Inorganic Chemistry

ARTICLE

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Interaction of VO^{2+} Ion and Some Insulin-Enhancing Compounds with Immunoglobulin G

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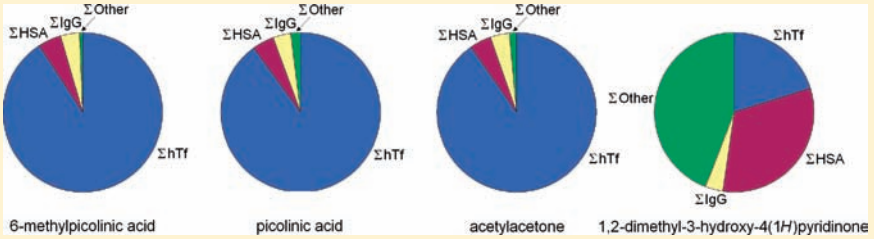
Supporting Information

ABSTRACT:



Complexation of VO^{2+} ion with the most abundant class of human immunoglobulins, immunoglobulin G (IgG), was studied using EPR spectroscopy. Differently from the data in the literature which report no interaction of IgG with vanadium, in the binary system VO^{2+}/IgG at least three sites with comparable strength were revealed. These sites, named 1, 2, and 3, seem to be not specific, and the most probable candidates for metal ion coordination are histidine-N, aspartate-O or glutamate-O, and serinate-O or threoninate-O. The mean value for the association constant of $(VO)_x$IgG, with $x = 3-4$, is $\log \beta = 10.3 \pm 1.0$. Examination of the ternary systems formed by VO^{2+} with IgG and human serum transferrin (hTf) and human serum albumin (HSA) allows one to find that the order of complexing strength is hTf ≫ HSA ≈ IgG. The behavior of the ternary systems with IgG and one insulin-enhancing agent, like $[VO(6\text{-mepic})_2]$, *cis*-$[VO(pic)_2(H_2O)]$, $[VO(acac)_2]$, and $[VO(dhp)_2]$, where 6-mepic, pic, acac, and dhp indicate the deprotonated forms of 6-methylpicolinic and picolinic acids, acetylacetone, and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, is very similar to the corresponding systems with albumin. In particular, at the physiological pH value, VO(6-mepic)(IgG)(OH), *cis*-$VO(pic)_2$(IgG), and *cis*-$VO(dhp)_2$(IgG) are formed. In such species, IgG coordinates nonspecifically VO^{2+} through an imidazole-N belonging to a histidine residue exposed on the protein surface. For *cis*-$VO(dhp)_2$(IgG), $\log \beta$ is 25.6 ± 0.6, comparable with that of the analogous species *cis*-$VO(dhp)_2$(HSA) and *cis*-$VO(dhp)_2$(hTf). Finally, with these new values of $\log \beta$, the predicted percent distribution of an insulin-enhancing VO^{2+} agent between the high molecular mass (hTf, HSA, and IgG) and low molecular mass (lactate) components of the blood serum at physiological conditions is calculated.

INTRODUCTION

Vanadium compounds exhibit a wide variety of pharmacological properties, and many complexes have been tested as antiparasitic, spermicidal, antiviral, anti-HIV, antituberculosis, and antitumor agents.[1] Among the metal ions with insulin-like effects, such as Mo, Zn, etc., vanadium has been proved to be one of the most efficient;[2] therefore, one of the most important potential applications of vanadium compounds is their use in the therapy of patients suffering from type II diabetes mellitus (DM), which affects worldwide 150 million people according to estimates of the World Health Organization.[3] Since type II diabetes goes along with an increasing lack of response to insulin, insulin injections can become ineffective and alternative methods of treatment are desirable. About 30 years ago, the insulin-enhancing action of vanadate was demonstrated and highlighted with respect to the possibility of oral administration.[4] After the initial use of vanadium(IV) and vanadium(V) inorganic salts, not easily applicable as antidiabetic drugs for their toxicity and very low absorption rate, several peroxovanadium(V) complexes with (N,N), (N,O), and (O,O) ligands[5] and neutral vanadyl species (VOL_2, where L is called organic carrier) with bidentate anionic ligands were tested;[2,6] the latter were found to be more effective in lowering the glucose concentration in blood serum than $VOSO_4$ and are well tolerated in all animal models of diabetes.[3] Generally, vanadium species stimulate the uptake and degradation of glucose by adipocytes (fat cells), glycogenesis in the liver, and inhibition of hepatic gluconeogenesis.[4]

The mechanism of action of vanadium compounds seems to be connected to the phosphate-like activity and/or interference with phosphatases[7] and mainly to the inhibition of protein tyrosine phosphatase (PTP), which is responsible of the

Received: January 14, 2011
Published: March 24, 2011

inhibition of the signal transduction pathways and of glucose transport into the cells in the absence of insulin.[2a,6,8] Instead, the active oxidation state, vanadium(IV) or vanadium(V), and the form with which an insulin-enhancing compound interacts with the cell in the target organs are not fully known. In vivo blood circulation monitoring-electron paramagnetic resonance (BCM-EPR) studies on rats showed that, almost independently of the initial oxidation state of the vanadium compound, the metal ion is transported in the blood in oxidation state +IV,[9] which can be further stabilized by interaction with the bioligands present in the bloodstream.

The conditions used in the experiments involving cell lines modeling diabetes can differ considerably from those existing in the living systems, where several biomolecules with high affinity for vanadium can partly or fully displace the original organic carrier during absorption in the gastrointestinal tract and the transport processes in the bloodstream and upon reaction with the intracellular components. Accordingly, the original carrier might be lost in these processes and the real biological/physiological activity might be due to a different chemical species.[1] In this context, one of the most important aspects to understand the action of a metallodrug is the form and efficiency of transport of the metal ion or of a complex in the blood serum. Moreover, more detailed knowledge of the biospeciation and pharmacokinetics of metallodrugs may promote planning for developing better compounds in the future.

