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New insights on vanadium binding to human serum transferrin

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ABSTRACT

The knowledge on the binding of vanadium ions and complexes to serum proteins and how vanadium might be transported in blood and up-taken by cells has received much attention during the last decade, particularly as far as the transport of $V^{IV}O^{2+}$ is concerned. In this work we revise and discuss some relevant aspects of previous research, namely the two main types of binding proposed for transport of $V^{IV}O(carrier)_2$ complexes. New results, obtained by circular dichroism (CD), EPR and gel electrophoresis, regarding the binding of vanadium to hTF in the oxidation states +5 and +3 are also presented. Namely, evidences for the binding of V^{V}-species to diferric-transferrin, designated by $(Fe^{III})_2$hTF, as well as to $(Al^{III})_2$hTF, are presented and discussed, the possibility of up-take of vanadate by cells through $(Fe^{III})_2$hTF endocytosis being suggested. It is also confirmed that V^{III} binds strongly to hTF, forming di-vanadium(III)-transferrin, designated by $(V^{III})_2$hTF, and gel electrophoresis experiments indicate that $(V^{III})_2$hTF corresponds to a 'closed conformation' similar to $(Fe^{III})_2$hTF.

1. Introduction

Due to its possible physiological role as insulin-enhancer [1–3], anticancer [4–6,7], antiparasitic [8–11,12,13] as well as anti-tuberculosis [14] agents, much interest has been given to the prospective therapeutic use of vanadium compounds.

To have a physiological role the metal ion or vanadium complex must first be transported and up-taken by cells. This process is most likely conducted by plasma proteins such as human serum transferrin (hTF), human serum albumin (HSA) and immunoglobulin G (IgG) [15–28]. Many studies focused on how vanadium is transported in blood serum, namely a few reviews [15–17,20], and most publications agree in that hTF is the main vanadium transporter in serum. In this study, besides presenting a short critical review of several issues associated to the binding of vanadium to hTF, some new results are presented particularly focused on the possibility of transport of vanadium in the oxidation states +5 or +3.

Transferrin is the primarily transporter of Fe^{III} ions in the blood. It is a glycoprotein which contains around 630 amino acids arranged in two similar lobes, designated as the N-terminal (hTF_N) and C-terminal (hTF_C) lobes [29–31]. Each lobe can reversibly bind a Fe^{III} ion, but also other metal ions, such as Bi^{III}, Ga^{III}, In^{III}, Al^{III}, Cu^{II}, Mn^{II}, Zn^{II}, Ni^{II}, Ru^{III} [29–31]. Conformational changes occur in hTF upon binding or release of Fe^{III} ions: in the apo-form the protein is in the 'open conformation' while, upon binding two Fe^{III} ions forming what we designate as $(Fe)_2$hTF, the protein adopts a structure which corresponds to the 'closed conformation' of hTF. This conformation can be recognized by the hTF receptors located at the cell surface, and iron up-take occurs by internalization of transferrin in a receptor-mediated 'endocytosis' process.

Each Fe-binding site of hTF is located in clefts in the protein, one on the hTF_N, the other on the hTF_C lobe, each Fe^{III} being bound to one N-atom from His, three O-atoms from one Asp and two Tyr residues, as well as to O-atoms from the carbonate anion, usually designated by 'synergistic anion' [31,32].

Transferrin is present in human blood plasma in ~37 μM concentration. In normal serum, only about 30% of the total binding sites are occupied by iron [32,33]; thus there are still sites available for other metal ions, without need to replace the strongly bound Fe^{III}. Vanadium, in the form of $V^{IV}O^{2+}$, can bind hTF and evidences indicate that this binding occurs mainly in the Fe-binding sites, although binding to histidine residues at the protein surface has also been suggested [15,17–28,31,34–38]. Recently, [39] geometry optimization calculations were carried out for the binding of $V^{IV}O^{2+}$ to the hTF_N lobe, indicating that in the presence of CO_3^{2-} or HCO_3^-, V^{IV} is bound to five atoms of the Fe-binding site in a distorted geometry. The ^{51}V (and ^{14}N) *A* tensors were also calculated by DFT methods and compared with the experimental values.

Abbreviations: hTF, human serum transferrin; Holo-hTF, holo-transferrin or di-ferric-hTF; Apo-hTF, apo form of hTF; hTF_N, N-terminal lobe of hTF; hTF_C, C-terminal lobe of hTF; CD, circular dichroism; ICP, inductively coupled plasma; MS, mass spectrometry.

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Globally, of all calculated $V^{IV}O$–hTF structures, the one that yielded lower calculated heats of formation and better agreement with the EPR data was the structure that includes CO_3^{2-} as synergistic anion (although the one with HCO_3^- cannot be ruled out). In this structure the V=O bond length is ~1.6 Å, and the V atom is also coordinated by the phenolate–O atom of Tyr188 (at ~1.9 Å), the aspartate-O of Asp63 (at ~1.9 Å), the His249 Nτ (at ~2.1 Å), and an $O_{carbonate}$ (at ~1.8 Å). The Tyr95 phenolic-O atom is at a long distance (~3.3 Å) from the V^{IV} center. All of the O atoms are able to establish dipolar interactions with groups of the protein.

It was demonstrated that when vanadium is administered in the form of a complex, *e.g.* $V^{IV}O(carrier)_2$, where carrier is an organic compound acting as a bidentate or tridentate ligand, often the doses needed to achieve the same therapeutic insulin-enhancing effect are significantly lower. Among the several $V^{IV}O$-complexes exhibiting insulin enhancing action, $V^{IV}O(maltolato)_2$ and $V^{IV}O(ethylmaltolato)_2$ have been extensively studied [18,40–44], as well as several $V^{IV}O(picolinato)_2$ and V^{V}-dipicolinato compounds [45]. The pyridinone, 1,2-dimethyl-3-hydroxy-4-pyridinone (Hdhp) has been used in the treatment of β-thalassaemia [42,46] and its insulin-like properties studied as well [47–51]. Long term *in vivo* insulin-enhancing properties of $VO(acac)_2$ (acac = acetylacetonato) and of acac derivatives in streptozotocin-induced diabetic Wistar rats were also reported [52].

Vanadium was found mostly to be bound to hTF even in the absence of carbonate [20,53], and either if it is supplied as $V^{IV}OSO_4$ or as a $V^{IV}O(carrier)_2$ complex, most evidences have been given to support that most of the vanadium in the serum is bound to hTF [15,17,18,22–28,34–36,53–55]. In particular, HPLC-ICP-MS studies made with blood serum samples indicated that vanadium is associated to the hTF fraction, irrespective of being introduced as $V^{IV}OSO_4$ or as a $V^{IV}O(carrier)_2$ complex [15].

