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METAL-INDUCED CONFORMATIONAL HETEROGENEITY OF TRANSFERRINS: A SPECTROSCOPIC STUDY OF INDIUM(III) AND OTHER METAL(III)-SUBSTITUTED TRANSFERRINS

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The conformation in solution of three different metal(III)-transferrins, namely aluminum(III), gallium(III) and indium(III) transferrin, was investigated by absorption, CD, ^{1}H NMR and ^{13}C NMR spectroscopies. The formation of the respective metal-transferrin complexes and the characteristic 2:1 metal-to-protein binding stoichiometry were unambiguously demonstrated, in all cases, through UV difference studies. The ^{13}C NMR spectra of these metallotransferrins in the carbonyl region are very similar to one another pointing out that the arrangement of the synergistic anion in the binding site must be essentially the same. However, the CD spectra in the near UV (aromatic region) reveal the occurrence of significant differences between indium transferrin, on one side, and the other two derivatives, on the other. Also, the ^{1}H NMR spectra exhibit a number of different features suggesting the occurrence of metal-induced conformational heterogeneity around the metal sites. Such metal-induced conformational heterogeneity probably affects the transferrin-receptor recognition process, resulting in a different metabolic fate of these metals in the organisms.

Transferrin (Tf hereafter) is the key protein of iron metabolism in higher organisms (1,2). The X-ray structure of transferrins has been recently solved to a good resolution and the main features of the two metal binding sites extensively documented (3-6). The protein is a single chain polypeptide comprising two lobes, each of them capable of binding, with high affinity, one iron(III) ion. In both lobes the virtually identical metal binding sites lie in a interdomain cleft; it is believed that the apoprotein, existing in an open conformation, wraps around the metal upon complex formation, with the assistance of the synergistic anion carbonate (7). Besides iron(III), transferrins bind, with relatively high affinity, several other metal ions, especially the tripositive ones, some of them being of relevant medical or toxicological interest (8,9). Given the fact that transferrin is an extremely abundant plasma protein and is normally no more than 30% iron-saturated (1), it follows that most tripositive metals present in the blood should primarily exist as transferrin complexes. In the case of aluminum(III) and gallium(III) ample evidence exists that metal binding to the protein is tight, occurs at the same binding sites of iron(III), invariably requires the assistance of the synergistic anion and causes deprotonation of at least two tyrosine

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residues per site (1,10,11). Similar features but lower stability constants were reported for bipositive metals such as cadmium(II) (12) and zinc(II) (13), and for big cations such as hafnium(IV) (14), thorium(IV) (15) and the lanthanides(III) (16).

Now a central point of transferrin chemistry is to understand ***whether*** and ***to what extent*** the nature of the central metal affects the conformation of the closed protein. An interesting study by Grossmann et al. using the small angle X-ray scattering technique (SAXS) suggested the occurrence of relevant differences in the overall solution conformation of various metallotransferrins depending on the nature of the metal (17,18). For instance indium transferrin, that closely reproduces the behavior of iron transferrin, has been reported to be significantly different from aluminum transferrin, and both of them markedly different from hafnium transferrin. The observed differences were mainly ascribed to the influence of the *size* of the metal ion on the protein closure mechanism, so that the small aluminum(III) ions are believed to induce a protein closure somewhat different from that of the bigger indium(III) and iron(III) ions, whereas the very large hafnium(IV) ions are thought to determine an incomplete closure (18). Surprisingly copper(II) transferrin behaves very similarly to iron(III) and indium(III) transferrins (18).

To better elucidate these aspects we discuss here a series of spectroscopic data on transferrin complexes of tripositive metals, namely aluminum, indium and gallium, that are of large medical concern (9). Indeed, aluminum is presumably involved in the etiopathogenesis of the Alzheimer's disease (19) and is toxicologically relevant for other clinical conditions such as dialysis encefalopathy (9). On the other hand, gallium and indium, beside being good radiotracers, have been shown to behave *in vitro* as antiproliferative agents through not yet completely identified mechanisms *-perhaps interference with the iron uptake route and subsequent inhibition of ribonucleotide reductase-* and are being considered for antitumor chemotherapy (20-23). Our study mainly focuses on the spectral results of indium transferrin for which only few studies exist (see ref.23 and references therein); for the cases of gallium and aluminum transferrin we will mostly refer to literature data. For comparison purposes we have performed some more spectral measurements on zinc(II) transferrin to monitor the effects of a typical bipositive metal on the protein conformation.

