in Figure 2. N1, N2, C1, and C2, domains 1 and 2 of the N- and C-lobes, respectively. [b] As in the X-ray structure of Fe_C-hTF (Zuccola, 1993). [c] 318 K, peak absent at 310 K.

RESULTS

Peak Assignments for hTF/2N. The 2D [^{1}H, ^{13}C] HMQC NMR spectrum of apo-hTF/2N is shown in Figure 2. As expected, five cross-peaks are resolved for the five Met residues in this lobe. One resonance is very sharp (peak 3), and one is very broad (peak 4). In the ^{1}H dimension, peaks 4 and 5 overlap, whereas in the ^{13}C dimension, all are resolved. The assignment of resonances for Met309 and Met313 was aided by the observation of 2D [^{1}H, ^{13}C] NMR spectra of the [ϵ-$^{13}CH_3$]Met-hTF/2N mutants Met309Ile and Met313Ile (Supporting Information, Figure S1). For M309I, peak 2 was missing, and for M313I, peak 1 was missing, hence establishing assignments for these peaks. The UV−visible spectra of Fe^{3+}-M309I and Fe^{3+}-M313I were the same as that of wild-type Fe^{3+}-hTF/2N (Woodworth et al., 1991), showing that the iron site was unperturbed by the mutations. To establish further assignments, HMQC-NOESY spectra of apo-hTF/2N were recorded. These showed NOE con-

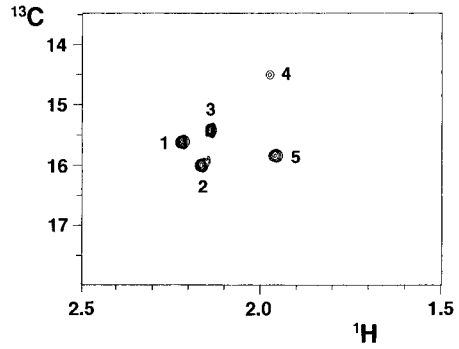

FIGURE 2: 2D [^{1}H, ^{13}C] HMQC spectrum of [ϵ-^{13}C]Met-hTF/2N showing cross-peaks for all five Met residues. For assignments see Table 1.

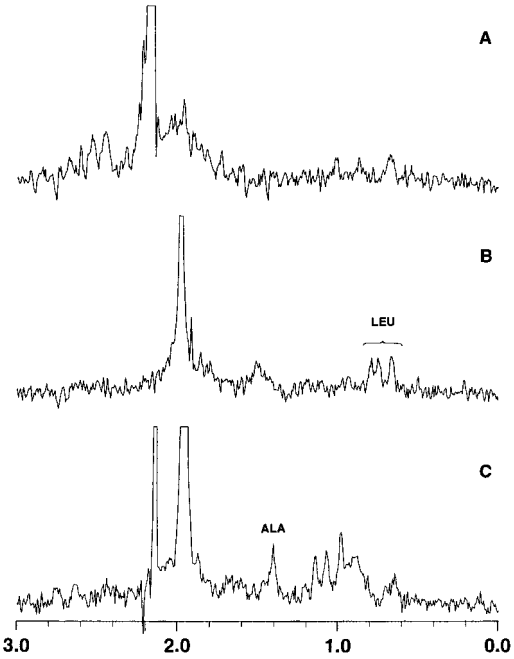

FIGURE 3: Slices through the 2D [^{1}H, ^{13}C] HMQC-NOESY spectrum of [ϵ-^{13}C]Met-hTF/2N at the ^{13}C chemical shifts of (A) peak 2, (B) peak 4, and (C) peak 5 of Figure 2. The assignments of NOE peaks to residue types are based on COSY and TOCSY data. The patterns of NOE's in (B) and (C) correlate with the Met environments shown in Figure 4 and hence provide assignments for the Met cross-peaks. In (C) the NOE peaks from 0.8 to 1 ppm are likely to arise from the methyl groups of Ile30 and Leu266 which are within 5 Å of ϵ-^{13}C of Met26.

nectivities to individual methionine ϵ-CH_3 groups. Peaks 1 and 3 showed no significant NOE connectivities, whereas peaks 2, 4, and 5 showed NOE peaks in the methyl region of the spectrum (Figure 3).