The blood plasma contains ca. 90% water, the rest being dissolved substances, such as albumin, immunoglobulins, fibrinogen, glucose, sodium chloride, and many other components such as transferrin, carbonate, amino acids, vitamins, urea, phosphate, lactate, citrate, etc.[1] The exact composition of the blood was reported by Harris;[10] the concentration range of the most abundant class of immunoglobulin, immunoglobulin G (IgG), is 7.7−20 mg/mL, which corresponds to a mean value of 84 μM.[11] To our knowledge, the most probable bioligands candidates for VO^{2+} binding in the blood serum are human serum albumin (HSA), lactate (lact), citrate (citr), and, above all, human serum transferrin (hTf).[12] It has been demonstrated that vanadium binds to both HSA and hTf proteins, although the binding is approximately 1000 times stronger to hTf than to HSA.[12−16]

The form with which an insulin-enhancing, kinetically labile, vanadium compound is transported in blood serum depends on the thermodynamic stability of the bis chelated complex and on its geometry in aqueous solution.[17] If the insulin-enhancing complex is not very stable it is mainly transported, independently of the geometry in water, as (VO)hTf and $(VO)_2$hTf and secondarily as $(VO)_2$[d]HSA ($(VO)_2$[d]HSA is the dinuclear species in which the two VO^{2+} ions are interacting and the spectrum is characteristic of an $S = 1$ spin state);[18] if the stability of the complexes increases, the insulin-enhancing compound can survive in its original form, ternary species VO^{2+}−carrier−lact/citr can be formed, and, when carrier is a synergistic anion (property connected with the presence of a carboxylate group in the structure),[19] a small amount of VO^{2+} can be present as (VO)hTf(carrier) and $(VO)_2$hTf(carrier)$_2$. In all systems, also (VO)hTf(lact), $(VO)_2$hTf(lact)$_2$, (VO)hTf(citr), and $(VO)_2$hTf(citr)$_2$ can be detected, in which lactate and citrate behave as synergistic anions.[20] Recent important insight, which follows the observations of Orvig and co-workers,[21] is that if the geometry of insulin-enhancing compound in aqueous solution is *cis*-octahedral (that is, with an equatorial−equatorial and equatorial−axial arrangement of the two ligands and an equatorial water molecule in the cis position with respect to the V=O bond), mixed complexes with albumin can be formed, in which HSA replaces the water molecule in one of the four equatorial positions with an imidazole-N of a histidine residue (*cis*-VO(carrier)$_2$(HSA)).[16,17] The recent proof about the formation of *cis*-VO(dhp)$_2$(hTf), with an equatorial histidine-N of transferrin, is in contrast with what was previously reported by other authors, which attributed to this species the stoichiometries (VO)hTf(dhp) and $(VO)_2$hTf(dhp)$_2$, with the coordination of dhp to vanadium in the specific iron binding sites.[15] The values of A_z (163×10^{-4} cm^{-1})[17] and log β (25.5)[16] of such a complex, comparable with those measured for *cis*-VO(dhp)$_2$(HSA) (162×10^{-4} cm^{-1} and 25.9)[16,17] and for the model *cis*-[VO(dhp)$_2$(1-MeIm)] (163×10^{-4} cm^{-1} and 25.4),[16,17] support our attribution; DFT calculations confirm that A_z^{calcd} for the *cis*-octahedral species with an equatorial imidazole-N is 161×10^{-4} cm^{-1}.[17] Furthermore, the other hypothesis, which considers coordination of dhp at the iron binding sites, remains doubtful since dhp is not a synergistic anion.

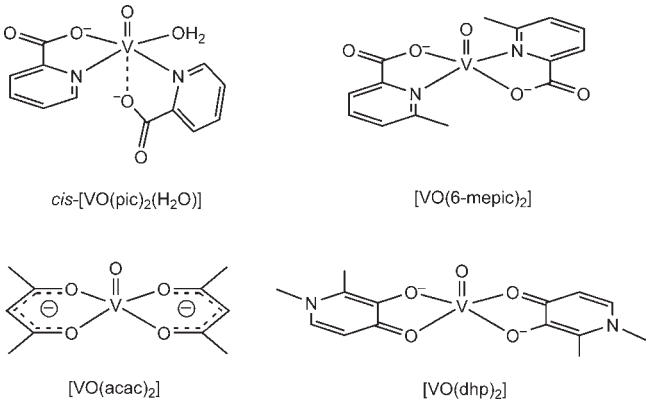
Immunoglobulins are glycoproteins having the capability to react in an immune response to foreign bodies introduced or inoculated into an organism. Immunoglobulins are divided into the classes IgA, IgD, IgE, IgG, and IgM; they differ in their biological properties, functional locations, and ability to deal with different antigens. IgG is the most abundant class in the blood and represents 75% of the total immunoglobulins. IgG monomer is a "Y"-shaped protein (ca. 150 kDa) that consists of four polypeptide chains, two identical heavy chains (50 kDa each one) and two identical light chains (25 kDa each one), connected by disulfide bonds. The arms of the "Y" contain the sites that can bind the antigens (Fab, fragment antigen binding region), whereas the base of the "Y" plays a role in modulating immune cell activity (Fc, fragment crystallizable region).

No interaction of IgG was found with $VOSO_4$ by EPR,[13] with $H_2VO_4^-$ by gel filtration and anion-exchange chromatography,[22] and with VO^{2+} or V^{3+} salts by FPLC-ICP-MS;[23] consequently, it was affirmed that among the high molecular mass (hmm) components, vanadium is complexed only to hTf and HSA.[12]

However, formation of adducts between IgG and Cu^{2+},[24] Mn^{2+},[25] Gd^{3+},[26] ^{99m}Tc,[27] and Ru^{2+} [28] has been reported. In particular, Dower et al. demonstrated that there are six specific Gd^{3+}-binding sites at pH 5.5, two very tight on the Fc fragment and two weaker on each of the Fab regions.[26]