In the case of $V^{IV}OSO_4$ most reports indicate that two vanadium ions are bound to apo-hTF at the Fe^{III} binding sites. The binding of $V^{IV}O$ to apo-hTF most certainly involves several amino acid residues of the Fe-binding site, and as concluded by urea gel electrophoresis experiments, the formation of $(V^{IV}O)_2$hTF species may occur with the closing of the hTF conformation as is the case in $(Fe^{III})_2$hTF [38], which is an essential feature for recognition by the transferrin receptor. If vanadium is introduced in blood in the form of a $V^{IV}O(carrier)_2$ complex, these carrier ligands and/or low molecular mass (lmm) bioligands may also participate in the transport of vanadium in blood [15,18,21–34,36,54–57], and two main types of binding have been proposed:

Type 1: the formulation $(V^{IV}O)$(hTF)(carrier) when the carrier is a synergistic anion, *cis*-$V^{IV}OL_2$(hTF) when it is not a synergistic anion and the V complex predominates in the octahedral *cis*-[$(V^{IV}O)(carrier)_2(H_2O)$] form at pH ~7.4 [22,24–26,56]. The formation of *cis*-$V^{IV}O(carrier)_2$(hTF) was explained with the replacement of the equatorial water molecule by an imidazole-N of an accessible His, or carboxylate-O^- of accessible Asp or Glu residues (presumably on the protein surface) with a nonspecific coordination similar to that of other proteins such as HSA and IgG (Fig. 1).

Type 2: in the systems containing $VO(carrier)_2$ (carrier = maltolate, 1-2-dimethyl-3-hydroxy-4(1*H*)-pyridinonato, picolinato, and pyrimidinonate derivatives) and hTF the formation of $(V^{IV}O)$(hTF)(carrier), $(V^{IV}O)_2$(hTF)(carrier) and $(V^{IV}O)_2$(hTF)$(carrier)_2$, not depending on the particular features of these carrier ligand (Fig. 1).

Concerning Type 1 binding, very probably it occurs. In fact some of us recently reported the characterization by X-ray diffraction of such a binding in the formation of $VO(picolinato)_2$-lysozime adducts [58]. In this complex, a carboxylate donor atom from the side group of Asp52 of lysozyme binds to V^{IV} and Asn46 interacts with the O_{oxido}, through an hydrogen bond.

Additionally, Sanna et al. [57] recently demonstrated, by EPR measurements, the interaction of $V^{IV}O^{2+}$ and of several insulin-enhancing $V^{IV}O(carrier)_2$ compounds with holo-hTF. It was shown that $V^{IV}O^{2+}$ can interact with e.g. surface sites of the protein, probably via the coordination of His-N, Asp-COO^- and Glu-COO^- donors; the residues of His-289, His-349, His-473, and His-606 were considered the most probable candidates for the complexation of the *cis*-$V^{IV}O(carrier)_2$ moieties studied. Since holo-hTF is recognized by the transferrin receptors, the formation of these complexes with holo-hTF may also be a way to transport vanadium compounds inside the cells.

However, it is questionable that this type of binding of a $V^{IV}O(carrier)_2$ to hTF justifies the high apparent binding constants determined for these type of species [20,25,26], even assuming that dipolar or hydrogen bond interactions of several atoms of $V^{IV}O(carrier)_2$ may be established with the protein. Moreover:

(i) It is normally considered that hTF binds two $V^{IV}O^{2+}$ ions [38,59–61]. If the relevant binding takes place at imidazole-N of surface His residues, as there are at least 12 histidines [62], there is no clear reason why hTF does not bind a significantly higher number of $V^{IV}O^{2+}$ ions.
(ii) If the binding is of type 1, it would be expected that hTF, HSA and IgG would all correspond to similar types of binding. However, although EPR spectra do not differ much, UV–Vis and circular dichroism spectra differ significantly. It is also known that HSA can bind more than two V^{IV}-centers in solutions containing vanadium-maltolato complexes [18,63,64], so why not also hTF?
(iii) The concentration of hTF in blood is ca. 37 μM, while that of HSA is ca. 630 μM and of IgG ca. 85 μM, and there are other proteins present. All these proteins have available N-imidazole atoms of His residues. However, it was found by HPLC-ICP-MS that in human serum blood samples the vanadium only binds to hTF [21,34]. Thus, the following question should be answered: 'what the His residues of hTF have so special that the $V^{IV}O^{2+}$ only binds to them, despite being present in much lower amounts than those of HSA or IgG'?

Thus, the issue of how V^{IV} is transported in the form of $V^{IV}O$-carrier complexes is not fully understood, as well as how relevant this is for up-take of vanadium by target cells. Additionally, vanadium in oxidation states +5 and +3 can also bind hTF [34,53,65,66], the binding of V^{III} to hTF being quite strong, probably almost as strong as that of Fe^{III} [20,34,53,66–68]. Not much attention has been given to the possibility of transport of vanadium in these oxidation states; thus, in this work we discuss aspects related to transport of vanadium in the oxidation states +5 and also +3.

2. Experimental

2.1. Buffer solutions

All measurements were carried out in aqueous buffered media. In some cases we included the major low molecular weight blood plasma constituents (e.g. citrate and lactate) in the buffer solutions used.

2.1.1. Hepes buffer

The composition of the Hepes buffer used is 50 mM Hepes (Sigma) and 25 mM carbonate added as $NaHCO_3$ (Sigma). This buffer system was adjusted to a pH of 7.4 using concentrated KOH. In some experiments $NaHCO_3$ was not added.

2.1.2. Hepes-S buffer

The composition of the Hepes-S buffer used is 50 mM Hepes (Sigma), 25 mM carbonate added as $NaHCO_3$ (Sigma), 1 mM phosphate added as $NaH_2PO_4{\cdot}H_2O$ (Merck) and 0.20 mM KCl (Merck).

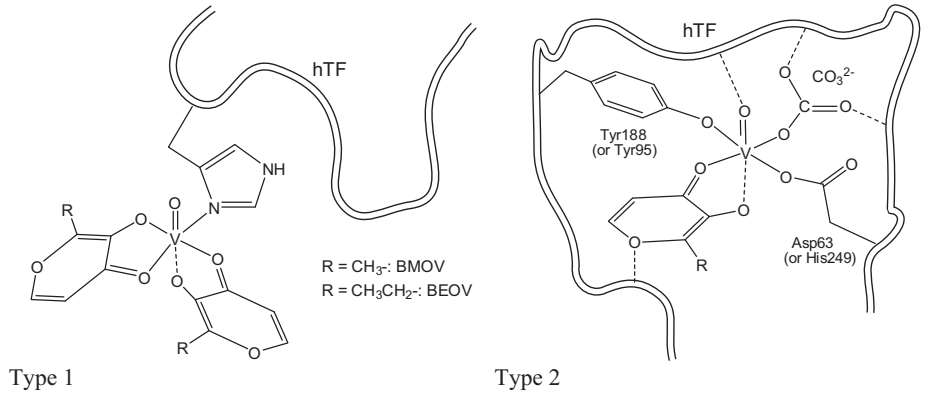

Fig. 1. Proposed binding of $V^{IV}O$-complexes to hTF. In the case of type 2 binding, the VO:carrier stoichiometry is 1:1; the actual donor atoms of the Fe-binding site that are coordinated to V^{IV} may be any two among the four: Asp-63, Tyr-95, Tyr-188, His-249. These two types of binding were recently designated by 'Description A' (type 2) and 'Description B' (type 1) by Sanna et al. [57].

