of the ^{13}C-coupled sequence to the structural analysis of the C_2 symmetric molecule.

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Sequential Binding of Aluminum(3+) to the C- and N-Lobes of Human Serum Transferrin Detected by ^1H NMR Spectroscopy

Gina Kubal and Peter J. Sadler*

Department of Chemistry, Birkbeck College
University of London
Gordon House and Christopher Ingold Laboratory
29 Gordon Square, London WC1H 0PP, U.K.

Robert W. Evans

Department of Biochemistry, UMDS
Guy's Hospital Medical School
St. Thomas Street, London SE1 9RT, U.K.

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The deposition of Al^{3+} in the brain is known to cause dialysis encephalopathy and may also be involved in other conditions of medical concern.[1] The major transport agent for Al^{3+} in the body is thought to be the Fe-binding protein transferrin,[2,3] and much attention is now focused on the design of chelating agents to remove Al^{3+} from this protein. Recent UV data[3] show that Al^{3+} binds strongly to the specific Fe^{3+} sites of human serum transferrin (HTF) with log K values of 13.5 (C-lobe) and 12.5 (N-lobe). Direct methods for detecting the binding of Al^{3+} to the individual lobes of intact HTF in solution are required. In the only previous high-resolution ^1H NMR study of HTF,[4] it was noted that the resolution and sensitivity did not allow the analysis of individual resonances, as might be expected for such a high M_r protein (79 kDa). We show here[5] that many individual resonances can be resolved in resolution-enhanced 500-MHz ^1H NMR spectra of intact HTF. This allows the study of sequential loading of Al^{3+} into the C- and N-lobes, Al^{3+}-induced structural changes in the protein, and the monitoring of Al^{3+} removal by therapeutic chelating agents.

Many resonances are resolved in both the aliphatic and aromatic regions of 500-MHz ^1H NMR spectra of apo-HTF,[6,7] when enhanced by combined application of exponential and sine-bell functions to the free induction decay, as shown in Figure 1. This procedure[8] removes broad resonances from the spectrum, leaving sharp peaks from protons in the most mobile regions of the protein. The most intense peaks, ca. 2.1 and 3.4–4 ppm (not shown), arise from glycan *N*-acetyl and sugar ring protons, respectively, in two biantennary chains in the C-lobe. High-field-shifted resonances (ca. 0.6 to −0.7 ppm), which are likely to arise from methyls close to the faces of aromatic side chains, are clearly visible, and in the aromatic region about six peaks assignable to His C2H protons can be seen.[9] Peaks for the majority of amino acids are absent from enhanced spectra because they are very broad, which may be related to the immobility of many regions of the apoprotein.

The effect of Al^{3+} addition to HTF[10] is shown in Figure 1. In the high-field methyl region, new peaks a and e appear after addition of 1 molar equiv of Al^{3+}, but these are little affected by further addition of Al^{3+}, whereas peak d disappears progressively. Peaks in the Lys/Arg $\epsilon/\delta CH_2$ region, ca. 3 ppm,[11] also disappear in the presence of Al^{3+}, but the sugar peaks are unaffected. In the His C2H region, new resonances such as h, o, and q appear on addition of Al^{3+}, increase in intensity until 1 equiv has been added, and change little on further addition, whereas peaks r and l disappear and appear, respectively, on addition of the second equivalent of Al^{3+}; see inset in Figure 1B. Peaks such as p (apparently a single His C2H on pH* titration) and d change their intensities on binding both the first and second equivalents of Al^{3+}. Other specific changes are notable in the region 6.2–7.4 ppm of the aromatic region involving His C4H peaks and perhaps other aromatic residues.

Thus Al^{3+} binding to intact HTF can be detected by ^1H NMR spectroscopy, and the data suggest that sequential binding of Al^{3+} to the C- and N-lobes of HTF can be followed together with Al^{3+}-induced structural changes. With the assumption that Al^{3+} binds more strongly to the C-lobe,[3] peaks h, o, and q can be tentatively assigned to His residues in the C-lobe, and peaks l and r to the N-lobe, although the possibility that binding to one lobe affects resonances of the other cannot be ruled out. Several peaks appear to be sensitive to the occupation of both lobes, and this may indicate that Al^{3+} binding involves interlobal communication. Peaks for >10 His residues appear to be seen in spectra of Al_2-HTF, but it is not clear yet whether these include His-249 and His-585 (Fe^{3+} ligands);[2] Al^{3+} would be expected to have a lower affinity for N ligands than Fe^{3+}. The changes in shift of high-field methyl groups imply that Al^{3+} binding affects the

* Author to whom correspondence should be addressed.