In this work the interaction between VO^{2+} ion and IgG, on one hand, and between an insulin-enhancing VO^{2+} compound and IgG, on the other, was re-evaluated. As representatives examples of insulin-enhancing vanadium complexes, [VO(6-mepic)$_2$],[29] *cis*-[VO(pic)$_2$(H$_2$O)],[8b,30] [VO(acac)$_2$],[31] and [VO(dhp)$_2$],[32] where 6-mepic, pic, acac, and dhp are the deprotonated form of 6-methylpicolinic and picolinic acid, acetylacetone, and 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (Scheme 1), were chosen. The goals of this work are (i) study of the binary system VO^{2+}/IgG to understand if some type of interaction between VO^{2+} ion and immunoglobulin G exists, (ii) study of the ternary systems VO^{2+}/IgG/hTf and VO^{2+}/IgG/HSA to evaluate the relative strength of the three high molecular mass bioligands toward VO^{2+}, and (iii) study at physiological pH of the ternary systems VO^{2+}/IgG/carrier, where carrier is 6-mepic, pic, acac, or dhp, to ascertain whether an insulin-enhancing vanadium compound can interact with IgG. Electron paramagnetic resonance (EPR) spectroscopy, which was

Scheme 1. Insulin-Enhancing Vanadium Compounds Studied in This Work



revealed to be an excellent tool to study the speciation of VO^{2+} ion in biological systems, was used as instrumental technique.[16] The results should allow further clarification of the biotransformation of an insulin-enhancing agent in the blood and the form with which it can be transported toward the target organs.

EXPERIMENTAL AND COMPUTATIONAL SECTION

Chemicals. Water was deionized prior to use through the purification system Millipore Milli-Q Academic. VO^{2+} solutions were prepared from $VOSO_4 \cdot 3H_2O$ following literature methods.[33]

Human immunoglobulin G, human serum apo-transferrin, and human serum albumin were purchased from Sigma. Immunuglobulin G (99%, Sigma G4386), apo-transferrin (98%, Sigma T4283), and albumin (97−99%, Sigma A9511) have a molecular weight of 155−160, 76−81, and 66 kDa, respectively. The concentration of the protein solutions was estimated from their UV absorption (ε_{280}(IgG) = 217 000 M^{-1} cm^{-1},[34] ε_{280}(hTf) = 92 300 M^{-1} cm^{-1},[35] and ε_{279}(HSA) = 35 300 M^{-1} cm^{-1} [36]). The solubility in water of immunoglobulin G, transferrin, and albumin is 50 (ca. 3.0 × 10^{-4} M), 20 (ca. 2.5 × 10^{-4} M), and 50 mg/mL (ca. 7.5 × 10^{-4} M), respectively.

6-Methylpicolinic and picolinic acids, acetylacetone, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, 1-methylimidazole (1-MeIm), $NaHCO_3$, NaCl, and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) were of the highest grade available and used as received.

Preparation of the Solutions. The solutions were prepared dissolving in ultrapure water the insulin-enhancing compound in order to obtain a VO^{2+} concentration between 3.0 × 10^{-4} and 1.0 × 10^{-3} M. Argon was bubbled through the solutions to ensure the absence of oxygen and avoid oxidation of VO^{2+} ion. To the solution containing the metal ion, an appropriate amount of HEPES and NaCl and, eventually, organic carrier (6-methylpicolinic and picolinic acids, acetylacetone, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone) was added. Subsequently, the pH was raised to ca. 5.5, and in the system with transferrin, $NaHCO_3$ was dissolved. To 1 mL of this solution, again carefully purged with argon, an appropriate amount of immunoglobulin G and eventually transferrin or albumin was added; readily, pH was adjusted to ca. 7.4.

The ligand to metal molar ratio between the organic carrier and VO^{2+} was 2. The final concentration of HEPES, NaCl, and $NaHCO_3$ was 1.0 × 10^{-1}, 1.5 × 10^{-1} (corresponding to the physiological osmolarity), and 2.5 × 10^{-2} M (corresponding to the concentration in the blood), respectively. The presence of $NaHCO_3$ as a synergistic anion is necessary for vanadium coordination.[19] EPR studies performed on model systems prove that HEPES, $NaHCO_3$, and NaCl do not interact with VO^{2+} ion in the conditions used for the experiments.

EPR spectra of all model systems (VO^{2+}/carrier and VO^{2+}/carrier/1-MeIm) were recorded as previously reported.[17]

EPR Spectroscopy. Anisotropic EPR spectra were recorded with an X-band (9.4 GHz) Bruker EMX spectrometer on frozen solutions at 120 K. Addition of DMSO was not necessary, and no improvement in the resolution of the spectra was obtained.

When the samples were transferred into the EPR tubes, the spectra were immediately measured. Only low-temperature EPR spectra were measured in order to minimize the oxidation of VO^{2+} ion to vanadium(V), which otherwise would take place very quickly, with a half-time between 5 and 13 min at room temperature.[35] To increase the signal-to-noise ratio, signal averaging was used.[16−18,20]

As usual for analysis of the EPR spectra,[37] in all the figures reported in the text only the high-field region, the part more sensitive to the identity and amount of several species in solution, is presented. Unless otherwise indicated, the estimated error in A_z is ±0.3 × 10^{-4} cm^{-1}. Complete spectra are reported in the Supporting Information (Figures S1−S7).

DFT Calculations. All calculations presented in this paper were performed with the Gaussian 03 program (revision C.02)[38] and density functional theory (DFT) methods.[39] The hybrid exchange-correlation B3LYP[40,41] and the half-and-half functional BHandHLYP as incorporated in the Gaussian 03 software were used.

As demonstrated in the literature, DFT simulations are a valid tool for predicting EPR parameters of VO^{2+} complexes.[42] Using the BHandHLYP functional and 6-311g(d,p) basis set it is possible to calculate the ^{51}V hyperfine coupling constant along the *z* axis (A_z) with a mean deviation from the experimental value lower than 3%.[43]

The VO^{2+} species used to model the three sites of immunoglubulin G were simulated using 1-methylimidazole for an imidazole-N of a histidine residue (N(His)), acetate for a COO^- group of a glutamate/aspartate residue (COO^-(Glu/Asp)), 4-methylphenolate for a tyrosine-O^- residue (O^-(Tyr)), methoxide for a serine-O^- or threonine-O^- residue (O^-(Ser/Thr)), and methyltiolate for a cysteine-S^- residue (S^-(Cys)).