MATERIALS AND METHODS

Human serum apotransferrin was purchased from the Sigma Chemical Company and further purified according to the reported procedure [24]. The lyophilized protein was dissolved in a buffer containing 10 mM NaH_2PO_4, and 4 mM $NaHCO_3$, pH 7.4. Protein concentration was determined by measuring the intensity of the characteristic UV band at 280 nm (ε_{280}= 91,200 M^{-1} cm^{-1}). Aluminum, gallium and indium chloride were of analytical grade.

The UV-visible spectra were recorded on a double beam CARY 17 D instrument operating at room temperature. The UV difference experiments were performed on the same instrument according to the procedure reported by Harris et al.(11). The CD spectra were run on a JASCO 200 D spectropolarimeter at room temperature. Samples for absorption and CD measurements were about $1x10^{-5}$ M in protein. When working in the 200-250 nm region the samples were

further diluted to about 1 μM protein concetration and analyzed with 1 mM pathlenght cuvettes in the presence of an intense nitrogen flow.

The ^{1}H NMR spectra were recorded on a Bruker AMX 600 instrument operating at 14 T. Samples for the ^{1}H NMR experiments typically consisted of deuterated solutions of 0.2 mM apotransferrin or M_2Tf in 10 mM phosphate buffer, pH 7.4, 4 mM $NaHCO_3$. The pH was checked before and after the measurements. The spectra were acquired with 90° pulses, 512 transients, 1-1.5 s relaxation delay, and presaturation to suppress the residual water signal. In order to improve spectral resolution temperature was set to 310 K. FIDs were processed using exponential functions equivalent to line broadenings of 1-3 Hz.

The ^{13}C NMR spectra were acquired on a Bruker AMX 400 instrument. Samples for ^{13}C NMR measurements were 1.5 mM in protein, 10 mM phosphate buffer, pH 7.4. The volume was about 0.5 mL with 10% D_2O. 6 mM ^{13}C enriched sodium bicarbonate was added to the samples before addition of two equivalents of the metal ion. Typical ^{13}C NMR acquisition parameters used in this study were as follows: 50° pulse length, 1.5 s repetition time, and a sweep width of 20,000 Hz. A line broadening of 5-10 Hz was applied to all ^{13}C data prior to processing.

RESULTS

Formation of the metal-transferrin complexes probed through UV difference spectroscopy.

UV difference spectroscopy is a powerful technique to demonstrate metal binding to transferrins by monitoring deprotonation of the tyrosine residues. So far, UV difference measurements have been extensively employed to determine the stoichiometry and the binding strength of several cations- and also anions- to the specific sites of apotransferrin (10-13). We have used this technique to monitor the binding of the above tripositive metals to apotransferrin. In the case of aluminum(III) and gallium(III) spectral patterns virtually superimposable to those already reported in the literature (10,11) were obtained (data not shown). The UV difference spectrum of apotransferrin after addition of two equivalents of a freshly prepared solution of indium(III) chloride, at pH 7.4, is shown in Figure 1. The obtained pattern is very similar, both in shape and intensity, to those of aluminum(III) and gallium(III) transferrin. No major spectral variation is observed upon addition of further indium(III) equivalents demonstrating that saturation is reached for a 2:1 metal-to-protein ratio and that the affinity of indium(III) for apotransferrin is high, as anticipated (23). Noticeably, the uptake of indium(III) ions by transferrin at room temperature is complete within 1-2 hours, even in the absence of the facilitating agent nitrilotriacetate, provided that a freshly prepared indium(III) solution is used.

CD spectra in the aromatic region.

Circular dichroism spectroscopy provides direct information on the solution structure of proteins, the occurrence of conformational variations being easily revealed (25-27 and refs. therein). We decided to use this technique to monitor the conformational changes of apotransferrin elicited by the binding of the above tripositive metal ions. The CD spectra in the near UV region (aromatic region -from 250 to 320 nm-) of apoTf, Al_2Tf, In_2Tf and Ga_2Tf are shown in Figure 2 -for comparison purposes the CD spectrum of Zn_2Tf is also reported-. It may be noted that metal binding to the protein of these three metals invariably causes small but significant changes in the aromatic region; of particular interest the case of indium(III) for which