This buffer system was adjusted to a pH of 7.4 using concentrated KOH.

2.1.3. Hepes-CL buffer

The composition of the Hepes-CL buffer used is similar to that of Hepes-S buffer, but it additionally contains 0.1 mM citrate and 1.5 mM lactate. This buffer system was adjusted to a pH of 7.4 using concentrated KOH.

2.1.4. Tris buffer

The composition of the Tris buffer used is 0.10 M Tris and 50 mM $NaHCO_3$. This buffer system was adjusted to pH 7.4 using concentrated HCl.

2.2. Metal transferrin solutions

Human apo-transferrin solutions were prepared by dissolving the protein (Sigma, T4382) in buffer at pH 7.4. The solutions were allowed to stand, gently swirled without strong agitation, for at least 1 h. The concentration of apo-transferrin solutions were determined by measuring the absorbance at 280 nm using an extinction coefficient of 92300 M^{-1} cm^{-1} [21,69,70]. Normally argon was bubbled through all solutions prior to use for measurements with V^{IV}-complexes to displace any oxygen that may be present, and the solutions were kept and manipulated under an argon atmosphere inside a glove bag.

Iron transferrin solutions were prepared by dissolving the Fe^{II} Mohr salt ($Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$) in the apo-hTF solution in buffer. The solution of Fe–hTF was kept for ca. 24 h so that Fe^{III} forms and binds to hTF yielding the Fe^{III}–hTF complex. For $(Fe)_2$–hTF an extinction coefficient of 113000 $M^{-1}cm^{-1}$ was used [71]. In some cases holo-hTF (Sigma, T4132) was used.

2.3. Desalting procedure with size exclusion columns

The solutions were prepared by adding (normally) an excess of molar equivalents of metal salts (5 to 10 times) to an apo-transferrin solution. These solutions were allowed to equilibrate from 1 to 24 h depending on the experiment. For solutions containing V^{IV} or V^{III} up to this point all manipulations were carried out in the absence of O_2, but the following steps were carried out allowing contact with air.

The samples were then passed through PD-10 MiniTrap Sephadex® G-25 exclusion columns (GE Healthcare) as described in [38]. The first component to elute from these size exclusion columns is the protein, which may contain (or not) bound metal ions.

The protein-metal solutions collected from these exclusion columns were used: (a) to determine the protein concentration by UV–Vis absorbance measurements, (b) to evaluate the metal content by ICP-AES, (c) gel electrophoresis experiments, (d) to do several spectroscopic measurements.

2.4. Materials and methods

2.4.1. Urea-polyacrylamide gel electrophoresis

The metal ligand binding status of the hTF was examined by urea gel electrophoresis using the Novex 6% tris(hydroxymethyl)aminomethane (Tris)–borate (TBE)–urea minigels and a XCell SureLock™ Mini-Cell (Invitrogen). Normally, 5 μL of sample was mixed with 5 μL of 2X Novex sample buffer and loaded in the gel. The electrophoresis was carried out for 3 h at 180 V voltage and 40 mA current. The protein bands were visualized by staining with Coomassie blue. It must be emphasized that in this work the sample and running buffers used were the Novex commercial buffers, including the presence of EDTA. In the presence of EDTA it was found that the electrophoretic results for solutions of hTF and V^{IV}-O-containing species differ from those obtained if it is not included [38].

2.4.2. Circular dichroism (CD)

The CD spectra were recorded with a JASCO 720 spectropolarimeter with quartz Suprasil® cells. In the 250–700 nm range the usual UV–Vis photomultiplier was used and 2 to 5 mm path length cells, while in the 400–1000 nm range 1, 2 or 5 cm path length quartz cells were used, and a red-sensitive photomultiplier (EXWL-308) was used as detector. For the 5 cm cells a special optical accessory system must be placed so that low-volume cells (sample volumes ≈ 500–700 μL) may be used in the spectropolarimeter.

2.4.3. Electron paramagnetic resonance (EPR)

The EPR spectra were recorded at 77 K (on glasses made by freezing solutions in liquid nitrogen) with a Bruker ESP 300E X-band spectrometer.

2.4.4. ICP

The quantification of metals was done by ICP-AES (inductively coupled plasma-atomic emission spectroscopy) analysis on a Horiba Jobin–Yvon apparatus, model Ultima. In ICP-AES metal concentration was determined in mg/L from calibration curves established on the same day.

3. Results and discussion

3.1. Systems containing hTF and V^{IV}/V^{V}

Many studies have indicated that $(V^{IV}O)_n$hTF species are susceptible to oxidation [55,70,72] and the presence of significant amounts (e.g. 1–3 mM) of glutathione, known to be a reducing agent for V^{V} [73,74], may delay this oxidation, but does not avoid it. It was also reported that oxidation proceeds faster in the presence of Fe^{III} [55]. This process could be more easily understood if V^{V}–hTF species had higher binding constants than V^{IV}–hTF species, but it is the opposite that is true. In order to get a better insight into the processes taking place, experiments were done by starting with solutions containing $V^{IV}OSO_4$ and hTF and adding $NaV^{V}O_3$, as well as starting from solutions containing $NaV^{V}O_3$ and hTF and adding $V^{IV}OSO_4$; CD, ^{51}V-NMR and EPR spectra were then recorded. Fig. 2 depicts some of the results obtained.

It is clear that the CD spectra of these solutions containing hTF, V^{IV} and V^{V} salts (spectra c–e) are quite distinct from the CD spectra of solutions containing hTF and V^{IV} salts (*e.g.* spectrum f, thus containing $(V^{IV}O)_n$hTF complexes). Remembering that V^{V} does not contain d electrons and no CD bands are expected to be observed in the visible range due to V^{V}–hTF species (see Fig. SI-1), the different spectra recorded for solutions containing $V^{IV}O$–hTF (spectrum f in Fig. 2) and when V^{V} is also present can only be due to the presence of distinct V^{IV}–hTF species from those existing in solutions only containing the $(V^{IV}O)_n$hTF complexes. One possibility is the presence of V^{V} close to the V^{IV}–hTF complexes, so that the chiral environment around V^{IV} is changed. Another possibility is the formation of mixed-valence (i.e. V^{IV}/V^{V}) species bound to chiral hTF residues. The appearance of bands with λ_{max} of ca. 900–1000 nm could be an indication for the presence of mixed-valence complexes.