The geometries of the VO^{2+} complexes investigated were first preoptimized at the B3LYP/sto-3g level and further optimized at the B3LYP/6-311g level of theory. For all structures, minima were verified through frequency calculations. The optimized structures were used to calculate the values of ^{51}V A_z at the BHandHLYP/6-311g(d,p) level of theory. It must be remembered that for a VO^{2+} species the A_z value is usually negative, but in the literature its absolute value is usually reported. This was done also in this work (Table 2).

RESULTS AND DISCUSSION

Binary System VO^{2+}/IgG. The data reported in the literature indicate that in the binary system VO^{2+}/IgG no interaction between the metal ion and the protein was observed.[1,13,22,23] However, taking into account that albumin is able to coordinate nonspecifically VO^{2+}, presumably through surface histidine and glutamate/aspartate residues, that immunoglobulin G has a high number of such residues on the protein surface,[25] and that it is present in the blood in an amount comparable to transferrin, we decided to re-examine the system VO^{2+}/IgG. Surprisingly, EPR spectra (see Figure 1) show that complexation of the metal ion by IgG takes place.

The intensity of EPR signals for the first two parallel resonances (M_I = −7/2 and −5/2), normalized for the scan number and vanadium concentration, changes slightly as a function of the ratio VO^{2+}/IgG. The maximum value is detected around the equimolar ratio. On this basis, in the ternary systems containing VO^{2+}, IgG, and carrier, which will be discussed below, a ratio of 1/1 between VO^{2+} and IgG was chosen.

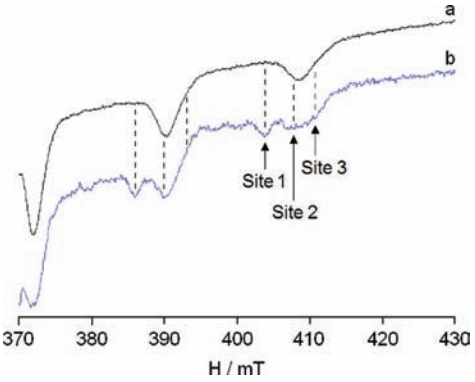


Figure 1. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/HSA 4/1 (VO^{2+} 1.0×10^{-3} M) and (b) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M). With dotted lines the resonances $M_I = 7/2$ and 5/2 of the three coordination sites of IgG are indicated.

EPR spectra indicate that VO^{2+} ion distributes on at least three distinct coordination sites (Figure 1), whose spectroscopic parameters are listed in Table 1 and demonstrate that in one site (which, for simplicity, will be named site 1) vanadium is bound to stronger donors than in the other two (named sites 2 and 3). This would suggest that the thermodynamic stability of the species formed in sites 1, 2, and 3 is comparable and vanadium distributes in a more or less uniform manner on the three sites. However, it cannot be ruled out that below the wide band around 409 mT there are resonances belonging to other sites; therefore, analogously to what we recently made for albumin, the stoichiometry of the binary species will be indicated as $(VO)_x$IgG with $x = 3-4$.[18]

It is evident that the coordination environment of vanadium in the three sites can be characterized only by a diffractometric study on a single crystal. However, to advance reasonable hypotheses, it can be useful to compare the spectra obtained in the system VO^{2+}/IgG (Figure 1b) with those of the system VO^{2+}/HSA (Figure 1a). It is noteworthy that the resonances of site 2 of IgG closely resemble those of $(VO)_x$HSA ($x = 5-6$), for which the coordination of imidazole-N of histidine residues (N(His)) and carboxylate-O^- of glutamic and aspartic acid residues (COO^-(Glu/Asp)) was proposed;[18] therefore, it is probable that this site of IgG is similar to those of albumin. Of course, this deduction implies that the coordinating residues are exposed on the protein surface. This was suggested by Nishihara et al., who, using the Mn^{2+} ion as an "EPR probe", demonstrated that the number of surface residues of glutamic acid, aspartic acid, and histidine in the Fab and Fc region is rather high (4, 3, and 3−5, respectively, in the Fab and 8, 5, and 8 in the Fc).[25] The X-ray three-dimensional structure confirms that the number of such residues is enough for metal ion coordination.[44]

Sites 1 and 3 are characterized by a smaller and larger value of A_z, respectively (Figure 1). For the additivity rule,[45] this means that in the first case in the coordination sphere of vanadium donors stronger than an imidazole-N (whose contribution to A_z depends on the orientation of the aromatic ring with respect to the V=O bond and is in the range $39.8-45.7 \times 10^{-4}$ cm^{-1} [46]) whereas in the second weaker donors than a carboxylate group (whose contribution to A_z is 42.1×10^{-4} cm^{-1} [47]) are bound to VO^{2+} ion. The increase of A_z for site 3 with respect to site 2 may depend on the presence of water molecules instead of histidine or glutamate/aspartate residues in the first coordination sphere of vanadium. About site 1, the binding of donors that significantly decreases the value of A_z, such as phenolate of tyrosine, alcoholate of serine or threonine, and thiolate of cysteine, can be supposed. In particular, it was demonstrated that Ser and Thr residues exposed on the protein surface are abundant in the Fab region and that their number is much higher than Tyr and Cys.[25] Moreover, one should remember that in immunoglobulins cysteines have a special role, because they stabilize the protein structure through formation of disulfide bridges and it is not clear if in native immunoglobulins there are free thiol groups or if those observed result from denaturation of the proteins.[48]

Table 1. EPR Parameters of the Three Sites of IgG

site	g_z	A_z[a]	probable donors
3	1.947	167.4[b]	N(His), COO^-(Asp, Glu), H_2O
2	1.951	163.6[b]	N(His), COO^-(Asp, Glu)
1	1.960	158.8	N(His), COO^-(Asp, Glu), O^-(Ser/Thr)/O^-(Tyr)/S^-(Cys)

[a] A_z measured in 10^{-4} cm^{-1}. [b] Estimated error $\pm 1.0 \times 10^{-4}$ cm^{-1}.

