four peaks to two at 38 °C ($\Delta\nu$ = 10 Hz) and two peaks to one at 94 °C ($\Delta\nu$ = 14 Hz).[14]

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^{205}Tl as an NMR Probe for the Investigation of Transferrin

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Abstract: Dithallium(III) transferrin and the monothallium derivative with bicarbonate as synergistic ion have been prepared and characterized. ^{205}Tl NMR spectroscopy is a reasonably good probe to monitor the occupancy of the two available binding sites. The signals for the two sites are well separated and have been assigned through titration with iron(III) at different pH values. Thallium shows larger affinity for the C-terminal site than for the N-terminal site all over the pH range investigated.

Human serum transferrin (TRN hereafter) is a glycoprotein of molecular weight 81 000[1] with two binding sites for iron(III).[1-5] The two sites are very apart in the rod-shaped protein and identified as C-terminal and N-terminal sites according to their location along the single polypeptide chain.[6,7] In vivo the two sites are occupied only at 30%.[4] The donor groups appear to be two tyrosinate anions and a number of histidines. Evidences of tyrosinate ligands stand on charge transfer spectra[8] and recently on a very appropriate model complex.[9] The presence of histidines is guessed from the observation of superhyperfine splitting in the EPR spectra of the copper(II) derivative[10] and from pulsed EPR techniques.[11] The bicarbonate ion is needed to form a stable ternary complex, and therefore it may be reasonably considered a donor group, as suggested also from the ^{13}C NMR data on the cobalt(III) derivative.[12] The overall coordination number presumably is at least six. Water proton NMR T_1^{-1} measurements, though they do not show definite evidence of exchangeable protons attached to the donor groups, are consistent with either H_2O or OH^- present in the coordination sphere.[9,13]

It is not clear whether the two sites are distinguishable from the physiological point of view;[1-5,14] however, the EPR spectra of the iron(III), copper(II), and oxovanadium(IV) derivatives have shown that the two sites are spectroscopically slightly nonequivalent,[10,15] whereas the distribution of a single metal ion over the two sites is dramatically pH dependent (for example at low pH only the C-terminal site is populated).[7] Substitution of iron(III) by a variety of metal ions has shown that also the size of the metal ion can distinguish between two sites; i.e., large ions such as neodymium(III) or praseodymium(III) can only enter the C-terminal site.[16]

Since the protein stabilizes the oxidation number three, we sought to introduce thallium(III) into the protein, not only to describe a new metal derivative but to provide a further spectroscopic tool for the investigation of the two sites. In principle, the ^{205}Tl NMR signal can be sensitive to the structural difference between the two sites, thus providing information on the metal distribution even in presence of other metal ions.

Experimental Section

Purified iron-free human serum transferrin (apo-TRN) was purchased from Sigma and was used without further purification. The lyophilized powder was dissolved in freshly bidistilled water, dialyzed twice against a 0.1 M $NaClO_4$ solution to remove possible traces of chelating agents,[17] and exhaustively dialyzed against bidistilled water. For spectroscopic studies apo-TRN was diluted up to a concentration of about 10^{-5} M.

The NMR samples were prepared by concentrating the apoprotein through ultradialysis up to $(1-2) \times 10^{-3}$ M. The concentration of apo-TRN was estimated spectroscopically, using $\epsilon_{278} = 9.23 \times 10^4$ M^{-1} cm^{-1}. All the experiments were performed in unbuffered solutions; the pH was adjusted to the required values by either addition of $NaHCO_3$ or by bubbling gaseous CO_2. The total concentration of bicarbonate and/or carbon dioxide was always in large excess with respect to the TRN sites concentration (0.03–0.3 M).

Thallium(III) solutions of approximate titer were prepared from $TlCl_3$ and adjusted to pH 3 by adding HCl. The actual thallium(III) concentration was determined by complexometric back-titrations. Excess EDTA was added to a known volume of $TlCl_3$ solution and the pH adjusted to 10 with ammonia buffer. Excess EDTA was then titrated by $MgCl_2$, using Eriochrome black T as indicator.