The EPR spectra of these solutions were recorded, showing some small difference from those of the $(V^{IV}O)_n$hTF complexes (see Fig. SI-2). However, as they do not differ much, particularly the g_z and A_z parameters obtained (Table 1) are very similar. Thus, probably the binding set of the new V^{IV}–containing species present may be involving similar donor groups of hTF residues.

The ^{51}NMR spectra recorded for the same solutions yielded resonances with chemical shifts very similar to those of solutions containing $NaV^{V}O_3$ and hTF, no new distinct peaks being detected except those corresponding to V_1 (Fig. SI-3). Thus, if new V^{V}–hTF species form, they are not detected by ^{51}NMR spectroscopy.

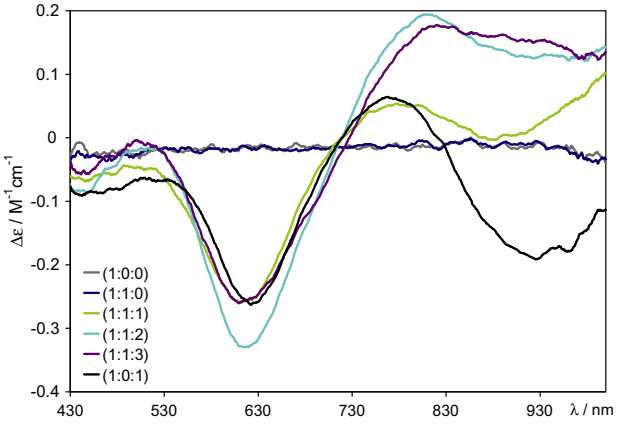

Fig. 2. CD spectra of a solution containing (a) hTF (750 μM), labeled (1:0:0); (b) hTF-$NaV^{V}O_3$ (1:1:0) in Hepes-CL buffer at pH 7.4 and of this solution upon progressive additions of $V^{IV}OSO_4$, so that the following hTF:V^{V}:$V^{IV}O$ molar ratios were obtained (c) (1:1:1), (d) (1:1:2), (e) (1:1:3). (f) The spectrum corresponding to hTF:V^{V}:$V^{IV}O$ molar ratios (1:0:1), obtained in similar conditions is also included. A 50 mm optical path quartz cell was used. The spectra recorded have similar pattern, but are not equal, if the initial solution is *e.g.* hTF-$NaV^{V}O_3$ (1:2:0).

Table 1
Spin Hamiltonian parameters (g_z and A_z) obtained by simulation [75] of the experimental spectra (recorded at 77 K) for the species formed in the system $V^{IV}O$-apo-hTF–V^{V} (aqueous solution in Hepes-CL buffer at pH 7.4). A* and B* designate species in the presence of V^{V}.

Species	g_z	$A_z/\times10^4$ cm^{-1}
A	1.938	167.7
A*	1.937	168.2
B	1.940	170.7
B*	1.940	171.1

3.2. Systems containing V^{V} and hTF

The direct competition observed between phosphate, bicarbonate and sulfate with vanadate, as well as the intense UV difference spectrum due to binding of these anions to apo-hTF was considered [61] to indicate that sulfate, phosphate, bicarbonate and vanadate anions are interacting with the same phenolic-O atoms. Furthermore, it was reported that binding of these anions is blocked in holo-hTF. A binding model was proposed which involves hydrogen bonding of the anions both to Arg residues and to the phenolic H atoms of the Tyr residues of the Fe-binding site (and possibly also Lys residues) [61]. The redox kinetics and complexation of V^{V} and V^{IV} with albumin and transferrin in serum, were also studied and the formation of a specific V^{V}:hTF (2:1) was confirmed [70]. EPR data revealed that the presence of reducing agents in fresh serum quantitatively converts V^{V} to V^{IV}. Half-lives for air oxidation of V^{IV}–hTF complexes and for the reduction of V^{V} by endogenous reducing agents in serum were determined to be in the range 5–30 min at pH 7.5. Thus, the inter-conversion between oxidation states may be considered rapid relative to the normal residence time of vanadium in the circulation [70]. This and other data suggested that vanadium may be present in both oxidation states in the bloodstream and carried by transferrin alone.

Apo-hTF was reported to be able to bind two equivalents of V^{V} in the presence or absence of the synergistic carbonate anions, which is necessary for the coordination of other metal ions [73]. As this was determined from difference UV spectra (removing the absorbance due to free vanadate), this should measure mainly V–hTF species formed close to aromatic residues, as several of those belonging to the Fe-binding site. Thus, V^{V} has been considered to occupy the same pockets as Fe^{III}. The molar differential absorbance of V^{V} coordinated to apo-hTF, measured by UV–Vis spectroscopy, suggested the deprotonation of the tyrosine side-chains, and this was considered to indicate the cationic coordination mode of V^{V} as VO_2^+ [61]. The lack of the synergistic anion was considered to suggest that all the coordination positions of the V^{V} are occupied, the two oxido groups preventing the V^{V}–carbonate interaction. The fact that the formation constants of V^{V}–hTF binding are ~3–4 orders of magnitude higher than those with inorganic anions (phosphate, sulfate, hydrogencarbonate) [23,61] was also taken as evidence of the non-anionic interaction of the binding.

Because apo-hTF does not absorb visible radiation and as V^{V} has no d-electrons, in solutions containing vanadate and apo-hTF at pH 7.4 no electronic bands show up in the visible range for $\lambda > 400$ nm (see *e.g.* Fig. SI-1). Only bands due to *e.g.* charge-transfer transitions may be recorded if intense enough. In the near UV range, several bands may be detected due electronic transitions of aromatic residues of hTF [39]. Upon additions of the solution of $NaV^{V}O_3$ the CD spectra show significant changes (see Fig. 3), much more important than in similar experiments by addition of $V^{IV}OSO_4$ [38,55]. It is not

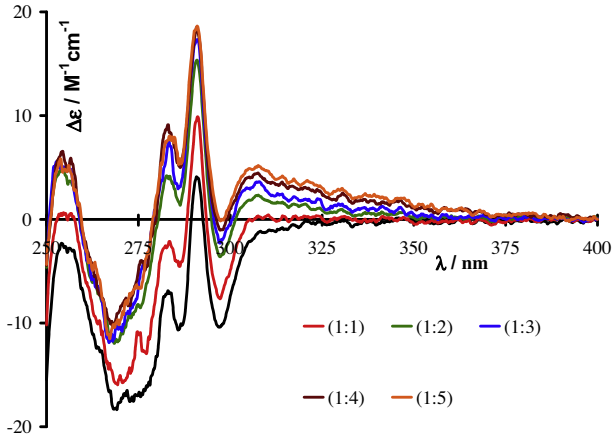

Fig. 3. CD spectra of a solution of apo-hTF (100 μM) in Hepes-CL buffer at pH 7.4 before (black spectrum) and after progressive additions of a $NaV^{V}O_3$ solution (apo-hTF:V ratios indicated). A 2 mm optical path quartz cell was used.