In order to obtain further insights about the donors coordinated to the vanadium in the three sites, DFT simulations were performed on model complexes with the aim of predicting their ^{51}V anisotropic hyperfine coupling constant (A_z). In recent years, several papers have been published on the possibility to calculate the hyperfine coupling constants in the EPR spectra of metal complexes[49] and in particular of VO^{2+} species.[42] Recently, the A_z value was calculated with Gaussian 03 software for 22 representative VO^{2+} complexes having different charge, geometry, and coordination mode at the BHandHLYP/6-311g(d,p) level of theory with a mean deviation of 2.7% from the experimental values.[43] Therefore, DFT methods may be used to predict A_z for a VO^{2+} species, and vice versa, to predict an unknown structure from its A_z value. DFT results have been reported in Table 2 (see also Experimental and Computational Section). Examination of the data allows one to advance the following hypotheses: (i) for site 3, coordination of two imidazole-N of histidine residues, of one COO^- group belonging to a glutamate or an aspartate residue, and of a H_2O molecule seems to be possible, whereas coordination of only one histidine-N is less probable; (ii) for site 2, H_2O ligands may be replaced by COO^- groups; and (iii) for site 1, the most probable candidate appears to be a serine-O^- or threonine-O^- rather than a tyrosine-O^- or cysteine-S^-. Interestingly, coordination of a deprotonated serine has been proposed for the active site of the reduced form of vanadium bromoperoxidase (VBrPO),[46] and as mentioned above, the number of surface Ser in the Fab region is higher than that of Tyr and Cys.[25]

Ternary Systems VO^{2+}/IgG/hTf and VO^{2+}/IgG/HSA. The two binary systems VO^{2+}/hTf and VO^{2+}/HSA have been examined previously,[1,16,35,50,51] and recently, the ternary system VO^{2+}/hTf/HSA has been studied.[18,20] The data indicate that transferrin forms with VO^{2+} ion more stable complexes than albumin, confirming what was reported in the literature.[12,35,52] However, given the higher concentration of HSA than hTf in the blood (630 vs 37 μM), albumin can compete with transferrin coordinating a non-negligible amount of total vanadium.[16,20]

The ternary system VO^{2+}/IgG/hTf was examined at molar ratio of 2/1/1; the high-field region of EPR spectra recorded at physiological pH is reported in Figure 2.

Table 2. EPR Parameters of the Three Sites of IgG Expected on the Basis of the "Additivity Rule" (A_z^{expct}), Calculated Through DFT Methods (A_z^{calcd}) and Experimental Measurements (A_z^{exptl})[a,b]

biological donor set[c]	A_z^{expct} [d,e]	A_z^{calcd} [f]	A_z^{exptl}	site
$N(His)^{\parallel}$, H_2O, H_2O, H_2O	176.6	182.6	167.4	site 3
$N(His)^{\parallel}$, $N(His)^{\parallel}$, H_2O, H_2O	171.0	170.7	167.4	site 3
$N(His)^{\parallel}$, $N(His)^{\perp}$, COO^-(Glu/Asp), H_2O	172.6	166.7	167.4	site 3
$N(His)^{\parallel}$, $N(His)^{\parallel}$, $N(His)^{\parallel}$, H_2O	166.1	164.3	163.6	site 2
$N(His)^{\parallel}$, $N(His)^{\perp}$, COO^-(Glu/Asp), COO^-(Glu/Asp)	169.1	161.3	163.6	site 2
$N(His)^{\parallel}$, $N(His)^{\perp}$, O^-(Tyr), COO^-(Glu/Asp)	165.8	158.9	158.8	site 1
$N(His)^{\parallel}$, $N(His)^{\perp}$, O^-(Ser/Thr), COO^-(Glu/Asp)	162.3	159.9	158.8	site 1
$N(His)^{\parallel}$, $N(His)^{\perp}$, S^-(Cys), COO^-(Glu/Asp)	157.9	156.5	158.8	site 1

[a] For the VO^{2+} complexes used as model of the three sites of IgG A_z^{calcd} and A_z^{exptl} (and, therefore, A_z^{expct}) are negative, but in the table the absolute values are reported. [b] A_z measured in 10^{-4} cm^{-1}. [c] $\parallel$, imidazole ring parallel to V=O bond (dihedral angle θ between the V=O and N−C bond, where C is the carbon atom bridging the two imidazole nitrogens, below 20°); $\perp$, imidazole ring perpendicular to V=O bond (dihedral angle θ between the V=O and N−C bond, where C is the carbon atom bridging the two imidazole nitrogens, above 70°). [d] Contribution to A_z: H_2O, 45.6 (ref 45a); COO^-(Glu/Asp), 42.1 (ref 47); N(His), $42.7 + 3.0 \times \sin(2\theta - 90)$ (ref 46); O^-(Tyr), 38.9 (ref 45a); O^-(Ser/Thr), 35.3 (ref 45a); S^-(Cys), 31.9×10^{-4} cm^{-1} (ref 45a). [e] A_z^{expct} determined with the value of the dihedral angle θ calculated by DFT simulations. [f] Value calculated at the level of theory BHandHLYP/6-311g(d,p).

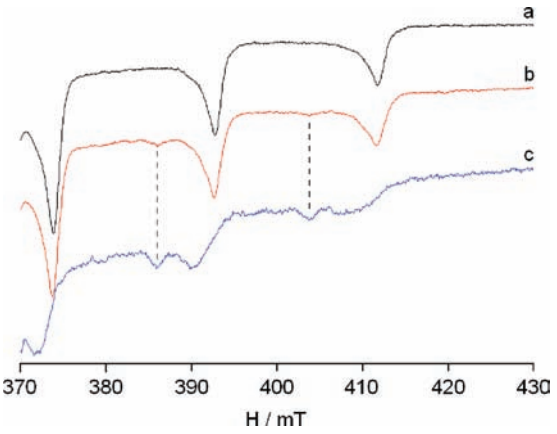


Figure 2. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/hTf 2/1 (VO^{2+} 5.0×10^{-4} M), (b) VO^{2+}/hTf/IgG 2/1/1 (VO^{2+} 5.0×10^{-4} M), and (c) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M). With dotted lines the resonances M_I = 7/2 and 5/2 of site 1 of IgG are indicated.

