RESEARCH COMMUNICATION

Uptake of Al^{3+} into the N-lobe of human serum transferrin

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We have studied the binding of Al^{3+} to human serum apotransferrin (80 kDa) and recombinant N-lobe human apotransferrin (40 kDa) in 0.1 M-sodium bicarbonate solution at a pH meter reading in ^2H_2O (pH*) of 8.8 using ^1H n.m.r. spectroscopy. The results show that for the intact protein, preferential binding of Al^{3+} to the N-lobe occurs. Molecular modelling combined with an analysis of ring-current-induced shifts suggest that n.m.r. spectroscopy can be used to probe hinge bending processes which accompany metal uptake in solution.

INTRODUCTION

Aluminium is widely consumed in drinking water, foods (especially herbs, tea, some baking powders and processed cheeses), and drugs (antacids), but does not appear to be an essential element. Its uptake into the body and deposition in tissues has been linked to conditions of medical concern, especially encephalopathy and osteomalacia associated with long-term dialysis [1,2]. The ultimate agent for transport of Al^{3+} in the blood is believed to be the iron transport protein serum transferrin [3,4], a single-chain, approx. 80 kDa glycoprotein. Transferrin has two structurally similar lobes each of approx. 40 kDa, linked by a short connecting peptide, and each lobe contains an interdomain high-affinity metal-binding site [5,6]. There is much interest in the functional differences between these sites, and numerous experiments have been reported in which attempts have been made to load either the N-lobe or the C-lobe selectively with a metal ion [7]. These experiments rely heavily on the ability of gel electrophoresis to separate the various metal-loaded transferrins (loaded in either N- or C-lobes, or in both lobes) and apotransferrin in 6 M-urea [8,9]. However, this procedure is not useful for Al^{3+}, and some other metal ions, because the complexes dissociate during electrophoresis ([10,11];¡R. W. Evans, personal communication). Here we use ^1H n.m.r spectroscopy to show that human apotransferrin can preferentially bind Al^{3+} in the N-lobe, and to probe interdomain hinge bending processes which accompany metal uptake.

MATERIALS AND METHODS

^1H-n.m.r. spectra (500 MHz) were obtained on a Bruker AM500 spectrometer, typically using 0.5 ml of solution in a 5 mm diameter tube at 310 K, 256–650 transients, 45° pulses, relaxation delay of 1.5–2 s, 8 k data points (zero-filled to 16 k), and gated secondary irradiation of HO^2H. The chemical shift reference was formate [8.462 p.p.m. relative to sodium trimethylsilylpropionate (TSP) at 0 p.p.m.]. Formate is always present in our samples as a minor impurity. Resolution enhancement was achieved by processing the free induction decays with exponential functions equivalent to line broadenings of 1–10 Hz combined with unshifted sine–bell functions.

The experiments with apo-human serum transferrin (HTF) (Sigma, Poole, Dorset, U.K.) were done as previously described [12]. Microlitre aliquots of a stock solution of $Al_2(SO_4)_3$ in ^2H_2O [pH meter reading in ^2H_2O solutions (pH*) 3.4] were added to a 0.35 mM solution of HTF in 0.1 M-Na^2HCO_3, pH* 8.78. A total of 2.5 mol equiv. of Al^{3+} was added in 0.25 mol equiv. steps with an average time of approx. 0.5 h between each addition. These conditions were chosen because control of pH* with bicarbonate buffers is difficult and slight changes in pH* can make the interpretation of His shifts difficult. pH* values of n.m.r. solutions were strictly monitored before and after n.m.r. runs using a micro combination electrode (Aldrich, Gillingham, Dorset, U.K.) and a Corning 145 pH meter (Halstead, Essex, U.K.).