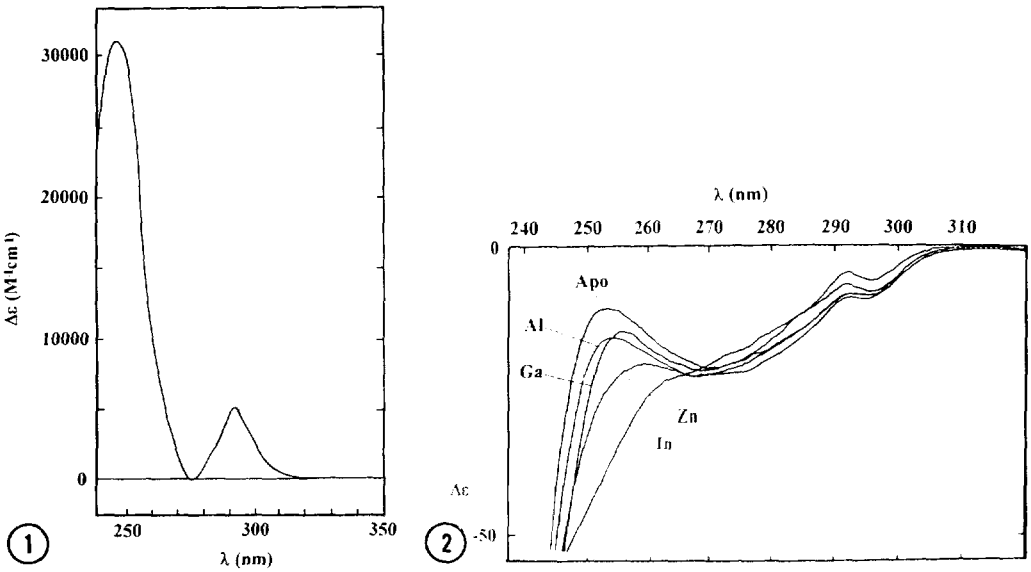

Figure 1. Difference UV spectrum obtained upon addition of two equivalents of indium(III) chloride to 3 mL of 1.5 x 10^{-5} M apotransferrin solution; 10 mM phosphate buffer, 4 mM sodium bicarbonate, pH 7.4.

Figure 2. CD spectra of various transferrin derivatives in the near UV region (aromatic region 320-250 nm).The CD spectra of apoTf, Zn_2Tf, In_2Tf, Al_2Tf and Ga_2Tf are shown. Protein concentration is 1x10^{-5} M, in 10 mM phosphate buffer, 4 mM sodium bicarbonate, pH 7.4.

an intense negative shoulder appears around 255 nm, a transition not observed in the other derivatives. The interpretation of this novel feature is not straightforward. However, since the main difference among aluminum(III), gallium(III) and indium(III) is the ionic radius (the respective values are: Al^{3+} 0.51 Å; Ga^{3+} 0.62 Å; In^{3+} 0.81 Å) one might assume that the relatively big indium(III) cations induce a peculiar perturbation of some aromatic residue. The CD studies of the various metallotransferrins were extended to the far UV region using far more diluted protein solutions (about 1μM) and 1mm pathlength cuvettes. No significant variations in the intensity and shape of the characteristic negative band at 208 nm were observed ruling out major changes in the secondary structure of the protein (26,27).

^1H NMR results.

To gain further insight into the solution conformation of the above metallotransferrins we recorded the respective high resolution ^1H NMR spectra at 600 MHz. Even if transferrin is characterized by a relatively high molecular weight (80 KD) that virtually prevents application of the classical 2D and 3D NMR techniques for structure determination, it is still possible to record reasonably well resolved 1D ^1H NMR spectra; the spectral resolution may be sensibly enhanced by adopting some simple tricks (high dilution, deuteration, high temperature). Even if the spectrum looks heavily overcrowded in several regions, it is still possible to extract valuable information from the ^1H NMR data by focusing on the analysis of a few spectral windows where signal crowding is not so severe and comparing the spectra of the different derivatives. Studies of

this kind have been recently reported by Sadler et al. (28,29). The monodimensional ^1H NMR spectra of Al_2Tf, Ga_2Tf and In_2Tf plus those of apotransferrin and Zn_2Tf, all recorded in D_2O at 37 °C, are shown in Figure 3. The details of two well resolved spectral windows, respectively located in the upfield region and in the aromatic region, are shown in Figures 4 and 5. From accurate inspection of the ^1H NMR spectra it emerges that although the main spectral features