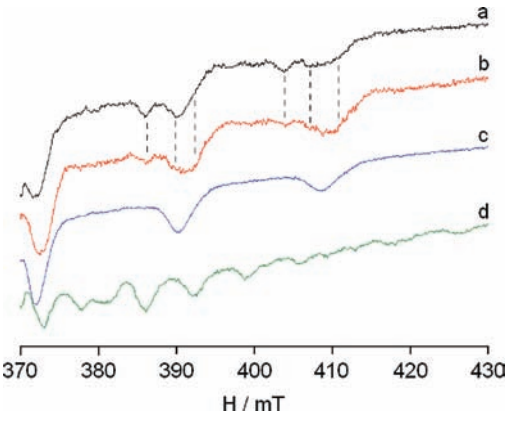


Figure 3. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M), (b) VO^{2+}/IgG/HSA 2/1/1 (VO^{2+} 5.0×10^{-4} M), (c) VO^{2+}/HSA 4/1 (VO^{2+} 1.0×10^{-3} M), and (d) VO^{2+}/HSA 1/1 (VO^{2+} 7.5×10^{-4} M). With dotted lines the resonances M_I = 7/2 and 5/2 of the three sites of IgG are indicated.

In the experimental conditions used, transferrin is potentially able to bind all of the metal ion present in the aqueous solution in the Fe^{3+} sites, forming $(VO)_2$hTf. In Figure 2 it is worth noticing that as a consequence of the high thermodynamic stability of hTf species most of VO^{2+} binds to the latter. However, besides the characteristic resonances of $(VO)_2$hTf, those of $(VO)_x$IgG also appear; in particular, the transitions of site 1 of IgG are easily observable. Of course, the greater stability of hTF complexes is related to the fact that it has two specific sites for metal ion binding, whereas IgG has only nonspecific coordination sites.

From the intensities of EPR signals for the resonances M_I = 7/2 and 5/2, it was possible to measure the percent of VO^{2+} bound to transferrin (95.5%) and immunoglobulin G (4.5%). These data allow one to obtain the constant for the displacement reaction of hTf by IgG; it must be highlighted, however, that because the stability of the three sites is comparable (see above), only a mean value for the association constant of $(VO)_x$IgG can be calculated, and using the notation of Kiss and co-workers on grounds of expediency[12,52] such a mean value will be referred to species (VO)IgG. Using the same procedure recently reported[16] and the values of 13.0 and 25.5 for $\log \beta_1$ and $\log \beta_2$ of (VO)hTf and $(VO)_2$hTf,[16] it is found that $\log \beta((VO)IgG) = 10.5 \pm 1.0$.

For the ternary system VO^{2+}/IgG/HSA two different molar ratios were examined, 2/1/1 and 4/1/1, since in the VO^{2+}/HSA system albumin is able to bind up to 5−6 equivalents of vanadium.[18,51] EPR spectra at a ratio of 2/1/1 are shown in Figure 3; although albumin is potentially able to bind all of VO^{2+} ion, it is observed that vanadium is distributed between the two species $(VO)_x$IgG and $(VO)_x$HSA, indicating that their affinity toward VO^{2+} is similar. This is not too surprising because complexation of albumin is nonspecific,[18] i.e., unlike transferrin coordination, sites specific for the metal ions do not exist; moreover, from the spectroscopic data, the donors which coordinate vanadium in HSA and IgG species should be similar (see above). This observation allows us to advance the hypothesis that vanadium can bind to any protein but on condition that there are residues of amino acids on the protein surface able to interact with the metal ion (particularly, histidine, glutamate, and aspartate residues).

dx.doi.org/10.1021/ic200087p |*Inorg. Chem.* 2011, 50, 3717–3728

Table 3. EPR Parameters of the Species Formed in the Ternary VO^{2+}−IgG−carrier and Related Systems

system	g_z	A_z[a]	species	donors	ref
VO^{2+}−IgG−6-mepic	1.948	163.6[b]	VO(6-mepic)(IgG)(OH)	(N, COO^-); N(His); OH^-	*c*
VO^{2+}−HSA−6-mepic	1.947	162.9	VO(6-mepic)(HSA)(OH)	(N, COO^-); N(His); OH^-	17
VO^{2+}−6-mepic−1-MeIm	1.950	162.9[d]	[VO(6-mepic)(1-MeIm)(OH)]	(N, COO^-); N(Imid); OH^-	17
VO^{2+}−IgG−pic	1.947	160.0	*cis*-$VO(pic)_2$(IgG)	(N, COO^-); (N, COO^{-ax}); N(His)	*c*
VO^{2+}−HSA−pic	1.950	159.7	*cis*-$VO(pic)_2$(HSA)	(N, COO^-); (N, COO^{-ax}); N(His)	17
VO^{2+}−pic−1-MeIm	1.943	158.8[e]	*cis*-[$VO(pic)_2$(1-MeIm)]	(N, COO^-); (N, COO^{-ax}); N(Imid)	17
VO^{2+}−IgG−dhp	1.944	162.6	*cis*-$VO(dhp)_2$(IgG)	(CO, O^-); (CO, O^{-ax}); H_2O	*c*
VO^{2+}−HSA−dhp	1.947	162.1	*cis*-$VO(dhp)_2$(HSA)	(CO, O^-); (CO, O^{-ax}); N(His)	17
VO^{2+}−hTf−dhp	1.947	163.3	*cis*-$VO(dhp)_2$(hTf)	(CO, O^-); (CO, O^{-ax}); N(His)	17
VO^{2+}−dhp−1-MeIm	1.947	163.0[f]	*cis*-[$VO(dhp)_2$(1-MeIm)]	(CO, O^-); (CO, O^-); N(Imid)	17

[a] Values measured in 10^{-4} cm^{-1}. [b] Estimated error $\pm 1.0 \times 10^{-4}$ cm^{-1}. [c] This work. [d] A_z value calculated by DFT methods (see ref 17): -161.1×10^{-4} cm^{-1}. [e] A_z value calculated by DFT methods (see ref 17): -156.6×10^{-4} cm^{-1}. [f] A_z value calculated by DFT methods (see ref 17): -160.7×10^{-4} cm^{-1}.