Recombinant N-lobe of HTF (HTF/2N) (residues 1–337) was expressed in baby hamster kidney cells using a pNUT plasmid, and was purified as previously described [13]. Iron was removed by treatment with nitrilotriacetate (1 mM) and EDTA (1 mM) in 0.5 M-sodium acetate, pH 4.9, followed by concentration in a Centricon 10 column (Amicon, Stonehouse, Glos., U.K.), and lyophilization after H–^2H exchange for 4 h at ambient temperature in 0.1 M-Na^2HCO_3 in ^2H_2O. Concentrations were determined using an ϵ_{280} of 38.6 $mM^{-1} \cdot cm^{-1}$. For n.m.r. spectroscopy, a 1.5 mM solution of apo-HTF/2N was prepared by dissolving the lyophilized protein in 20 mM-Na^2HCO_3 (0.45 ml) and the pH* was adjusted to 8.85 with 0.5 M-^2HCl (38 μl). Al^{3+} additions were made as above in 0.25 mol equiv. steps.

The model of the N-lobe of Fe–HTF was based on the crystal co-ordinates of rabbit Fe_2–transferrin [14] modified by sequence alignment, appropriate changes in residues, and energy minimization of side-chains using Polygen QUANTA in the CHARMm force-field.

Ring-current calculations [15] were carried out using the program RCCAL provided by Dr. S. J. Perkins (Royal Free Hospital, London, U.K.).

RESULTS AND DISCUSSION

First we studied ^1H-n.m.r. spectra of apo-HTF. It is a very large protein for high resolution studies, but with the aid of resolution-enhancement procedures well-resolved peaks are observable from the more mobile regions [12]. The easiest region of

Abbreviations used: COSY, shift-correlated n.m.r. spectroscopy; HTF, human serum transferrin; HTF/2N, recombinant N-lobe of human serum transferrin; pH*, pH meter reading in ^2H_2O solutions.

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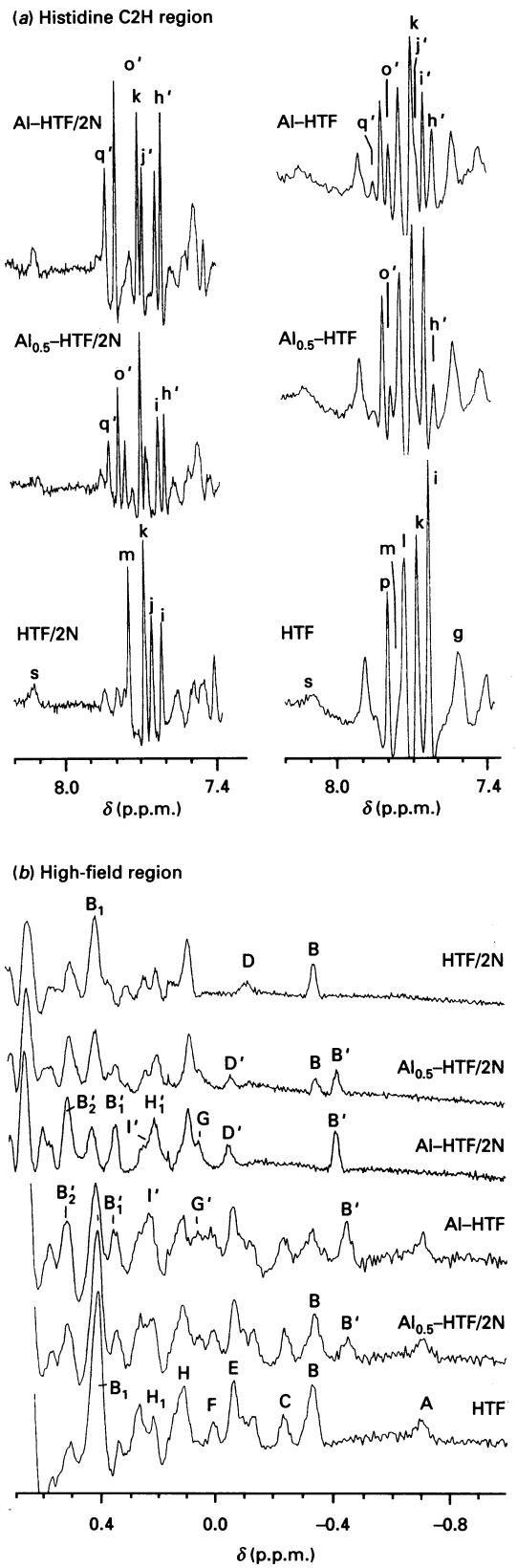