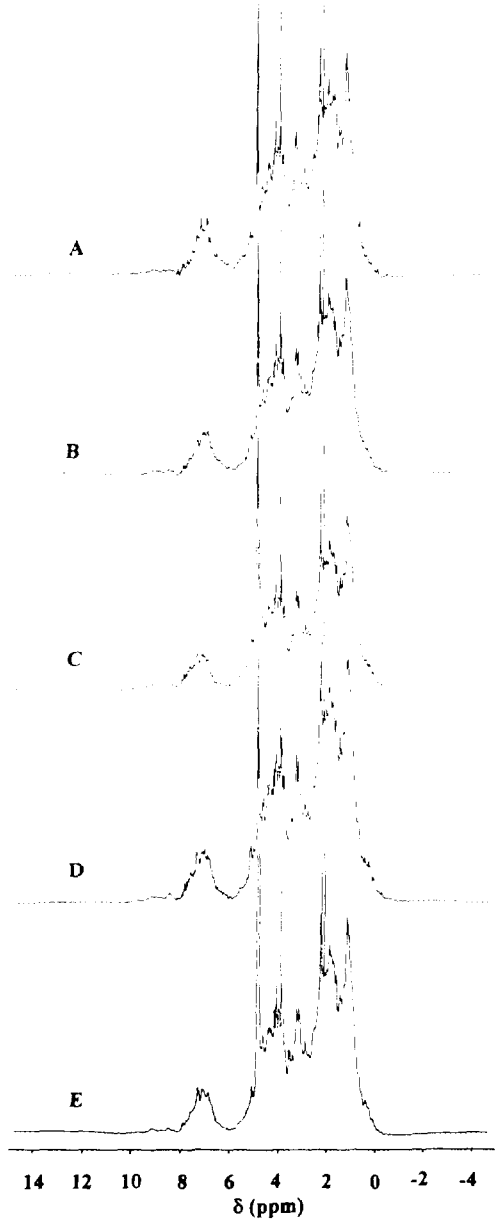

Figure 3. 600 MHz ^1H NMR spectra of a series of metallotransferrins. A: apoTf; B: Al_2Tf; C: Ga_2TF; D: In_2Tf; E: Zn_2Tf. Conditions: 0.2 mM protein in a 10 mM phosphate and 4 mM bicarbonate, deuterated buffer, pH 7.4, T= 310 K.

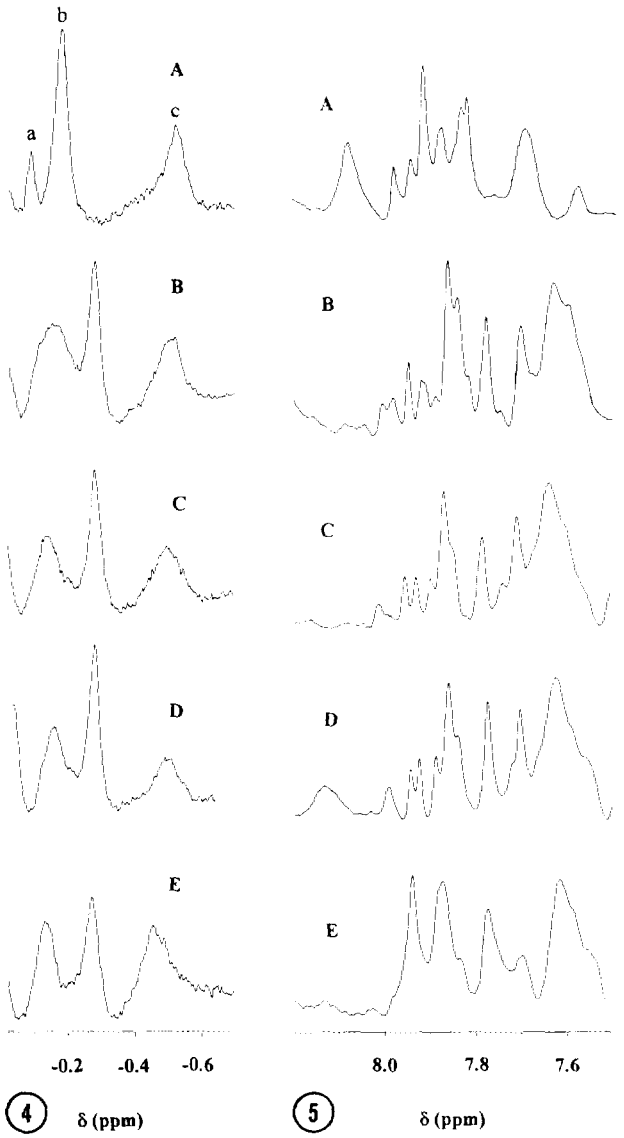

Figure 4. 600 MHz ^{1}H NMR spectra of a series of metallotransferrins: detail of the upfield region (0.0/-0.7 ppm); A: apoTf; B: Al_2Tf; C: Ga_2TF; D: In_2Tf; E: Zn_2Tf.