The electronic spectra were recorded on a Cary 17D spectrophotometer through the difference UV spectroscopy technique. ^{205}Tl NMR measurements were performed with a CXP Bruker spectrometer equipped with a 1.41-T electromagnet operating at 34.7 MHz; quadrature detection and a standard phase alternated pulse sequence (PAPS) were employed.

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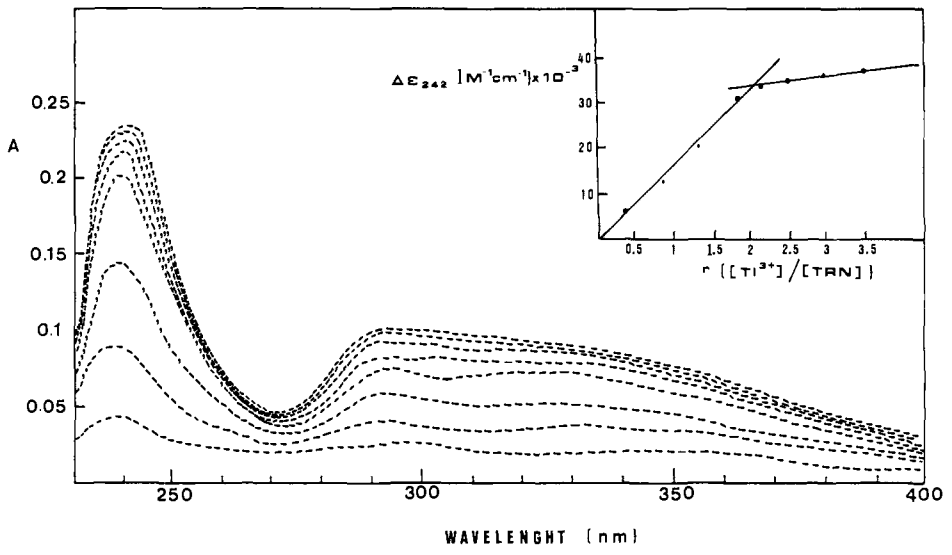

Figure 1. Difference UV spectra of 3 mL of apo-TRN solutions 6.9×10^{-6} M, pH 8.4, 0.2 M HCO_3^-, upon stepwise additions of 10 μL of $TlCl_3$ 8.9 $\times 10^{-4}$ M. Final dilution less than 3%. The inset shows the variation of ϵ_{242} (corrected for dilution) as a function of the metal/apoprotein ratio.

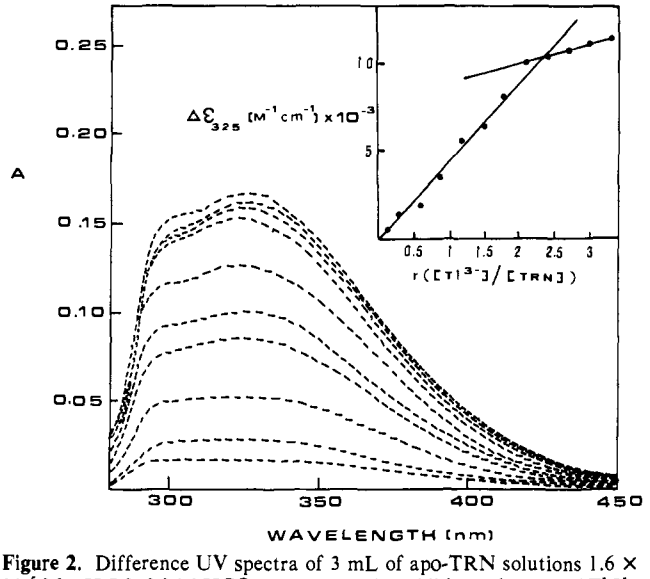

Figure 2. Difference UV spectra of 3 mL of apo-TRN solutions 1.6×10^{-5} M, pH 7.8, 0.2 M HCO_3^-, upon stepwise additions of 15 μL of $TlCl_3$ 9.6×10^{-4} M and contemporary additions of 15 μL of $Al(NO_3)_3$ 9.6×10^{-4} M in the reference cell. Final dilution 5%. The inset shows the variation of ϵ_{325} (corrected for dilution) as a function of the metal/apoprotein ratio.