(1) Articles in the following: *Met. Ions Biol. Syst.* **1988**, 24.

(2) HTF:678 amino acids in two structurally homologous but chemically distinct lobes; two domains in each lobe form the Fe-binding clefts with two Tyr, one His, one Asp, and CO_3^{2-} as ligands. Lys and/or Arg residues may stabilize bound CO_3^{2-}. The crystal structure of HTF has not been reported. X-ray structure of rabbit serum transferrin: Bailey, S.; Evans, R. W.; Garratt, R. C.; Gorinsky, B.; Hasnain, S.; Horsburgh, C.; Jhoti, H.; Lindley, P. F.; Mydin, A.; Sarra, R.; Watson, J. L. *Biochemistry* **1988**, *27*, 5804–5812. Related proteins: melanotransferrin, ovotransferrin, and lactotransferrin. See: Baker, E.; Rumball, S. V.; Anderson, B. F. *Trends Biochem. Sci.* **1987**, *12*, 350–353. Crichton, R. C. *Adv. Protein Chem.* **1990**, *40*, 281–363.

(3) Harris, W. R.; Sheldon, J. *Inorg. Chem.* **1990**, *29*, 119–124 and references therein.

(4) Woodworth, R. C.; Williams, R. J. P.; Alsaadi, B. M. In *Proteins of Iron Metabolism*; Brown, E. B., Aisen, P., Fielding, J., Crichton, R. R., Eds.; Grune and Stratton: New York, 1977; p 211. Better resolution has been obtained from the N-lobe, and the effect of pH and Ga^{3+} on the His C2H peaks has been reported: Valcour, A. A.; Woodworth, R. C. *Biochemistry* **1987**, *26*, 3120–3125. However, it is also important to study intact transferrin because there is thought to be a functionally-significant association between the N- and C-lobes in solution: Brown-Mason, A.; Brown, S. A.; Butcher, N. D.; Woodworth, R. C. *Biochem. J.* **1987**, *245*, 103–109.

(5) Presented in part at ICBIC-5, Oxford, August 1991: Bell, J. D.; Evans, R. W.; Kiang, W.; Kubal, G.; Radulovic, S.; Sadler, P. J.; Williams, G. *J. Inorg. Biochem.* **1991**, *43*, 488.

(6) Apo-HTF was purchased from Sigma (Catalog No. T0519, Batch 67F9454). Batches sometimes contain bound citrate (Evans, R. W.; Kubal, G.; Sadler, P. J.; Williams, G., unpublished), and these were avoided for Al^{3+} work. Protein concentrations were determined from ϵ_{280} (Luk, C. K. *Biochemistry* **1971**, *10*, 2838–2843). The pH* values of NMR solutions were strictly monitored before and after NMR runs. Control of pH* with bicarbonate buffers is difficult, and pH* 8.78 was chosen to minimize problems from shifts of His peaks which occur at lower pH* values with slight pH* drift. pH* is the pH meter reading in D_2O solutions.

(7) ^1H NMR spectra (500 MHz) were recorded on Bruker AM500 and JEOL GSX500 spectrometers, typically using 0.55 mL in a 5-mm tube, 310 K, 512–650 transients, 45° pulses, relaxation delay 1.5–2 s, 8k data points (zero-filled to 16k), and gated secondary irradiation of HOD. FIDs were processed using exponential functions equivalent to line-broadenings of 1–3 Hz combined with unshifted sine-bell functions.

(8) Sadler, P. J.; Tucker, A. *Biochem. Soc. Trans.* **1990**, *18*, 923–924.

(9) Assignments of His C2H and C4H peaks of apo-HTF have been confirmed by pH titrations (Kubal, G.; Sadler, P. J., unpublished). Over the range pH* 3–11, titration curves for ca. 14 His C2H peaks can be followed (i.e., several His C2H peaks overlap at pH* 8.78). There are 19 His residues in HTF; 10 in the C-lobe and nine in the N-lobe. Sugar protons are seen clearly in 2D COSY spectra, together with cross peaks for several amino acid residues.