Figure 5. 600 MHz ^{1}H NMR spectra of a series of metallotransferrins: detail of the aromatic region (8.2-7.5.ppm); A: apoTf; B: Al_2Tf; C: Ga_2TF; D: In_2Tf; E: Zn_2Tf.

are conserved, the spectra of these derivatives differ in many details. Indeed, the *upfield region* shows marked changes upon passing from the apoprotein to the metal-loaded proteins: the relative positions and the intensities of signals **a**, **b**, and **c** significantly change upon metal binding. In the apoprotein these three signals are located at -0.07, -0.15 and -0.52 ppm, respectively, whereas their positions through all the metal(III) loaded transferrins are virtually identical with the following shift values: -0.12, -0.26 and -0.48 ppm. A similar behavior is observed in the case

of zinc(II) saturated transferrin. So, at first glance, the positions of these three signals may be considered as a rough index of metal binding to transferrins. The analysis of the *aromatic region* of the spectrum (more precisely the portion comprised between 8.2 and 7.5 ppm), that exhibits a greater number of signals, is less straightforward but, perhaps, more informative. Apparently the ^1H NMR spectra of indium, gallium and aluminum transferrin are strictly similar to one another but significantly different from those of both apotransferrin and zinc transferrin. Remarkably, out of aluminium, indium and gallium transferrins, the spectrum of indium transferrin is almost superimposable to that of gallium transferrin but slightly different from that of aluminum transferrin.

^{13}C NMR studies.

The carbonyl region of the ^{13}C NMR spectrum of In_2Tf is reported in Figure 6. The spectrum is characterized by the usual natural abundance envelope of carbonyl signals from the protein, the signal of free bicarbonate at 161.4 ppm and a signal at 167.2 ppm that reasonably corresponds to the signal of protein bound carbonate, under slow exchange conditions (30). The ^{13}C NMR signal of bound carbonate does not look completely homogeneous, probably corresponding to the sum of two not resolved, slightly inequivalent ^{13}C NMR signals (31,32). The shape of the spectrum and the position of the signal of protein bound carbonate closely reproduce the pattern previously found for aluminum and gallium transferrin (31,32), suggesting that the arrangement of the synergistic anion through these metallotransferrins is virtually the same.

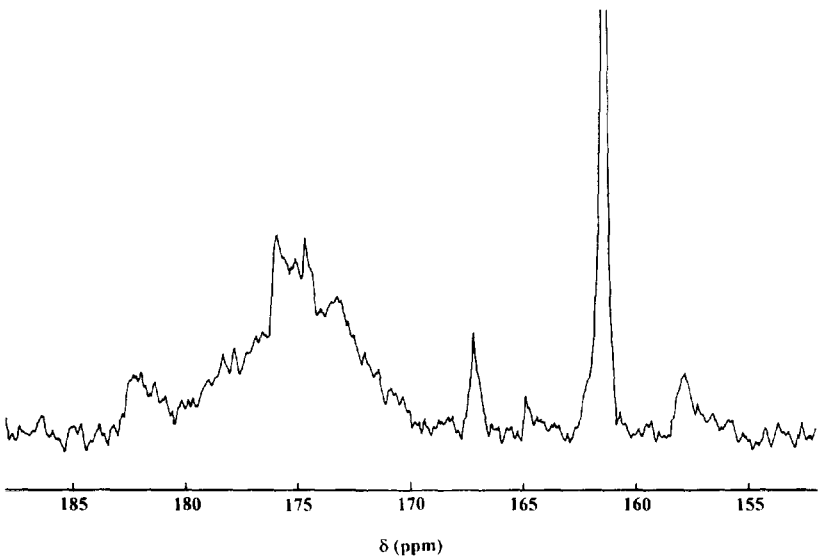

Figure 6. 100.3 MHz ^{13}C NMR spectrum in the carbonyl region of In_2Tf. Conditions: 1.5 mM protein concentration, 10 mM phosphate buffer, 6 mM ^{13}C enriched sodium bicarbonate, pH 7.4; 10% D_2O; 300 K.

DISCUSSION

The Conformation of Metallotransferrins.