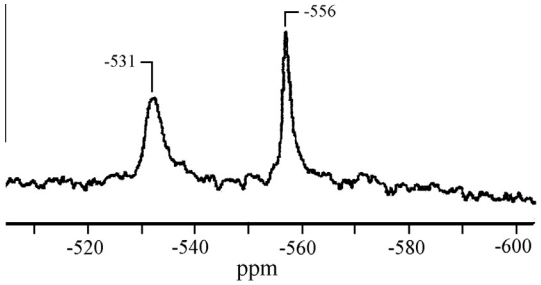

Fig. 4. ^{51}V NMR spectra of a solution containing apo-hTF (*ca.* 0.60 mM), $NaV^{V}O_3$ (*ca.* 1.2 mM), in buffer Hepes, without carbonate, at pH 7.4. The resonance at *ca.* −531 ppm corresponds to the formation of V^{V}-hTF (probably mostly V^{V}-hTF_N) and the one at *ca.* −556 ppm corresponds to the free V^{V} in solution ($H_2VO_4^-/HVO_4^{2-}$, often designated by V_1).

clear why these changes occur (see also below), but they are the result of interaction of V^{V}-species with chiral centers from hTF. The bands at ca. 300–350 nm are probably due to phenolate-O^- to V^{V} charge transfer transitions. Harris [61] proposed the binding of V^{V} close to the Fe–hTF binding sites.

The binding of V^{V} to apo-hTF may also be detected by ^{51}V NMR. The two V^{V} binding sites are characterized by ^{51}V NMR chemical shifts at −529 and −531 ppm and these shifts were assigned to the C- and N-terminal sites [77]. The resonance at −529 ppm is observed as a shoulder of the −531 ppm band, but normally it is not clearly visible. At relatively high V^{V} concentrations and at high ratios of V^{V}/hTF, conditions under which it is expected that a large fraction of the V^{V} is present as V-oligomers, the ^{51}V resonance of hTF-bound V^{V} is not observed, possibly owing to interference by the oligomeric species [77]. Under conditions of tight V^{V} binding, the sharpness of the protein-bound resonances is a consequence of the motional characteristics of hTF, which place the hTF-bound V^{V} outside the extreme narrowing limit but within the motional narrowing limit [77].

Fig. 4 depicts ^{51}V NMR spectra of a solution containing apo-hTF and $NaV^{V}O_3$ in a Hepes buffer without carbonate. The resonance at −531 ppm corresponds to V^{V} bound to apo-hTF, and the one at −556 ppm to monovanadate ($V_1 = H_2VO_4^-/HVO_4^{2-}$). It is thus clear that the V^{V}–hTF resonance can be detected in solutions in the absence of carbonate.

Another interesting aspect is that gel electrophoresis data indicate that V^{V} bound to hTF does not yield the 'closed conformation', as is the case with $(V^{IV}O)_2$hTF and $(V^{III})_2$hTF (see below).

Knowing that carbonate anions are necessary for the binding of most metal ions (but not for V^{V}), that at pH 7.4 in dilute solution V^{V} is present in solution as $H_2VO_4^- + HVO_4^{2-}$ and that $(V^{V})_2$hTF does not yield the closed conformation, several questions may then be raised:

a) if vanadate may act as a 'synergistic anion' or even 'if it may substitute carbonate' in the binding of other metal ions.
b) if indeed it is the VO_2^+ cation that is bound to hTF, or if it is $H_2VO_4^-$ or HVO_4^{2-}.
c) if the binding is at the Fe-binding site, or if simply involves one/two residues from this site, as suggested previously (see above [61]).

3.3. Solutions containing hTF and vanadate and/or Al^{III}

The binding of Al^{III} to hTF at the Fe-binding sites is much stronger that of V^{V}. In fact the $\log K_1$ (13.5) and $\log K_2$ (12.5) [78] are several orders of magnitude higher than those corresponding to V^{V}: $\log K_1$ (6.0) and $\log K_2$ (5.5) [23]. Fig. SI-5 depicts CD spectra of solutions containing apo-hTF and $AlCl_3$ in the presence or absence of V^{V}, before and after passing the solutions through desalting size exclusion columns. Fig. SI-5A depicts CD spectra of solutions containing (1) apo-hTF at pH 7.4, of (2) apo-hTF + Al^{III} (1:5 M ratio), and (3) of a solution containing apo-hTF + Al^{III} + V^{V} (1:5:5 M ratios). Fig. SI-5B depicts CD spectra of the same solutions after passing them through desalting size exclusion columns.

It can be seen that the CD spectra (4), (5) and (6) almost coincide in this λ range. Even interacting with hTF and apparently depicting bands at 400–600 nm, it doesn't yield a clear induced CD spectrum. Thus, in the spectrum obtained with solutions (4 and 5) no interaction of V^{V} with hTF is clearly detected by CD, although before passing the solution through the exclusion column a positive CD band is detected at ca. 300–350 nm (spectrum 3 in Fig. SI-5A). Possibly these bands are due to V^{V}–hTF species that no longer exist after passing through the exclusion column, presumably because the binding is not strong enough.

Although it is expected that the solution contains $(Al^{III})_2$hTF, with both Fe-binding sites occupied by Al^{III} ions, a significant amount of V was analyzed by ICP in the sample corresponding to spectrum 6 in Fig. SI-5B, corresponding to a V:Al molar ratio of 0.5:2, thus indicating that V^{V} is also bound to hTF. With a solution containing apo-hTF + Al^{III} + V^{V} (1:5:2 M ratio, C_{hTF} = 0.75 mM), resonances at δ_V = −531 ppm, corresponding to V^{V}–hTF species could also be clearly detected (Fig. SI-4). As it is not expected that in these experimental conditions V^{V} binds at Fe-binding sites displacing Al^{III}, either vanadate is acting as a 'synergistic anion' in the binding of Al^{III} to apo-hTF, or it is binding at a distinct site, close to the Fe-binding site.

3.4. Solutions containing hTF and vanadate and/or Fe^{III}

The binding of Fe^{III} to hTF at the Fe-binding sites is also much stronger that of V^{V}. In fact the $\log K_1$ (21.5) and $\log K_2$ (20.5) [78] are many orders of magnitude higher than those corresponding to V^{V}. Fig. 5 depicts CD spectra of solutions containing apo-hTF at pH 7.4 and after additions of Fe and/or V^{V}, and of solutions of holo-hTF and after additions of Fe and/or V^{V}, before and after the solutions were passed by exclusion columns. The spectra are all quite similar, except for some differences for $\lambda > 550$ nm; it is not clear why significantly distinct CD signals are recorded in this range.