To allow the assignment of the HMQC resonances, DQF-COSY and TOCSY spectra were recorded under the same conditions as the HMQC-NOESY spectrum. From these it was possible to determine the type of residue to which the HMQC peaks showed NOESY connectivities, and then by examining the crystal structure of the protein, it was possible to assign the HMQC peaks to particular methionine groups. For instance, there is a NOESY connectivity from HMQC peak 5 to a peak at 1.40 ppm (Figure 3C). At this chemical shift in the DQF-COSY and TOCSY spectra, there are cross-peaks characteristic of an alanine residue. Cross-peak patterns characteristic of the various amino acid side chains have been described by Wüthrich (1986). Therefore, this indicates that the resonance at 1.40 ppm is from an alanine

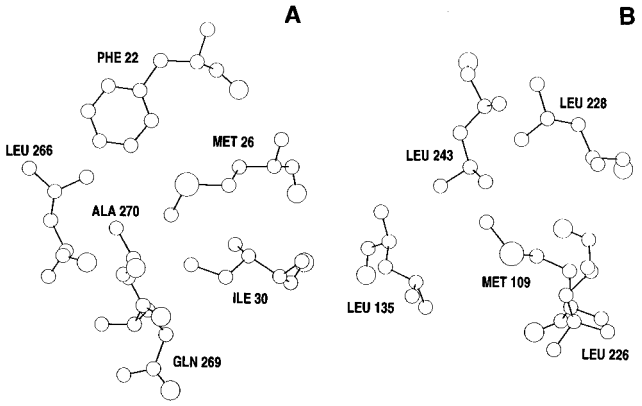

FIGURE 4: Amino acid residues within 5 Å of ϵ-CH_3 of (A) Met26 and (B) Met109. The close contacts to methyl groups of Ala, Leu, and Ile residues allow NOE assignments to be made for these two Met residues (Zuccola, 1993).

methyl group. The X-ray crystal structure (Zuccola, 1993) of Fe_C-hTF indicates that Met26 is the only methionine group within 5 Å (maximum NOE distance) of an alanine CH_3 group (Figure 4A). This must therefore mean that HMQC peak 5, which shows the NOESY connectivity to the alanine methyl resonance, must be that of Met26. The other resonances to which peak 5 has NOESY connectivities are in crowded regions of the DQF-COSY and TOCSY spectra but appear to be compatible with cross-peak patterns of leucine and isoleucine residues, groups which are also within 5 Å of Met26. Similarly, peak 4 shows NOESY connectivities to three resonances between 0.6 and 0.8 ppm (Figure 3B). These resonances are all associated in the DQF-COSY and TOCSY spectra with cross-peak patterns characteristic of leucine residues. Met109 is within 5 Å of three leucine residues (Figure 4B), and thus HMQC peak 4 can be assigned to this methionine group. This leaves assignment of peak 3 to Met256, which is highly consistent with its small line width, a feature expected for a highly solvent-accessible and mobile residue.

Peak Assignments for hTF. The 2D [^{1}H, ^{13}C] HMQC spectrum of apo-hTF is shown in Figure 5A. This spectrum was recorded at 310 K and shows cross-peaks for eight of the nine Met residues. When the temperature was raised to 318 K, a ninth peak appeared at 2.02/15.49 ppm (this peak also appeared on adding oxalate; see Figure 5B). HMQC-NOESY spectra of this full-length protein showed no detectable NOE connectivities, attributable to the necessarily low concentration used (about one-tenth of that of the N-lobe—limited by sample availability) and the broadness of the resonances. Therefore, we have based peak assignments on other considerations.

Three of the [^{1}H, ^{13}C] cross-peaks for apo-hTF have the same shifts as peaks for apo-hTF/2N (peaks 3, 4, and 5: Met256, 109, and 26, respectively). None of these is close to the C-lobe, and it is reasonable to assume that they are unperturbed by its presence. One of the [^{1}H, ^{13}C] peaks for apo-hTF is strongly high-field shifted in the ^{1}H dimension (δ 1.38) (Figure 5A). This would be expected for Met464 which, in the X-ray structure (Zuccola, 1993), lies directly above Trp460 within helix 5 of the C-lobe and is also close to the face of the phenyl ring of Phe476.