In recent years, following the appearance of the crystal structure of lactoferrin, and then of serum transferrin, deep insight has been gained into the structural features of these proteins, including the identification of the metal ligands, the arrangement of the synergistic anion, and the mechanism of metal binding. These progresses have prompted an extensive reinterpretation of the numerous spectroscopic studies appeared on transferrins during the last decades. Since transferrins are able to bind, apart from iron(III), a large number of cations different in size and in charge, the crucial question raises *whether all the resulting metallotransferrins follow the physiological cycle of iron-transferrin to deliver the respective metals to the tissues or are recognized as anomalous and discarded at some stage of the cycle.* This issue seems particularly important as several among the cations that bind transferrin *in vivo* are of large medical or toxicological concern. To answer such a question one should preliminarily demonstrate whether all these metallotransferrins are characterized by the same gross conformation in solution or differ to some degree; then the investigations might be extended to the details of the protein/receptor interaction.

A rapid analysis of the literature data suggests that almost all metallotransferrins exhibit a number of conserved, fundamental features such as metal binding to the iron sites, the 2:1 metal-to-protein binding stoichiometry, and the absolute requirement of the synergistic anion. Moreover, the UV difference studies invariably demonstrate the involvement of two tyrosine residues *per site* in metal binding, while the ^{13}C NMR studies indicate a substantially identical arrangement of the synergistic anion. From these facts it might be argued that the metal binding sites of transferrin, even if not preformed, are highly conserved ones, independently of the nature of the metal. Thus, the main difference among the various metallotransferrins would reside in the different strength of the metal-protein interaction: while the tripositive cations similar in size to iron(III) are characterized by large affinity constants, the bipositive metals and the big tripositive or tetrapositive cations display far lower affinities; when the metal-protein interaction is strong, the metal binding region is endowed with a high conformational stability and a high resistance to perturbations (pH, ionic strength, etc) (33). Nevertheless, in contrast to the above unified picture of transferrin conformation, a number of structural differences among metallotransferrins have been revealed through spectroscopic and crystallographic studies (1,17,18,34). Briefly, two main kinds of structural differences have been documented: i) *local conformation differences* (or active site differences); ii) differences in the *general solution conformation*. Whereas the former are predominantly dictated by the *nature* of the metal and by its coordination preferences, the latter differences are highly sensitive to the *size* of the metal. A nice example of structural differences of the first kind is provided by the crystal structure of copper lactoferrin (32) when compared to that of iron lactoferrin. In this case the local conformation of the active site ligands is much different between copper and iron but the overall conformation is virtually the same. Conversely, the recently reported small angle X-ray scattering studies of indium, copper, iron aluminum and hafnium transferrins (17,18) provide good examples for structural differences of

the second kind; according to this technique the overall conformation of metallotransferrins in solution depends critically on the size of the metal, that modulates the protein closure process.

Conformational Differences among Indium, Gallium and Aluminum Transferrins.

The aim of our study has been that of addressing the above issue- *i.e.* the effect of the central metal on the overall solution structure- by analyzing and comparing the spectral properties of a series of M(III) transferrins (Al, Ga and In); simultaneously the local conformation of the metal site has been carefully monitored. In agreement with previous investigations our spectroscopic results suggest that these three metallotransferrins are almost indistinguishable when investigating the local conformation through difference UV and ^{13}C NMR spectroscopy, *i.e.* when looking at the phenolate groups and the synergistic anion; on the other hand, significant spectral differences appear when looking at the whole protein. In fact, the CD spectra in the UV region of indium transferrin highlight a significant perturbation of the aromatic region with respect to aluminum and gallium whereas the ^{1}H NMR spectra reveal small but significant differences through metal(III) transferrins. (In turn the ^{1}H NMR spectra of Metal(III) transferrins exhibit far more conspicuous differences with respect to either zinc(II) transferrin or apotransferrin). Thus, our data provide support to the view that even if the arrangement of the metal ligands in indium, aluminum and gallium transferrin is roughly the same, the respective conformations of these derivatives in solution may differ significantly, suggesting that transferrin is able to discriminate among these very similar metal ions just on the basis of their *different size*. The observed differences are interpreted in terms of variations in the *tertiary structure*, probably originating from a different closure around the metal, and of specific perturbation of some aromatic residue in the metal site region. The CD studies in the far UV region rule out major changes in the *secondary structure* among the various metallotransferrins. The observed conformational differences probably reflect into substantial differences in the recognition process by the specific receptor, and might account for the different biological fate of these metals in higher organisms.

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