The amounts of Fe and V were also analyzed in these samples by ICP: significant amounts of V were obtained, lower in the solution of holo-hTF + V^{V} (obtained Fe:V molar ratios of 2:0.06), than for apo-hTF + Fe + V^{V} (obtained Fe:V molar ratios of 2:0.11), and higher for solution of holo-hTF + Fe + V^{V} (obtained Fe:V molar ratios of

2:0.29). Thus a small but significant amount of V^V is also present in solutions expected to contain $(Fe^{III})_2$hTF, as described above for solutions containing $(Al^{III})_2$hTF. A small amount of V^V, up to ca. 2:0.14 (Fe:V) was also estimated by UV difference spectroscopy in solutions containing $(Fe^{III})_2$hTF by Harris and Carrano [76].

Fig. 6 depicts CD spectra of solutions containing hTF and Fe^{III} (molar ratio of hTF:Fe^{III} of 1:0.6, thus ~30% of the Fe-binding sites should be occupied by Fe^{III}) and after progressive additions of NaV^VO_3. The CD bands at ca. 310–360 nm that show up in the presence of V^V are due to the binding of V^V to hTF, probably involving residues belonging or close to the Fe-binding sites. Binding of V^V to hTF was confirmed also by ^{51}V NMR due to the presence of the $\delta_V = -531$ ppm band, but no new resonance was detected.

Recently it was reported that $V^{IV}O^{2+}$ may bind to holo-hTF; the confirmation of the binding of a small amount of V^V to holo-hTF may also be relevant for the up-take of vanadium by cells. Whether vanadate is acting as a synergistic anion, partially substituting carbonate, or is bound in a distinct site, cannot be predicted at this stage.

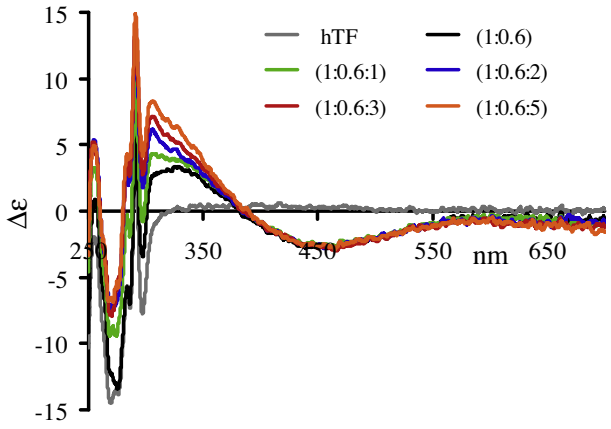

Fig. 6. CD spectra in the 250–700 nm range of solutions containing (1) apo-hTF (100 μM) in Hepes-S buffer at pH 7.4, (2) after addition of iron (hTF:Fe^{III} molar ration of 1:0.6) and equilibration for several hours, and after progressive additions of NaV^VO_3 so that the indicated hTF:Fe^{III}:V^V are obtained. The spectra were recorded using a quartz cell of 2 mm optical path.

3.5. Solutions containing hTF and V^{III}

The binding of V^{III}, V^{IV}, and V^V to hTF in blood serum samples was studied using anion-exchange chromatography directly connected to a high-resolution inductively coupled plasma-mass spectrometer for vanadium-51 detection. [15,34,53,66]. Namely it was stated that, in affinity to hTF, the three metal ions were ranked $V^{III} > V^{IV} > V^V$ in the presence of carbonate and $V^{III} \approx V^{IV} > V^V$ in its absence. V^{IV} and V^V were bound to the N-lobe site in the "closed form" and "open form," respectively, but in the absence of carbonate the hTF assumes the 'open' form [53].

No studies have been reported on the determination of V^{III}–hTf binding constants. However, based on the correlation of the first binding constant of hTF for divalent and trivalent metal ions with that for hydroxide binding, we predicted for V^{III} a value of *ca.* $\log K_1 = 20 \pm 1.5$ [20].

Beside the work of Bertini et al. [67,68], to our knowledge no other publication reported spectroscopic studies of V^{III}-complexes with hTF. In contrast with the V^{III}-species formed with human lactoferrin [65], which are rapidly oxidized by O_2, the $(V^{III})_2$hTF complex was reported to be quite stable to atmospheric O_2. The absorption spectrum of $(V^{III})_2$hTF showed a band at *ca.* 375 nm, assigned to a tyrosinato-O to metal charge transfer transition, and d–d bands at 444 and 637 nm. The $(V^{III})_2$hTF complex was reported to be extremely stable to dialysis against water or buffer solutions, and was not easily substituted by Fe^{III} [67,68]. A few additional studies involving V^{III}–hTF complexes have been determinations either by HPLC-ICP-MS and gel electrophoresis with solutions containing apo-hTF and V^{III} [34,53], or HPLC-ICP-MS and MALDI-TOF determinations with solutions containing apo-hTF and V^{III}, as well as human blood serum samples incubated with $V^{III}Cl_3$ or $V^{IV}OSO_4$ [53,66]. In all cases the results showed that both V^{III} and V^{IV} associate to hTF with the synergistic effect of carbonate ions.

3.6. Gel electrophoresis studies

To compare the binding pattern of vanadium and iron ions with transferrin, urea-gel electrophoresis experiments were carried out. To ensure complete metal ligand binding, solutions containing an excess of metal ions were prepared, and later samples collected after passing them through exclusion columns, were used in gel