The assignments of the remaining five Met residues for apo-hTF can be made tentatively on the following grounds. Met499 would be expected to give rise to a sharp peak since

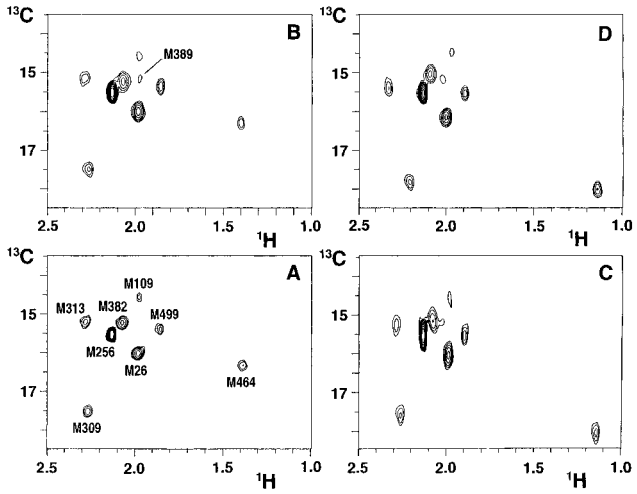

FIGURE 5: 2D [^{1}H, ^{13}C] HMQC spectra of apo-[ϵ-^{13}C]Met-hTF (A) before and (B) after addition of 10 mol equiv of potassium oxalate, (C) followed by addition of 1 mol equiv of Ga^{3+} and (D) a further addition of 1 mol equiv of Ga^{3+} (total 2 mol equiv). The missing cross-peak for Met389 in (A) appears on binding oxalate (B). Specific shifts of cross-peaks on binding either the first or second equivalent of Ga^{3+} are notable; e.g., Met464 in the C-lobe shifts on binding the first equivalent but not on binding the second (see Figure 6).

it is a surface residue. The ϵ-CH_3 group lies over the aromatic ring of Tyr514 and so may be high-field shifted, suggesting assignment to the cross-peak at 1.86/15.41 ppm. This leaves four cross-peaks to be assigned; of these one is broader than the rest and is observable only at higher temperatures, or with oxalate bound (vide infra). We assign this to Met389 which is buried at the base of the C-lobe metal binding site (see Figure 1) and would be expected to be relatively immobile. The two cross-peaks with the lowest field ^{1}H shifts are similar to peaks 1 and 2 observed for the N-lobe (cf. Figure 2) but slightly shifted. These can be assigned to Met309 and Met313, and the changes in chemical shift can be attributed to contact with the C-lobe. Since Met309 is close to Phe676 in the C-lobe, it may experience the greater shift, and this has guided our tentative assignment. The remaining cross-peak is assigned to Met382, a residue which lies in the hinge region of the C-lobe in contact with the N-lobe and yet is partially exposed to solvent in the X-ray structure. This cross-peak is relatively sharp and has shifts similar to the peak assigned to the fully solvent-exposed residue Met256.

In summary, confident assignments can be made for the N-lobe residues Met26, Met109, and Met256 of hTF, and for Met464 in the C-lobe, and reasonable assignments for Met499 and Met389 in the C-lobe. The assignments made for Met309, Met313, and Met382 are ambiguous, but notably, all these residues are in the region of contact between the two lobes. Therefore, with assigned peaks as markers for both lobes we were able to study the lobe preference for Ga^{3+} uptake.

Synergistic Anion (Oxalate) Binding. Addition of up to 5 mol equiv of oxalate to the half-molecule apo-hTF/2N at pH* 7.2 produced no observable effects on any of the [^{1}H, ^{13}C] NMR cross-peaks. In contrast for the whole protein apo-hTF, the cross-peak which was too broad to observe at 310 K became visible in the spectrum when 10 mol equiv of oxalate was added (Figure 5B). There were no significant shifts of the other peaks.