Results

The titration of apo-TRN solutions with $TlCl_3$ in the presence of 0.1 M $NaHCO_3$ in the 7–8.5 pH range was followed through difference spectroscopy in the 200–400-nm region. The increase of the difference absorption bands at 242 nm typical of binding of tyrosine groups to metal ions was found to be linear up to 2:1 metal:protein ratios (Figure 1). The molar absorbance values ($\epsilon_{242} = 3.2 \times 10^4$ M^{-1} cm^{-1}) are in the range observed for other transferrin derivatives with trivalent metal ions[9] and consistent with the binding of two tyrosine residues per metal ion. A further ill-resolved absorption is present around 325 nm, which could be assigned to a ligand–metal charge transfer. Difference spectra were recorded by using the aluminum(III) derivative rather than apo-TRN in the reference cell in order to better resolve the latter band. The absorption spectrum of such derivative in the 240–300-nm region is known[18] and is very similar to that of the present system, but no charge-transfer bands are present. The spectra in Figure 2 show the titration of apo-TRN with Tl^{3+} ions performed by adding each time equimolar amounts of Al^{3+} in the reference cell. From the 2:1 spectrum a molar absorbance of 1.0 $\times 10^4$ M^{-1} cm^{-1} per TRN dimer is calculated. The same titrations performed at pH 5.5–5.8 showed that the binding of thallium(III) was complete at 1:1 molar ratio. The break in the 242-nm ab-

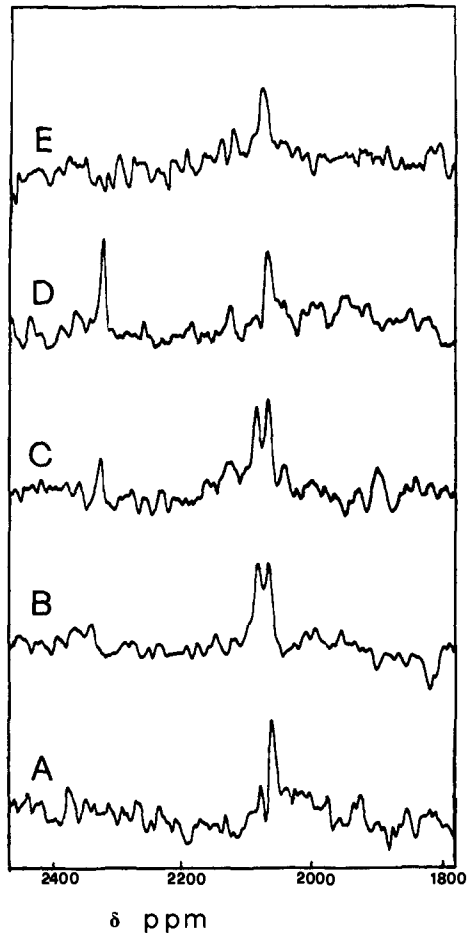

Figure 3. ^{205}Tl NMR spectra: TlTRN, pH 7.2 (A); Tl_2TRN, pH 7.2 (B); Tl_2TRN, pH 8.5 (C); Tl_2TRN (C) brought to pH 6.4 (D); Tl_2TRN, pH 8.5 (C) after addition of 1 equiv of Fe^{3+} (E). Spectra have been obtained with 3–4-μs pulse width corresponding to 60–70° flip angles, 500-μs deadtime, 5-s repetition rate, and 10000–20000 scans. Exponential line-broadening factors corresponding to 100–200 Hz have been applied for sensitivity enhancement.