Fig. 1. ^{1}H-n.m.r. spectra of HTF, HTF/2N and their Al^{3+} complexes

Resolution-enhanced 500 MHz ^{1}H-n.m.r. spectra of HTF, HTF/2N and their Al^{3+} complexes are compared: (*a*) His C2H region (*b*) high-field region. The peaks have been labelled (with a prime for the Al^{3+} complex) merely to illustrate the correspondence in features between the two proteins. Resolution enhancement has removed the very broad peaks from the spectrum, including one at approx. −0.64 p.p.m. in the normal spectrum of apo-HTF/2N, which, together with peak D, may form part of the spin system for Ile-132 (see text). For HTF, peak B appears to lie on top of a C-lobe peak.

the spectrum to analyse is that between 7.5 and 8 p.p.m. which contains peaks for the His C2H resonances. The high-field region, between 0 and −1 p.p.m., contains peaks with unusual shifts which are likely to arise from methyl groups of aliphatic side-chains close to the faces of the aromatic rings of Phe, Tyr or Trp residues. Other workers (e.g. [16]) have observed relatively sharp resonances even from high-molecular-mass proteins. However, in the present case the resonances seem to arise from localized mobile regions in different parts of the protein, rather than from a single mobile domain, since pH titration curves for 14 of the 19 His residues can be constructed (results not shown) and these are distributed throughout the protein. On addition of Al^{3+}, new peaks for Al–HTF appear and those for apo-HTF decrease in intensity, i.e. apo-HTF and Al–HTF are in slow exchange on the n.m.r. timescale, which is characteristic of strong metal binding. As we have described previously [12], the stepwise changes in the intensities of the His C2H peaks suggest that the lobes of apo-HTF are sequentially populated with Al^{3+} and plots for the high-field shifted peaks conform this picture (Fig. 1).

In order to investigate, using n.m.r. methods, which lobe of apo-HTF takes up Al^{3+} first we next studied the titration of apo-HTF/2N with Al^{3+}. It seemed reasonable to suppose that ^{1}H-n.m.r. spectra of the intact apo- and metal-loaded proteins would, for the most part, be the sum of the spectra for the individual N- and C-lobes, since interlobe contacts in the X-ray crystal structure of rabbit Fe_2–TF involve only a specific cluster of hydrophobic contacts, and the crystal structures of rabbit Fe_2–TF and Fe–TF/2N are almost identical [14,17].

The effect of Al^{3+} on the ^{1}H-n.m.r. spectrum of HTF/2N is also shown in Fig. 1. Again the resonances of the apo-protein disappear and a new set assignable to Al–HTF/2N appear gradually during the course of the titration, which is consistent with strong Al^{3+} binding, and slow exchange between HTF/2N and Al–HTF/2N on the n.m.r. timescale. Interpretation of the high-field methyl region was aided by a phase-sensitive double-quantum-filtered two dimensional shift-correlated (COSY) n.m.r. spectrum in which two spin systems assignable to Val or Leu residues are prominent.

While it must be recognized that specific unambiguous assignments for resonances of HTF and HTF/2N cannot be made at this stage, and that there is great difficulty in fully analysing spectra which consist of a large number of broad overlapping peaks, there are sufficient similarities in the behaviour of HTF on titration with Al^{3+} in comparison with those of HTF/2N for us to conclude that the first mol equiv. of Al^{3+} added to HTF enters the N-lobe. These similarities are highlighted in Fig. 1, and cover peaks in both the high-field region and the His C2H region. In the high–field region, three peaks assignable to a single Val or Leu spin system (labelled B) shift further to high field (by approx. 0.06 p.p.m.) on titration with Al^{3+} (labelled B′ in the Al^{3+} complexes), and new peaks G′ and I′ appear. Not all high-field-shifted peaks are affected by Al^{3+} binding (e.g. H). A phase-sensitive double-quantum-filtered two-dimensional COSY spectrum of Al–HTF/2N showed clear connectivities for B peaks (−0.400, 0.346/0.511 p.p.m.) and H peaks (0.096, 0.212/0.819 p.p.m.) typical of Val or Leu spin systems. Other peaks in the high-field region of HTF can be tentatively assigned to the C-lobe, e.g. C and E, and these decrease in intensity on addition of the second equiv. of Al^{3+} to Al–HTF to form Al_2–HTF (i.e. N- and C-lobe both populated; results not shown).