Ga^{3+} Binding. Ga^{3+} additions to ox-hTF/2N (5 mol equiv of oxalate, pH* 7.2) were made in steps to give Ga^{3+}:protein mol ratios of 0.33, 0.5, 0.67, 1.0, and 1.5. This led to the gradual appearance of a new set of cross-peaks for Met26, Met109, and Met309 assignable to metal-bound protein in slow exchange (on the NMR time scale) with peaks for unbound protein and to the disappearance of peaks for the apoprotein (Supporting Information, Figure S2). The metal-induced shifts are small (Figure 6A−E), but it is notable that none of these Met residues is in direct contact with the metal site.

Ga^{3+} was added to solutions of ox-hTF (10 mol equiv of oxalate, 5 per lobe) to give metal:protein mol ratios of 1, 2, and 3, at pH* 7.1. The addition of the first mol equiv of Ga^{3+} gave rise to a dramatic upfield ^{1}H and downfield ^{13}C shift of the cross-peak assigned to Met464 and smaller shifts for Met499 and Met389 (Figure 5C). Other peaks were not significantly shifted. When the second mole equivalent of Ga^{3+} was added, there was no further change to these three cross-peaks, but shifts were now observed for five of the others (Figure 5D). Two of these, which we assigned to N-lobe residues Met26 and Met109 in spectra of hTF, behaved in the same way as the corresponding peaks 4 and 5 when Ga^{3+} was added to the N-lobe alone (Figure S2), and the other three shifted cross-peaks are for those tentatively assigned to the interlobe contact residues Met309, Met313, and Met382. No further significant shifts were observed on addition of excess Ga^{3+}; i.e., the shifts observed above are due to occupation of the two specific metal ion binding sites. The Ga^{3+}-induced ^{1}H and ^{13}C chemical shift changes are illustrated graphically in Figure 6F−N.

DISCUSSION

Transferrin is a metal transport protein which is recognized and internalized by specific cell surface receptors (Dautry-Varsat, 1986). It binds strongly to its receptor at pH 7.4 when both iron sites are occupied, whereas the apoprotein dissociates from the receptor at this pH. Such a recognition probably depends intimately on metal-induced structural changes in the protein. For the related protein lactoferrin it has been shown that N-lobe metal binding causes hinged closure of domains N1 and N2 involving a 54° rotation about a hinge formed by two interdomain β-strands (Anderson et al., 1987, 1990; Gerstein et al., 1993). The conditions under which the C-lobe can open and close are not clear since in one crystal form of apolactoferrin the C-lobe is closed and in another there is a C-lobe opening of ca. 15° (Baker, 1994).

A wide variety of measurements have shown that the N-lobe and C-lobe sites are not equivalent; for example, conditional binding constants of log $K = 20.3$ and 19.3 have been reported (Harris & Pecoraro, 1983) for Ga^{3+} binding to hTF in 27 mM bicarbonate at pH 7.4, and Fe^{3+} binds about 20 times more strongly to one lobe than the other (Aisen et al., 1978). It is also clear that the two lobes recognize low-molecular-mass metal complexes differently. For example, at pH 7.4, Fe^{3+} complexed with citrate preferentially transfers the metal to the N-lobe, whereas Fe^{3+} complexed with NTA loads the C-lobe (Harris & Aisen, 1989).

We have recently shown that, despite the high molecular mass of transferrin, high-resolution ^{1}H NMR spectroscopy can be used to monitor the uptake of Al^{3+} and Ga^{3+} by