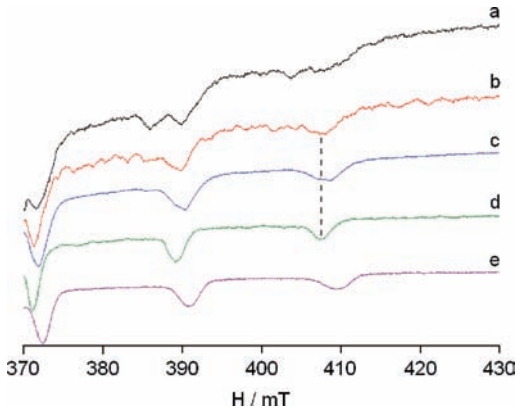


Figure 4. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M), (b) VO^{2+}/IgG/6-mepic 1/1/2 (VO^{2+} 3.0×10^{-4} M), (c) VO^{2+}/HSA/6-mepic 4/1/8 (VO^{2+} 1.0×10^{-3} M), (d) VO^{2+}/6-mepic/1-MeIm 1/2/4 (VO^{2+} 1.0×10^{-3} M), and (e) VO^{2+}/6-mepic 1/2 (VO^{2+} 1.0×10^{-3} M). With dotted line the resonance $M_I = 7/2$ of VO(6-mepic)(IgG)(OH) is indicated.

The percent concentration of IgG species, calculated from the intensities of the resonances $M_I = 7/2$ and 5/2 is 66.3%, whereas that of HSA is 33.7%, with a ratio between them close to 2:1. With a procedure similar to that discussed above, it is possible to find in this case too the value of the displacement reaction of albumin by immunoglobulin G, which allows one to get a value of $\log \beta$((VO)IgG) of 10.1 ± 1.0. It is noteworthy that this value is comparable to that calculated from examination of the ternary system VO^{2+}/IgG/hTf. Therefore, the final value for the stability constant of (VO)IgG can be taken as the mean between those found in the two different ternary systems

$$\log \beta((\mathrm{VO})\mathrm{IgG}) = 10.3 \pm 1.0 \qquad (1)$$

Ternary System VO^{2+}/IgG/6-mepic. Anisotropic EPR spectra recorded at physiological pH on the ternary system formed by VO^{2+}, IgG, and 6-mepic show the presence of a species with g_z = 1.948 and $A_z = 163.6 \times 10^{-4}$ cm^{-1} (Table 3). The spectral parameters are intermediate between those of mono- and bis-chelated complexes of 6-methypicolinate ($A_z = 168 \times 10^{-4}$ and 161×10^{-4} cm^{-1}, respectively).[53]

A deeper look reveals that the spectrum can be compared with those obtained in the ternary systems containing 1-methylimidazole and albumin (Figure 4).[17] EPR spectra of the systems with HSA and 1-MeIm were interpreted supposing formation of a mixed complex, in which HSA binds VO^{2+} ion with an imidazole-N of a histidine residue of the polypeptide chain, 6-mepic with the donor set (N, COO^-), whereas a OH^- ion completes the equatorial coordination sites. The analogous spectroscopic response obtained in the system with IgG can be explained in the same manner: this implies that IgG can coordinate the metal ion with an imidazole-N nitrogen of a side chain of a histidine probably present on the protein surface (Scheme 2a).

The stoichiometry [VO(6-mepic)(1-MeIm)(OH)] for the model compound formed by 1-methyimidazole was confirmed by pH-potentiometry, EPR spectroscopy, and DFT calculations.[16,17] Therefore, the spectrum recorded in the system VO^{2+}/IgG/6-mepic can be interpreted in the same way: this implies that IgG can coordinate VO^{2+} with a histidine-N, probably on the protein surface, forming the species VO(6-mepic)(IgG)(OH). Its stability constant, not measurable from EPR intensities, should be comparable to that of [VO(6-mepic)-(1-MeIm)(OH)] ($\log \beta = 3.82$).[16]

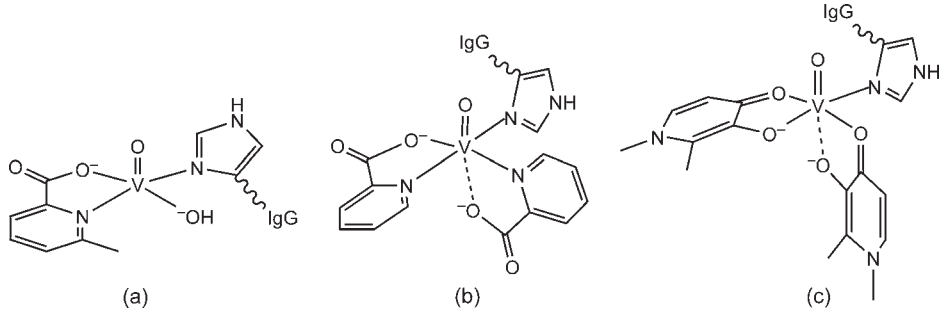
$$\log \beta(\mathrm{VO(6\text{-}mepic)(IgG)(OH)}) \approx 3.8 \qquad (2)$$

Ternary System VO^{2+}/IgG/pic. Anisotropic EPR spectra of the ternary system VO^{2+}/IgG/pic are characterized by the intense resonances of a species with g_z = 1.947 and $A_z = 160.0 \times 10^{-4}$ cm^{-1} (Figure 5b). In this case too, the spectral pattern closely resembles that of the mixed complexes formed in the systems with 1-methyimidazole and albumin, *cis*-[$VO(pic)_2$(1-MeIm)], and *cis*-$VO(pic)_2$(HSA), respectively; in the case of 1-MeIm and HSA, the correct composition was established through the combined application of spectroscopic studies, potentiometric titrations, and DFT calculations.[16,17]

Therefore, IgG, like HSA, can replace the water molecule in the equatorial plane of *cis*-[$VO(pic)_2(H_2O)$] with an imidazole-N of a histidine, forming an analogous species *cis*-$VO(pic)_2$(IgG) (Scheme 2b).