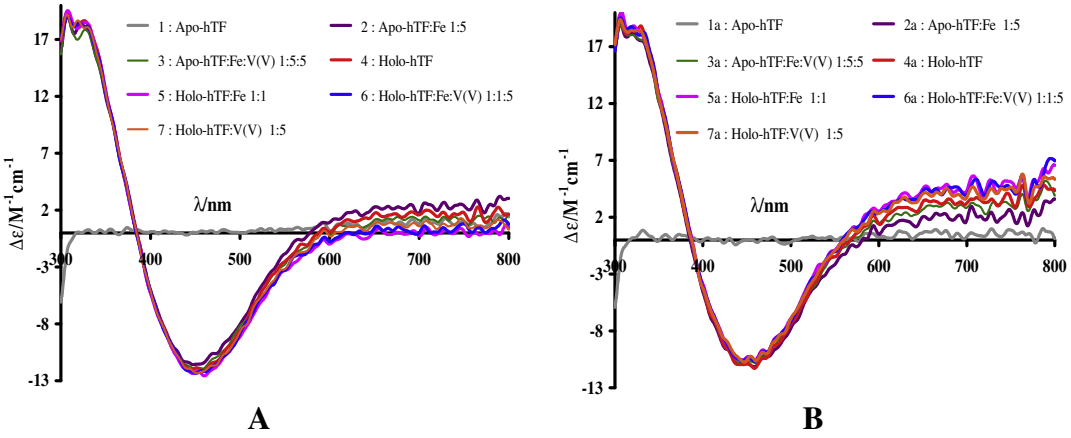

Fig. 5. CD spectra in the 300–800 nm range of solutions at pH 7.4 containing (1) apo-hTF (~150 μM) in tris buffer (containing 25 mM HCO_3^-/CO_3^{2-}), (2) the same solution after addition of $AlCl_3$ (hTF:Al molar ratio of 1:5), (3) solution of (1) after additions of $AlCl_3$ and NaV^VO_3 (hTF:Al:V molar ratios of 1:5:5). (4) holo-hTF (~150 μM) in tris buffer (containing 25 mM HCO_3^-/CO_3^{2-}), (5) the solution of (4) after addition of $FeCl_2$ (holo-hTF:Fe molar ratio of 1:1), (6) solution of (5) after addition of NaV^VO_3 (holo-hTF:Fe:V molar ratios of 1:1:5), (7) solution of (4) and addition of NaV^VO_3 (holo-hTF:V molar ratios of 1:5). Solutions of (1–7) were incubated for ca. 24 h and then the spectra of Fig. 5A were run. Then each solution was introduced into a Sephadex PD-10 exclusion column, eluted with tris buffer and the corresponding spectrum recorded; the corresponding spectra are depicted in Fig. 5B, where the concentrations of either hTF or holo-hTF are ~110 μM.

electrophoresis. Binding of metal ions to transferrin depends on several factors such as pH, temperature, etc. It has therefore been proposed that after loading to urea gels, during electrophoresis fully Fe^{III}-saturated hTF also gets denatured to some extent and releases some iron from the C and/or N-terminals, and give bands for all forms. In the denaturation of the mono-ferric hTF complexes the unfolding of the iron-free domain is assumed to be responsible for the decrease in electrophoretic mobility, since the di-ferric protein complexes appear to be quite resisting to unfolding.

It is clearly indicated from the electrophoresis picture (Fig. 7) that the binding pattern of V^{III}–hTF and Fe^{III}–hTF are quite similar and differ from those of V^{IV}–hTF (see note below) and V^{V}-hTF. This should be due to the similar closed configuration of transferrin upon binding with V^{III} or with Fe^{III}. So these results indicate that upon binding of V^{III} to hTF, the protein is converted into the closed form similar to that existing with holo-transferrin. Similarly to the case of fully saturated Fe^{III}–hTF we found bands for all forms of transferrin, namely C-mono-vanadium–hTF, N-mono-vanadium-hTF, and di-vanadium-hTF.

On comparison of commercially available holo-hTF from Sigma and fully saturated holo-hTF prepared in the lab, it was found that both gave similar gel-electrophoresis bands.

Note: The loading and running buffers used to carry out the experiment of Fig. 7 is the commercially available one and contain EDTA. In these conditions the $V^{IV}O^{2+}$ ions bound to hTF are sequestered by EDTA and what is presented in lane C corresponds mostly to apo-hTF. If the same experiment is done with similar buffers but not containing EDTA, a pattern of electrophoretic bands similar to that of holo-hTF is found. With V^{V}–hTF the pattern of bands is similar to that of apo-hTF either if EDTA is present or not. These observations confirm that the binding of hTF to V^{III} is very strong, and that even EDTA, present in relatively high amounts cannot remove V^{III} from $(V^{III})_2$hTF.

To evaluate the concentration of hTF and metal ions in the samples loaded to the electrophoretic lanes, UV and ICP-AES measurements were carried out with those solutions. The results are depicted in Table 2. As the hTF concentration cannot be accurately determined in these samples, the V/hTF molar ratios obtained in the samples after passing the exclusion columns should be considered approximate values.

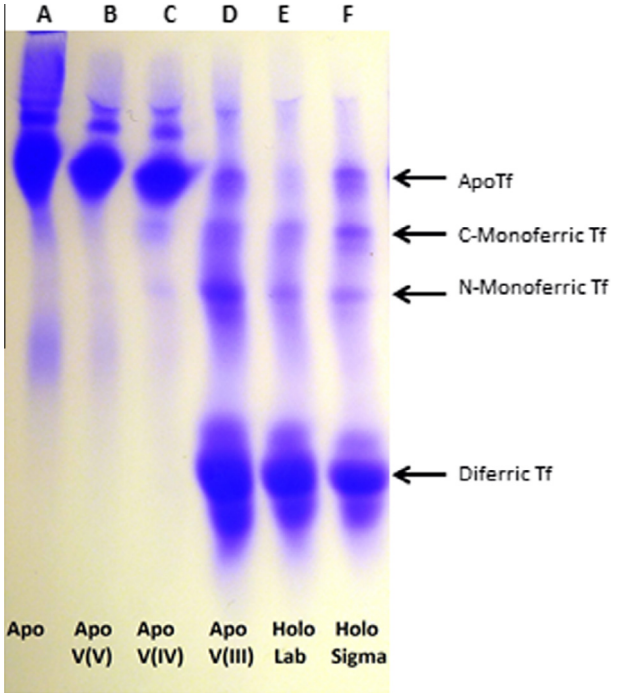

Fig. 7. Gel picture of apo-hTF with vanadium and Fe^{III} ions (A) apo-transferrin, (B) apo-transferrin-V^{V}, (C) apo-transferrin-V^{IV}, (D) apo-transferrin-V^{III}, (E) apo-transferrin-Fe^{III} (F) holo-transferrin from Sigma.

3.7. Circular dichroism

In a container placed inside a glove bag purged with argon, a solution of apo-hTF (C_{hTF} ~400 μM) was prepared in Hepes-S buffer, and carefully purged with argon. Solid VCl_3 was added so that upon dissolving the vanadium concentration is ~0.80 mM. It was observed that the CD spectra for $\lambda > 550$ nm (Fig. 8) change with time; initially it includes bands of V^{III}–hTF and V^{IV}–hTF (formed immediately after addition of VCl_3 to the buffered solution of apo-hTF), but after 24 h the bands due to V^{IV}O–hTF disappeared. This suggests that after *ca.* 24 h the V^{IV}O-species present were converted to V^{III} (the bands due to V^{IV}O–hTF are no longer present). The EPR spectrum of the solution after 1 h of addition of VCl_3 depicts signals corresponding to an appreciable amount of V^{IV}O-species (Fig. 9). However, after 24 h of gently stirring the solution, keeping it inside the glove bag purged with argon, the EPR signal due to V^{IV}O-species disappeared and no V^{V}-species were detected in this solution by ^{51}V NMR spectroscopy. This suggests that all vanadium is in the form of V^{III} bound to hTF.

Globally it was also observed that CD spectra of solutions containing V^{III}–hTF species are quite intense compared to those of solutions containing $V^{IV}OSO_4$ and apo-hTF; the pattern of the CD bands of $(V^{III})_2$hTF is also different from that of $(Fe^{III})_2$hTF.