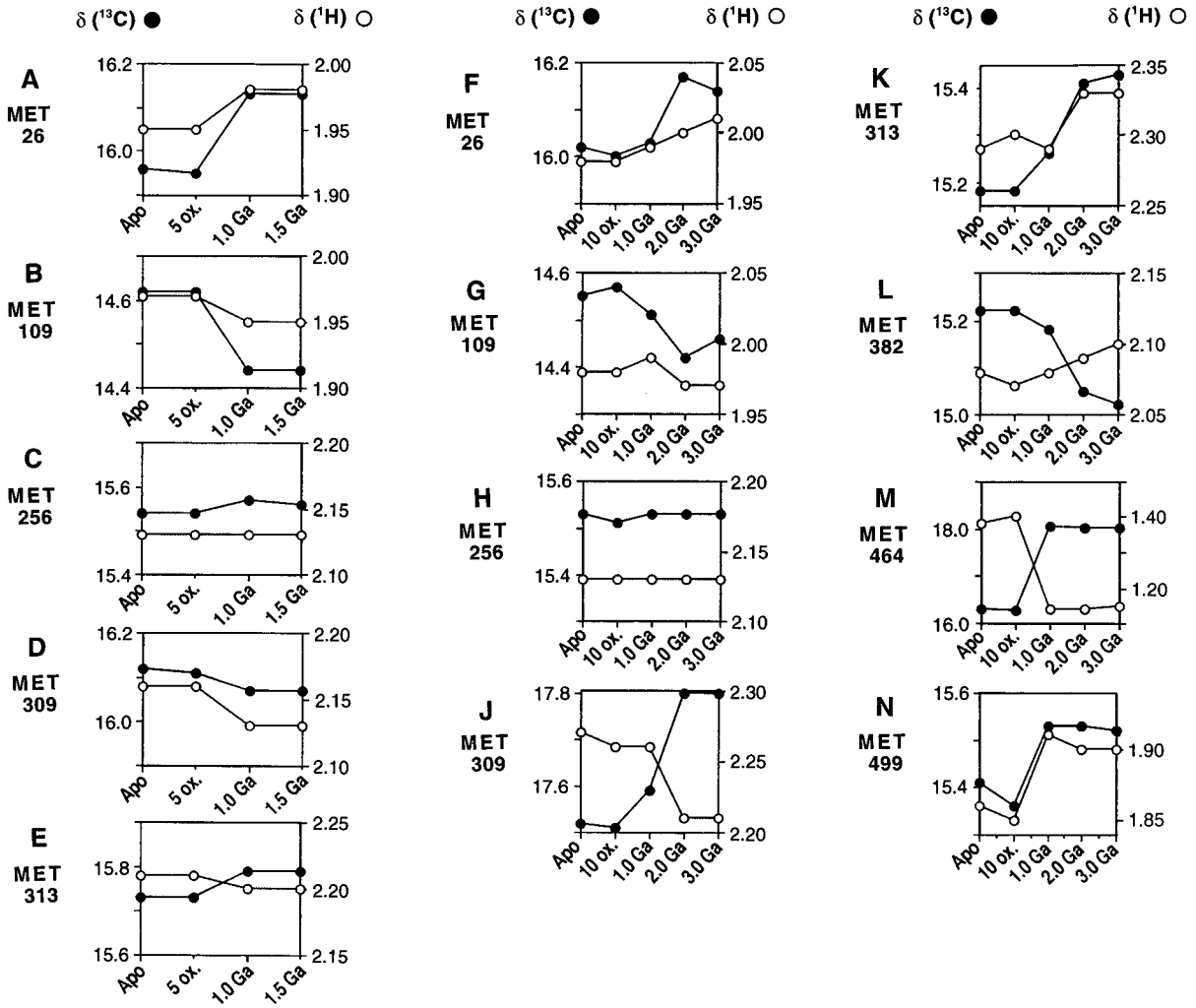

FIGURE 6: Graphs showing the changes in ^{1}H (right-hand axis, open circles) and ^{13}C (left-hand axis, filled circles) NMR chemical shifts of the methionine [ϵ-$^{13}CH_3$]Met resonances of (A−E) apo-hTF/2N and (F−N) apo-hTF on addition of oxalate (5 mol equiv per lobe) and various mole equivalents of Ga^{3+}, as indicated. The ^{1}H, and ^{13}C, δ scales for all the graphs are the same, except for Met464 for which $\delta(^1H)$ is ÷ 4 and $\delta(^{13}C)$ is ÷ 10 due to the larger shifts. Notable features are that oxalate has little effect on any of the resonances and that for hTF/2N only the first mole equivalent of Ga^{3+} induces shifts as expected (one binding site). In general for hTF, C-lobe Met residues are strongly affected by the first mole equivalent of Ga^{3+} while N-lobe residues are affected by the second equivalent (data for Met389 are not shown because the peak is not visible until oxalate is added and is weak and obscured by the resonance of Met382; see Figure 5).