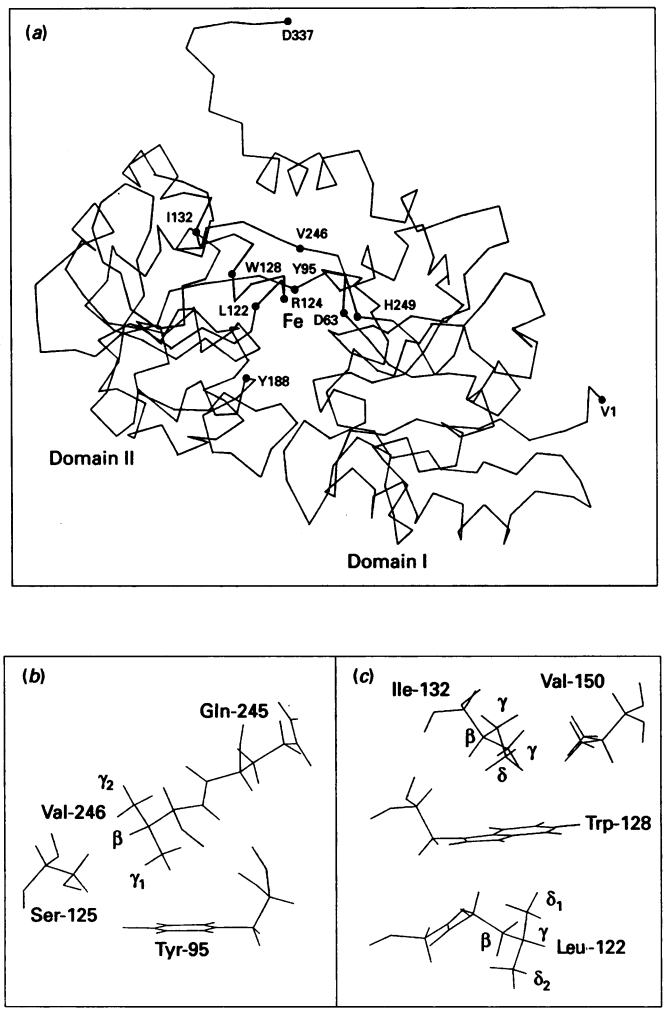

Fig. 2. Models of the N-lobe of transferrin and two of its hydrophobic patches

(*a*) Model of the N-lobe of Fe–HTF (C_α backbone shown). The Fe^{3+} ligands are provided by Asp-63 (domain I), Tyr-95 and His-249 (interdomain strands), Tyr-188 (domain II) and carbonate (synergistic anion). The positions of some other residues in the interdomain hinge and metal-binding region are also indicated. (*b*) and (*c*) Regions of the N-lobe predicted (on the basis of the above model) to give rise to the largest high-field ring-current-induced ^{1}H-n.m.r. shifts. The pseudocentre of the γ_1-CH_3 protons of Val-246 is approx. 0.31 nm (3.1 Å) from the centre of the ring of the metal-ligand Tyr-95, and the pseudocentres for δ-CH_3 of Ile-132 and δ_1-CH_3 of Leu-122 are approx. 0.31 (0.36) nm [3.1 (3.6) Å], and 0.34 (0.37) nm [3.4 (3.7) Å], from the centres of the six-membered (and five-membered; in parentheses) ring respectively, of Trp-128.

Again, while there is not an exact correspondence of all His C2H peaks in the apo- or Al- forms of HTF and HTF/2N, some similarities are notable: the appearance of new peaks h′, o′ and q′ and the lack of effect of Al^{3+} on peaks i and k. The lack of exact correspondence of peaks between HTF and HTF/2N is likely to be the result of interactions between the N- and C-lobes which is known to have a functional significance [18].