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Table I. ^{205}Tl Chemical Shift Values (δ)[a] for Thallium(III) Complexes with Nitrogen and Oxygen Ligands

compd	donor set	δ
$[Tl(oxalate)_3]^{3-}$	O_6	1951
Tl_2TRN	N_2O_4 (?)	2055
	N_2O_4 (?)	2075
$[Tl(EDTA)]^-$	N_2O_4	2281
$[Tl(8\text{-hydroxyquinoline-5-sulfonate})_3]^{3-}$	N_3O_3	2526
$[Tl(1,10\text{-phenanthroline})_3]^{3+}$	N_6	2564

[a] The shifts are given in ppm downfield with respect to $^{205}Tl^+$ ions at infinite dilution.

sorption rise was less pronounced than at alkaline pH, owing to the tail of an intrinsic absorption of the free thallium(III) ion in this region.

^{205}Tl NMR (34.7 MHz) spectra of concentrated solutions (1–2 mM) of the Tl_2TRN derivative at pH values of 7.2 and 8.5 showed two signals (Figure 3B,C) of similar intensity and line width (~100 Hz) separated by 700 Hz (~20 ppm). Their chemical shifts are 2075 and 2055 ppm downfield from an aqueous solution of Tl^+ at infinite dilution.[19] Stepwise additions of $TlCl_3$ to apo-TRN solutions containing 3×10^{-1} M $NaHCO_3$ at pH 7.5 resulted in the appearance and growth of the lower frequency signal (2055 ppm) up to 1:1 metal:protein ratios (Figure 3A) and in the subsequent development of the higher frequency signal up to 2:1 ratios (Figure 3B). Further additions of Tl^{3+} ions yielded a third narrower signal at somewhat higher frequency (2329 ppm) in a position similar to that of thallium(III) chloride in aqueous solutions of the same concentration. The latter signal disappeared upon leaving the sample at room temperature for 1 or 2 days; correspondingly, a new signal was observed in the frequency region typical of Tl^+ ions, indicating that excess thallium(III) was slowly reduced, probably by oxidation of some protein residue. On the other hand, there was no evidence of reduction of thallium(III) bound to the native coordination sites of the protein over a period of more than a week.

By decreasing the pH of concentrated Tl_2TRN solutions below 6.0 through microliter additions of 0.1 M HCl, the higher frequency signal disappeared (Figure 3D); an increase of pH from 8.3 to 9.5 with 0.1 M NaOH caused complete loss of both the NMR signals. The same results were obtained by starting from apo-TRN at pH 5.7 and 9.5, respectively, and adding up to 3:1 molar ratio of Tl^{3+} ions. The pH 9.5 sample contained 3×10^{-1} M total concentration of the HCO_3^-–CO_3^{2-} species; the sample at pH 5.7 was saturated with CO_2 under $P_{CO_2} = 10^5$ Pa ($\sim 3 \times 10^{-2}$ M).

Addition of $FeCl_3$ in a 1:1 ratio to a sample of Tl_2TRN at pH 8.3 caused disappearance of the higher frequency signal (Figure 3E); the lower frequency signal was also almost completely abolished by addition of a 2nd equiv of $FeCl_3$.

As indicated by the electronic spectra, substitution of Tl^{3+} with Fe^{3+} requires a few hours in order to reach completion; the samples were therefore left standing several hours after each addition of $FeCl_3$ before recording the ^{205}Tl NMR spectra.

Transferrin is known to bind only one Fe^{3+} ion at pH below 6; the binding occurs selectively at the C terminal site,[7] the N terminal site having much lower affinity at this pH. Monothallium(III) transferrin prepared at pH 5.5 was reacted with Fe^{3+} in a 1:1 ratio; the sample developed the characteristic orange-red color of the iron(III) derivative and the single (low frequency) ^{205}Tl signal disappeared.