(10) Added as microliter aliquots of a stock solution of $Al_2(SO_4)_3 \cdot 16H_2O$ (BDH) in D_2O (pH* 3.4). UV spectra of similar HTF solutions (after 50× dilution in the same buffer) were also recorded. New maxima were observed at 240 and 288 nm for Al-HTF with intensities similar to those reported,[3] and characteristic of coordinated tyrosines, showing that Al^{3+} binds to the high-affinity metal sites under our conditions.

(11) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

0002-7863/92/1514-1117$03.00/0

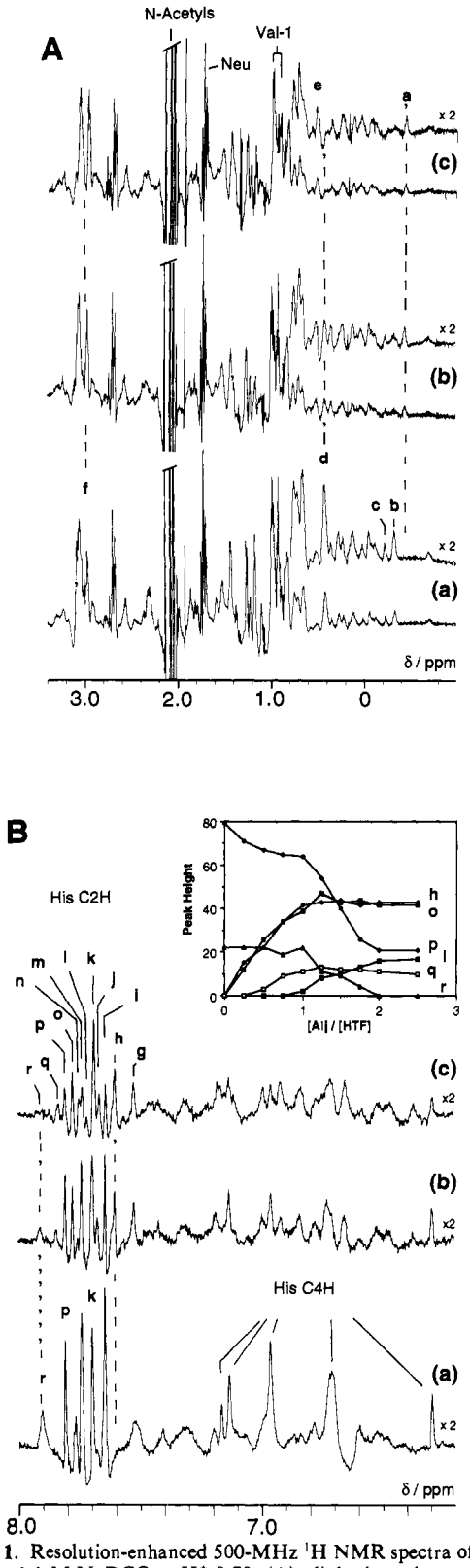

Figure 1. Resolution-enhanced 500-MHz ^{1}H NMR spectra of 0.56 mM HTF in 0.1 M $NaDCO_3$, pH* 8.78, (A) aliphatic region and (B) aromatic region: (a) before and (b) after addition of 1.25 molar equiv of Al^{3+} and (c) 2.5 molar equiv of Al^{3+}. Assignments: Val-1, valine-1 CH_3; *N*-acetyls, MeCO of NAcGlc and NAcNeu of glycan chains (C-lobe); Neu, NAcNeu; a-f, peaks perturbed by Al^{3+} binding; g-r, His C2H peaks. The intensities of His C2H peaks h, l, o, p, q, and r are plotted in the inset in part B; these data are from a second (and more complete) titration using 0.35 mM HTF. With broad overlapping peaks, low S/N ratios (low protein concentrations), and the use of resolution enhancement, peak heights (or areas) are subject to error from the choice of base lines. The data indicate slow exchange of HTF with Al-HTF on the NMR time scale.

orientation of these methyls with respect to certain aromatic side chains of other residues, perhaps due to closure of the metal-binding cleft.[12]