apotransferrin (Kubal et al., 1992, 1993). In the case of Al^{3+} with bicarbonate as synergistic anion at pH* 8.8, there was preferential binding to the N-lobe, whereas with Ga^{3+} and oxalate at pH* 7.3, the C-lobe was occupied first. The latter experiments relied on identification of markers for N-lobe binding derived from investigations of metal binding to recombinant N-lobe alone followed by identification of similar features in spectra of intact apotransferrin. In particular, it was possible to assign high-field-shifted resonances from the Leu122-Trp128-Ile132 hydrophobic patch in helix 5 of the N-lobe. However, the complexity and broadness of ^{1}H NMR spectra of hTF complicate further attempts to make residue-specific assignments.

In the present work we have shown that ^{13}C-labeled SCH_3 groups of methionine residues of both hTF/2N and hTF give rise to well-resolved 2D [^{1}H, ^{13}C] NMR cross-peaks. Using 2D [^{1}H, ^{13}C] HMQC NMR methods they can be detected even at micromolar concentrations. For the N-lobe, peaks for all five Met residues were readily detected and assigned using a combination of NOE effects, crystallographic structural data, and point mutations (M309I and M313I). These approaches provided reasonable assignments for all of the methionines.

For the intact protein, apo-hTF, NOE effects were weak, and other assignment procedures were necessary. Peaks for three of the N-lobe Met residues (Met 26, Met109, and Met256) appeared to be unperturbed in spectra of hTF compared to that of hTF/2N, consistent with these residues being well removed from the interlobe contact region. The presence of a high-field-shifted [^{1}H, ^{13}C] cross-peak was expected in view of the position of Met464 (directly above the indole ring of Trp460) within the hydrophobic patch Val454-Trp460-Met464 in helix 5 of the C-lobe. This is the analogue of the Trp128 hydrophobic patch in helix 5 in the N-lobe and is a region which appears to be highly conserved in both lobes of transferrins (Baldwin, 1993).

We have made other observations which support the above assignments. Pt(II) forms strong bonds to the sulfur of methionine, and when the methionine-specific reagent [Pt(en)Cl_2] (where en = ethylenediamine) reacts with hTF, only resonances assigned to the surface methionine residues 256 and 499 are affected (K. J. Barnham, M. C. Cox, J. D. Hoeschele, A. B. Mason, P. J. Sadler, and R. C. Woodworth, unpublished).

The small changes in shifts of the resonances for Met26, Met109, and Met309 when Ga^{3+} binds to the half-molecule

hTF/2N show that metal-induced structural changes penetrate to regions of the structure well away from the metal site, consistent with the proposed hinged lobe closure (Baker, 1994). However, they also indicate that domain movements do not merely involve the rotation of solid bodies around an axis but include more subtle perturbations of domain structure.

The shifts observed when 1 mol equiv of Ga^{3+} binds to hTF are consistent with preferential binding to the C-lobe. The assignment of [^{1}H, ^{13}C] peaks for Met464 provides the first unambiguous NMR assignment for a C-lobe amino acid residue, which is important because, thus far, it has not been possible to prepare recombinant C-lobe alone. When Ga^{3+} binds to oxalate−hTF, the ^{1}H NMR resonance of Met464 shifts to high field by ca. 0.2 ppm, consistent with the movement of this group nearer to the face of Trp460 or nearer to the center of the indole ring, so giving rise to an increase in the ring-current shift. It is noticeable, however, that the Ga^{3+}-induced ^{13}C shift is in the opposite direction to the ^{1}H shift. This may be a consequence of a through-bond electronic effect which is likely to influence carbons more than the protons. Movement of residues within helix 5 is also observed in the N-lobe as monitored by the ^{1}H NMR shifts of the corresponding residue Ile132 which lies over the face of Trp128 (Kubal et al., 1992). These effects may be related to the domain closures which, in the solid state (of lactoferrin), involve the pivoting of helix 5 on helix 11 and a ca. 50° domain movement in the N-lobe and possible 15° movement in the C-lobe (Baker, 1994). Helix 5 backs onto the metal and anion binding site, and the N-terminal residues of this helix (Ala126 and Gly127 in the N-lobe; Ala458 and Gly459 in the C-lobe) hydrogen-bond to the metal-coordinated synergistic anion.