It is known that in aqueous solution at pH 7.4 V^{III}-species are normally not stable to oxidation. It is also known that in solutions containing $V^{IV}OSO_4$ and apo-hTF so that there is enough hTF to bind all V^{IV} present, the V^{IV} is progressively oxidized to V^{V}. Complexes $(V^{III})_2$hTF are very stable, and as stated above, its 1st binding constant is ~10^{21}. Thus it is possible that in solutions already containing a relatively high amount of V^{III}–hTF species and a low

Table 2
Concentration determination of metal bounded transferrin by UV and metal ions by ICP-AES analysis. The concentration of hTF was estimated by UV, being ~92 μM.

Sample	V Conc. by ICP (μM)	Ratio (V/hTF)
hTF + V^{V}	0.239	~2.6
hTF + V^{IV}	0.196	~2.1
hTF + V^{III}	0.254	~2.8

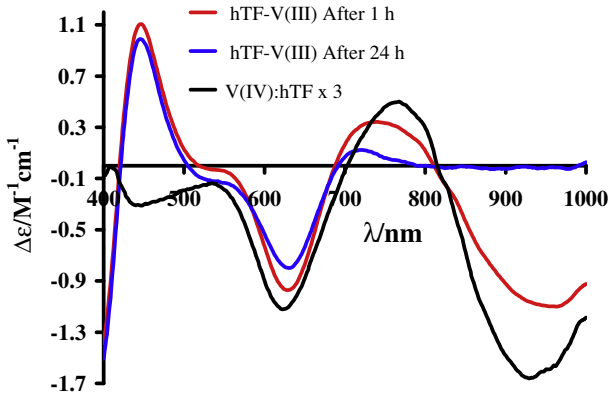

Fig. 8. CD spectra of a solution of apo-hTF (C_{hTF} ~400 μM) in Hepes-S buffer, purged with argon, to which solid VCl_3 was added (apo-hTF:V molar ratio of 1:2). The CD spectra for $\lambda > 550$ nm change with time (see text). The spectrum of a solution containing hTF and $V^{IV}OSO_4$ (1:2 molar ratio) is also included for comparison, but the corresponding $\Delta\varepsilon$ values are multiplied by 3.

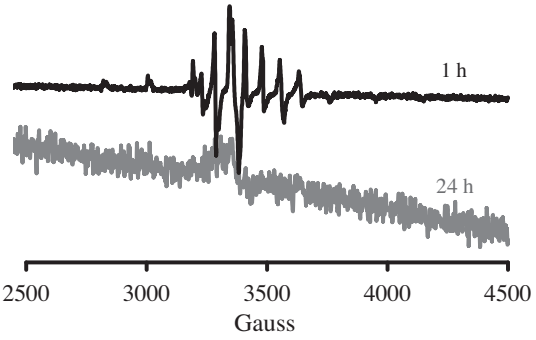

Fig. 9. 1st derivative X-band EPR spectra of a solution in Hepes-S buffer containing apo-hTF (C_{hTF} ~400 μM) and V^{III} ($C_V \approx 800$ μM) at pH 7.4, after 1 h and 24 h of addition of solid VCl_3.

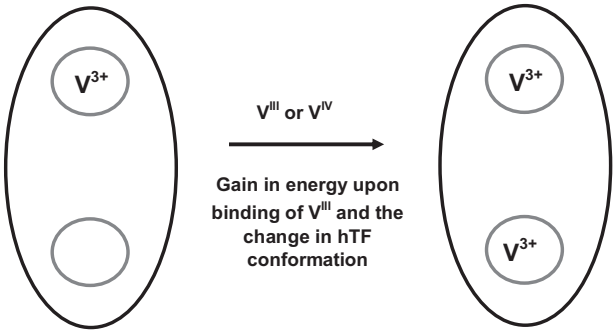

Fig. 10. Schematic explanation of the reduction process where the driving force is the formation of the highly stable $(V^{III})_2$hTF.

amount of V^{IV}–hTF, the high stability of $(V^{III})_2$hTF possibly drives the reduction of V^{IV} to V^{III} so that $(V^{III})_2$hTF, corresponding to the closed conformation, may be formed (see Fig. 10). Once $(V^{III})_2$hTF is formed, it is quite stable to oxidation, as previously reported by Bertini et al. [67,68], and *e.g.* electrophoretic experiments can be carried out without significant oxidation of the hTF bound V^{III}. It is feasible that in blood serum a similar situation might occur in the presence of Fe^{III} bound to hTF, *i.e.* the possible formation of hTF bound simultaneously to one Fe^{III} and one V^{III}, forming what may be designated by (Fe^{III}, V^{III})hTF. This probably corresponds to a 'closed conformation' of hTF, should be quite stable and its formation is plausible.

4. Conclusions

The knowledge on the binding of vanadium ions to serum proteins and how it might be transported in blood and up-taken by cells had very significant progresses in the last decade, particularly as far as transport of $V^{IV}O^{2+}$ is concerned. In this work we outlined and discussed the most relevant aspects of previous research, namely the two main types of binding proposed for transport of $V^{IV}O(carrier)_2$ complexes.

Besides absorption in the gastro-intestinal track, the up-take of vanadium by cells may possibly be the crucial step determining the efficacy of insulin enhancing vanadium compounds. Recent relevant observations are that $V^{IV}O^{2+}$ may bind to holo-hTF [57] and that $(V^{IV}O)_2$hTF probably corresponds to a closed conformation of hTF, although much less stable than that of $(Fe^{III})_2$hTF or $(V^{III})_2$hTF [38]. Previous studies by HPLC-ICP-MS gave contradictory conclusions: in one case it was considered that $(V^{IV}O)_2$hTF yields the closed conformation of hTF [53] and in another one hTF was considered to be in the open conformation [34].

Human serum transferrin is accepted as the main vanadium transporter in blood serum and recent research has mainly focused on $V^{IV}O^{2+}$, the understanding of the binding of vanadium in the oxidation states +5 and +3 having been must less explored. In this work we discussed several aspects concerning the probable relevance of binding of vanadate ions to apo-hTF and holo-hTF. It is possible that a small amount of vanadate may act as a synergistic anion or bind close to the Fe-binding site; thus besides the possibility of up-take of vanadate by cells through phosphate channels, its up-take through holo-hTF endocytosis cannot be ruled out.

It is also confirmed that V^{III} binds strongly to hTF and $(V^{III})_2$hTF corresponds to a 'closed conformation' similarly to holo-hTF. Whether $(V^{III})_2$hTF or (Fe^{III} V^{III})hTF complexes may form or not in blood serum, thus having the possibility of being up-taken by receptor-mediated endocytosis, being a relevant mechanism of vanadium up-take by cells, is a subject that needs and deserves further research to be fully clarified.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ica.2013.11.025.

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