These methods can now be used to test both the thermodynamic and kinetic effectiveness of therapeutic chelating agents designed to remove Al^{3+} from HTF and the body, such as desferrioxamines, hydroxypyridinones, and catecholates.[13-16] For example, incubation of an Al_2-HTF sample with 3 molar equiv of desferrioxamine (Ciba) for 4 h at 310 K completely reversed the effects of Al^{3+} on the spectrum, and the spectrum of apo-HTF was regenerated. It should also be possible to study the sequential uptake and release of other metal ions, e.g., Ga^{3+}, In^{3+}, and perhaps Fe^{3+}, by transferrin, and with longer accumulation times it may even be possible to do experiments at HTF concentrations close to those found in vivo (ca. 37 μM).

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(12) An X-ray structure of apolactoferrin with the N-lobe open and the C-lobe closed has been reported: Anderson, B. F.; Baker, H. M.; Norris, G. E.; Rumball, S. V.; Baker, E. N. *Nature* **1990**, *344*, 784-787.
(13) Leung, F. Y.; Hodsman, A. B.; Muirhead, N.; Henderson, A. R. *Clin. Chem. (Winston-Salem, N.C.)* **1985**, *31*, 20-23.
(14) Dobbin, P. S.; Hider, R. C. *Chem. Br.* **1990**, *26*, 565-568.
(15) Zhang, Z.; Rettig, S. J.; Orvig, C. *Inorg. Chem.* **1991**, *30*, 509-515.
(16) Kretchmar, S. A.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 1436-1441.

Drug-Induced Reactions of Bovine Serum Albumin: ^{1}H NMR Studies of Gold Binding and Cysteine Release

Orla M. Ni Dhubhghaill, Peter J. Sadler,* and Alan Tucker

Department of Chemistry, Birkbeck College
University of London
Gordon House and Christopher Ingold Laboratory
29 Gordon Square, London WC1H 0PP, U.K.

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The protein albumin plays a major role in the transport of drugs and metal ions in the blood.[1] For example, in patients treated with gold antiarthritic drugs, over 80% of the circulating gold in blood serum is carried on albumin.[2,3] However, the chemistry of serum albumin is poorly understood. Progress in this area is likely to arise from a combination of X-ray crystallography[4] and high-resolution NMR spectroscopy.[5] Albumin (MW 66.5 kDa) is a large molecule for ^{1}H NMR spectroscopy, but with high-field spectrometers and resolution-enhancement methods, resonances from the more mobile regions of the protein are well-resolved and can be used to probe drug-induced structural changes. We report here[6] evidence for specific structural changes in bovine serum albumin (BSA) on binding to an antiarthritic gold drug and the accompanying release of Cys from the protein. The fate of released Cys is determined by a ligand on the gold drug.

* To whom correspondence should be addressed.

(1) Peters, T. Jr. *Adv. Protein Chem.* **1985**, *37*, 161-245.
(2) Finkelstein, A. E.; Walz, D. T.; Batista, V.; Mizraji, M.; Toisman, F.; Misher, A. *Ann. Rheum. Dis.* **1976**, *35*, 251-257.
(3) Blodgett, R. C.; Heuer, M. A.; Pietrusko, R. C. *Semin. Arthritis Rheum.* **1984**, *13*, 255-272.
(4) A recent low-resolution (4-Å) crystal structure shows that HSA has three structurally-homologous domains in a circular arrangement: Carter, D. C.; He, X. *Science* **1990**, *249*, 302-303.
(5) Peaks for about 80 spin systems are observable in 2D COSY spectra. Through a comparison of 1D and 2D COSY spectra of bovine, human, porcine, and rat albumins, resonances for the three N-terminal amino acids can be assigned: Sadler, P. J.; Tucker, A. *Biochem. Soc. Trans.* **1990**, *18*, 923-924. Sadler, P. J.; Tucker, A., submitted for publication.
(6) Ni Dhubhghaill, O. M.; Radulovic, S.; Sadler, P. J.; Tucker, A. Presented in part at the 201st National Meeting of the American Chemical Society, Atlanta, GA, 14-19 April 1991; Abstract INOR 456.

0002-7863/92/1514-1118$03.00/0