No significant changes in N-lobe [^{1}H, ^{13}C] cross-peaks of hTF were observed when the C-lobe was loaded. However, subsequent loading of the N-lobe caused a perturbation of the cross-peak for one of the C-lobe residues, Met382. This residue is situated in the interlobe contact region (Figure 1). The N-lobe Met residues in this region (309 and 313) are also perturbed on binding Ga^{3+} to Ga_C-ox-hTF in a similar manner as when Ga^{3+} binds to ox-hTF/2N. Therefore, we can conclude that a mechanism for interlobe communication exists in transferrin and could form part of a trigger mechanism leading to binding of the fully loaded protein to cell surface receptors. There is evidence that Ga^{3+} transferrin as well as Fe^{3+} transferrin can bind to transferrin receptors (Ward & Taylor, 1988). Although we have detected N- to C-lobe communication as part of the second stage of Ga^{3+} binding to hTF/2N with oxalate as the synergistic anion, it may form part of the first stage of binding with carbonate as the anion. It has been shown for ovotransferrin by ^{27}Al and ^{71}Ga NMR that there is preferential binding of both Al^{3+} and Ga^{3+} to the N-lobe when carbonate is the synergistic anion (Aramini & Vogel, 1993; Aramini et al., 1994b), whereas with oxalate the C-lobe is preferentially loaded with Al^{3+} and there is no preference with Ga^{3+}. We have not used carbonate in the present work, since it appeared from our ^{1}H NMR studies (Beatty, 1995) that Ga^{3+} loading of hTF/2N in the presence of carbonate did not lead to complete lobe closure at pH 7.

It is interesting to note that only one Met residue appeared to be significantly affected by addition of the synergistic anion oxalate to the hTF, Met389. The appearance of the NMR cross-peak for this methionine only with addition of oxalate to apo-hTF or with increase in temperature suggests that it has a restricted mobility in the apoprotein or is slowly interconverting between two conformational states (giving rise to exchange broadening of the resonance). This result suggests that oxalate binds to the C-lobe even in the absence of metal ion, in line with current thinking on the mechanism of anion and metal uptake (Baker, 1994). Oxalate is known to bind to the N-lobe (log $K = 4.04$) under conditions similar to those used here (Kubal et al., 1992).

There are advantages in choosing to label the ϵ-CH_3 group of Met in proteins: the ^{1}H resonance is a singlet arising from a reasonably mobile group at the end of a long side chain, and the relatively low abundance of Met residues in proteins allows resolution of these resonances even in large proteins. For transferrin, selective labeling experiments of the type described here should now allow detailed investigations of selective lobe loading with a wide variety of metal ions and synergistic anions. Selective ^{13}C labeling combined with inverse NMR detection is a general approach which can be applied to other large proteins.

CONCLUSION

Reasonable assignments can be made for the resonances of all nine methionine ϵ-$^{13}CH_3$ groups (five in the N-lobe and four in the C-lobe) of recombinant human transferrin (80 kDa), providing the first specific assignment of C-lobe NMR resonances of transferrin. By use of these resonances as probes, it can be shown that Ga^{3+} preferentially loads the C-lobe when oxalate is the synergistic anion and that subsequent binding of Ga^{3+} to the N-lobe is communicated to the C-lobe, specifically affecting Met382, a C-lobe residue in the interlobe contact region.

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SUPPORTING INFORMATION AVAILABLE

[^{1}H, ^{13}C] NMR spectra of the hTF/2N mutants M309I and M313I (Figure S1) and oxalate−hTF/2N half-loaded with Ga^{3+} (Figure S2) (2 pages). Ordering information is given on any current masthead page.

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