^{205}Tl NMR spectra of a few thallium(III) complexes in aqueous solution were also recorded, in order to have an estimate of the differences in chemical shift to be expected by changing the nature of the donor atoms. The resonance frequencies of the ^{205}Tl signals are reported in Table I, together with those observed for Tl_2TRN. All of the above complexes have been prepared by adding large excess of ligands to $TlCl_3$ water solutions; for all of them the reported values of the stability constants[20] ensure complete formation of the complexes under the present conditions.

Discussion

The present data indicate that the $^{205}Tl^{3+}$ ion is a suitable NMR probe for trivalent metals bound to transferrin. Its high sensitivity allows one to record reasonably good NMR spectra in the millimolar concentration range even when the lines are relatively broad, as in the present case. The high affinity of the protein for trivalent metal ions is apparently effective in stabilizing the 3+ oxidation state of the above metal, which otherwise is a strong oxidative agent ($E_0(Tl^{3+}/Tl^+) = 1.247$ V). This is not uncommon for transferrin, which is able to bind metal ions such as manganese(III)[21] or vanadium(III).[22]

Although not identical, the two metal binding sites in transferrin impart absolutely similar spectroscopic properties to the bound metal ions; slight differences have only been recognized in the EPR spectra of some derivatives;[10,15] on the other hand, it is well-known that the relative affinities of the two sites are different and also display different pH dependences.[7,10,15] Transferrin derivatives in which a single site has been selectively occupied by a chosen metal ion may be therefore prepared. It is however a difficult taks to identify such monometallic derivatives through any kind of spectroscopy. Thallium(III) seems to solve at least part of the problem, since the two sites give rise to two separate ^{205}Tl resonances. This sensitivity can be explained by considering the large range of chemical shifts covered by thallium(III) complexes (Table I): the resonance signals of $Tl(o\text{-phen})_3^{3+}$ and $Tl(oxalate)_3^{3-}$ are separated by hundreds of ppm. On this scale, the sizeable separation between the two ^{205}Tl resonances in transferrin (20 ppm) may be considered extremely small and a further evidence of the chemical similarity of the two sites. From systematic studies on ^{113}Cd complexes,[23] a correlation seems to exist between the number and chemical nature of the ligands and the metal chemical shift. This kind of studies is only at the beginning on ^{205}Tl, the chemical shifts of its halide complexes having been only recently analyzed in detail;[24] we note however that the chemical shifts observed for $^{205}Tl^{3+}$ in transferrin as compared with the data in Table I may be consistent with a N_2O_4 donor set, as often proposed.

Thanks to the NMR properties of the Tl^{3+} ion, several conclusions may be drawn on the present biological system: (1) ^{205}Tl NMR spectroscopy allows easy discrimination between the two protein sites; (2) from pH 5.5 to 8.3 a monothallium derivative can be prepared by simply adding 1 equiv of $TlCl_3$ to the apoprotein; (3) in this derivative the thallium ion occupies the C-terminal site, since its NMR signal is abolished when the C-terminal site is selectively occupied by iron(III) at low pH; (4) the affinity of thallium(III) for the N-terminal site is much lower, as shown by its inability to bind at pH <6 and by the nonrandom distribution in the two sites at pH 7.2–8.5; (5) owing to such difference in affinity, iron(III) selectively substitutes thallium(III) at the N-terminal site at pH 8.3, where the native metal has comparable affinity for both sites when empty.

Monothallium(III) transferrin seems therefore to represent an easy way to prepare mixed-metal derivatives of nonrandom distribution, by keeping in mind that thallium(III) has a large preference for the C-terminal site at every pH.