Molecular modelling provides an insight into the possible assignment of the high-field-shifted methyl groups. Since the X-ray structure of HTF has not been published, a model of Fe–HTF/2N was constructed based on the crystal co-ordinates of rabbit Fe_2–transferrin [14]. Calculations of the shifts of ^{1}H-n.m.r. resonances induced by the ring currents of aromatic side-chains in the N-lobe were then carried out [15], and only three side-chains had predicted high-field shifts large enough (> 1.1 p.p.m.), and in the correct pattern, to account for the observed peaks: Leu-122, Ile-132, and Val-246. Peaks labelled B (or B′) satisfy those predicted for Leu-122 (δ_1 -CH_3 > γ -CH > δ_2 -CH_3), and those labelled H′ are consistent with predictions for Val-246 (γ_1 -CH_3 > β -CH > γ_2 -CH_3). In the spectrum of apo-HTF/2N there is an additional very broad peak at −0.64 p.p.m. which shifts to −0.84 p.p.m. in the spectrum of Al–HTF/2N (results not shown in Fig. 1) which disappears with the resolution enhancement. Such a peak could not be seen at all in spectra of HTF, presumably because it is even broader (does not correspond to peak A of HTF, Fig. 1*b*, which is likely to be a C-lobe peak). The peak at −0.64 p.p.m. and peak D may form part of the spin system for Ile-132.

Leu-122 and Ile-132 lie below and above the plane of the indole ring of Trp-128, and Val-246 is above Tyr-95, which is a metal-binding ligand in the N-lobe (Fig. 2). The residues in these hydrophobic patches show a high degree of conservation in transferrins and lactoferrins [5], and our modelling of these regions of the protein and the associated ring-current calculations should therefore be reasonably good. In crystalline transferrin (and lactoferrin), several residues from the peptide -C-H-T^{120}-G-L-G-R-S^{125}-A-G-W-N-I^{130}-P- (HTF numbering) appear to play important roles in stabilizing the region around bound metal (Fe^{3+}) and its co-ordinated carbonate anion, via an H-bonding network [14], including Thr-120, Arg-124 (which is at the terminus of helix 5), Ser-125, Ala-126 and Gly-127. The hydrophobic patches involving Tyr-95 and Trp-128 are both in the interdomain hinge region, and may play key roles in controlling domain rotation (opening and closing of the interdomain metal-binding cleft) and the formation of the metal-binding site. Small movements of Leu-122 and Ile-132 towards Trp-128 may account for the observed changes in ^{1}H n.m.r. shifts of these aliphatic residues on Al^{3+} binding. Indeed, for lactoferrin [19], the analogous residues Leu-119 and Ile-129 move closer to Trp-125 by approx. 0.03–0.05 nm (0.3–0.5 Å) on Fe^{3+} binding, accompanied by a rotation of domain II by 53° relative to domain I. Domain movements subsequent to metal binding have also been detected by small-angle X-ray scattering experiments on transferrins [20].

It might have been expected that Al^{3+} would bind preferentially to the C-lobe of HTF since this is thought to form thermodynamically more stable metal complexes than the N-lobe [7]. Thus Harris & Sheldon [21] assigned the larger Al^{3+} binding constant (log K values 13.5 and 12.5, determined by u.v. methods and use of mono-Fe^{3+}–HTFs) to C-lobe binding. In blood plasma, transferrin is only approx. 30% saturated with Fe^{3+} and this is thought to reside in the N-lobe [22], as is the case with Al^{3+} loading described here. It is possible that the products observed in our experiments are kinetic ones (although stable over a period of hours). Such products may be those of most biological significance since interlobe transfer reactions are thought to be very slow (days). Calorimetric studies on hen ovotransferrin [23] have also suggested that preferential binding of Al^{3+} to the N-lobe occurs.

These n.m.r. experiments will allow a large range of new kinetic and thermodynamic investigations to be made into the sequential loading of transferrins with a wide variety of metal ions, at different pHs, with different synergistic anions and metal-donating complexes. In particular they will allow critical comparisons of the sequential order of lobe loading as determined by n.m.r. with those from other methods such as gel electrophoresis (carried out at a similar pH to the present experiments), e.p.r. (using paramagnetic ions such as VO^{2+} and Cr^{3+}), and the rates of acid dissociation [7]. N.m.r. spectra also appear to provide sensitive indicators of atomic movements around the metal binding site which are probably associated with domain rotations.

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