The comparison of the affinity of thallium(III) for the two sites with that of the other metal ions allows some comments. The preference for the C-terminal site spans a larger range of pH than for instance iron(III) and copper(II).[7,10] This consideration qualitatively fits with the concept that large ions do not enter at all the N-terminal site.[16] The ionic radius of thallium(III) lies

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on the borderline between the set of metals capable of binding to both sites and those which only bind to the C-terminal site.[9,16,25]

A final comment is due on the general utility of thallium(III) as a probe in biochemical problems. From the pulse delay needed to avoid saturation of the signal, T_1 may be estimated of the order of seconds. From the reported rotational correlation time of transferrin of 2×10^{-7} s at 25 °C,[26] the T_1/T_2 ratio at 34.7 MHz is expected to be larger than 10^3. Indeed, the line width at half-peak height is of the order of 100 Hz, which sets T_2 3 orders of magnitude shorter than T_1. It appears therefore that a molecular weight of about 80 000 such as that of transferrin is close to be an upper limit for the practical use of ^{205}Tl as an NMR probe. On the other hand, proteins of lower molecular weight are expected to give rise to much sharper signals and much better signal-to-noise ratios.

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Registry No. Thallium, 7440-28-0; thallium-205, 14280-49-0.

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Mechanism of T_1 Relaxation in ^{13}CO Complexed to an Iron Porphyrin: Implications for CO Bonding in Heme Proteins

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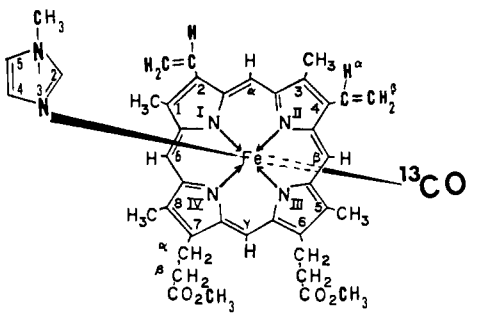
Abstract: Chemical shifts and spin-lattice relaxation parameters for ^{13}CO bonded to the complex 1-methylimidazole-iron protoporphyrin dimethyl ester are reported. The ^{13}CO chemical shifts are much different from those observed in the carbonyl vertebrate hemoglobins, but remarkably similar to that observed for the monomeric hemoglobin component from *Glycera dibranchiata*. The relaxation parameter, T_1, is shown to change dramatically upon ligation to both the hemin model system and to hemoglobin A. The mechanisms of spin-lattice relaxation are dominated by chemical shift anisotropy for ^{13}CO bound to both hemins and proteins, but, in the latter, dipole-dipole forces make an additional contribution to the overall relaxation rate.

Heme prosthetic groups function as the active sites in a number of proteins including ligand-binding enzymes such as catalase and peroxidase, oxygen transport proteins such as hemoglobin, and oxygen storage proteins such as myoglobins. Many of these proteins also bind carbon monoxide, which has provided a basis for infrared and nuclear magnetic resonance spectroscopic studies of the molecular aspects of ligand binding in these heme proteins.[1-11]

In the case of infrared studies, interpretation of results from work on the carbonyl hemoglobins[4,6,8-10] has been greatly aided by observations on various carbonyl heme models that have revealed how cis[2,11] and trans[1,12,13] effects influence the vibrational modes of the CO ligand. These results have enhanced our understanding of interactions in the heme proteins themselves.

In contrast, a less firm basis of model studies exists for understanding the molecular effects which influence the ^{13}C chemical shifts of heme-bound CO, although chemical shifts of carbonyl myoglobins and hemoglobins reconstituted with modified hemes have been shown[14-16] to be sensitive to substitutions around the periphery of the heme. To provide a firmer basis for interpreting NMR spectroscopic properties of ^{13}CO bound to heme proteins, we have studied the NMR properties of the carbonyl hemochrome derived from ferrous (Fe^{2+}) protoporphyrin IX dimethyl ester (I). These data are compared with the NMR properties of adult human hemoglobin (Hb A) and the monomeric hemoglobin fraction from the marine annelid *Glycera dibranchiata*.

The chemical shift studies are aimed at understanding to what degree the protein alters, in a spectroscopically detectable manner,



1-Melm-I-CO

the environment of heme-bound CO. Several views[3,11,17-19] have been expressed with regard to the functional specificity of heme

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