Unfortunately, on the basis of EPR spectra, no exact value of the stability constant β of this species can be measured; however, it can be suggested that in this case too $\log \beta$ should be similar to *cis*-[$VO(pic)_2$(1-MeIm)] ($\log \beta = 14.96$), determined through

Scheme 2. Ternary Complexes Formed by IgG: (a) VO(6-mepic)(IgG)(OH), (b) *cis*-VO(pic)$_2$(IgG), and (c) *cis*-VO(dhp)$_2$(IgG)



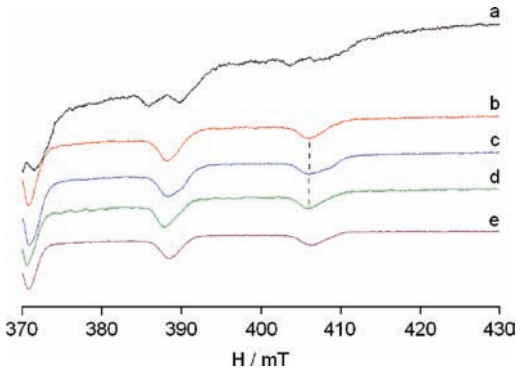


Figure 5. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M), (b) VO^{2+}/IgG/pic 1/1/2 (VO^{2+} 3.0×10^{-4} M), (c) VO^{2+}/HSA/pic 4/1/8 (VO^{2+} 1.0×10^{-3} M), (d) VO^{2+}/pic/1-MeIm 1/2/4 (VO^{2+} 1.0×10^{-3} M), and (e) VO^{2+}/pic 1/2 (VO^{2+} 1.0×10^{-3} M). With the dotted line the resonance $M_I = 7/2$ of *cis*-VO(pic)$_2$(IgG) is indicated.

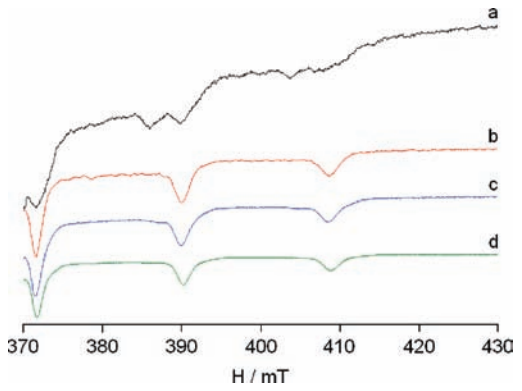


Figure 6. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M), (b) VO^{2+}/IgG/acac 1/1/2 (VO^{2+} 3.0×10^{-4} M), (c) VO^{2+}/HSA/acac 4/1/8 (VO^{2+} 1.0×10^{-3} M), and (d) VO^{2+}/acac 1/2 (VO^{2+} 1.0×10^{-3} M).

pH titrations.[16]

$$\log \beta(\textit{cis}\text{-VO(pic)}_2\text{(IgG)}) \approx 15.0 \qquad (3)$$

Ternary System VO^{2+}/IgG/acac. Potentiometric and spectroscopic studies indicate that in the system VO^{2+}/acetylacetone

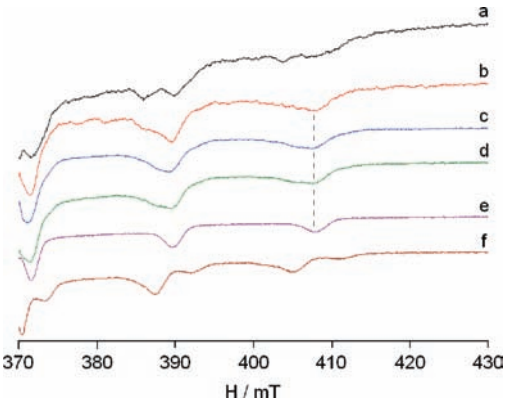


Figure 7. High-field region of the X-band anisotropic EPR spectra recorded at pH 7.4 on frozen solutions (120 K) containing (a) VO^{2+}/IgG 1/1 (VO^{2+} 3.0×10^{-4} M), (b) VO^{2+}/IgG/dhp 1/1/2 (VO^{2+} 3.0×10^{-4} M), (c) VO^{2+}/HSA/dhp 4/1/8 (VO^{2+} 1.0×10^{-3} M), (d) sum of the spectra obtained in the systems VO^{2+}/dhp/1-MeIm (trace e) and VO^{2+}/dhp (trace f), (e) VO^{2+}/dhp/1-MeIm 1/2/4 (VO^{2+} 1.0×10^{-3} M), and (f) VO^{2+}/dhp 1/2 (VO^{2+} 1.0×10^{-3} M). With the dotted line the resonance $M_I = 7/2$ of *cis*-VO(dhp)$_2$(IgG) is indicated.

around the physiological pH VO^{2+} ion is present as neutral complex [VO(acac)$_2$] with a very low hydrolysis degree.[54]

A comparison of the anisotropic EPR spectra recorded at pH 7.4 on the systems VO^{2+}/IgG, VO^{2+}/acac, and VO^{2+}/IgG/acac reveals that in the ternary system only the set of signals belonging to [VO(acac)$_2$] can be distinguished (Figure 6). Therefore, the spectroscopic measurements suggest that, unlike what was observed in previous systems, formation of mixed complexes can be ruled out. This is confirmed by examination of the spectra recorded in the ternary systems with 1-methylimidazole, which are practically indistinguishable from those of the binary system VO^{2+}/acac; the results cannot be explained only with the stability of [VO(acac)$_2$] but also considering that in such a species the absence of water molecules coordinated in the equatorial position to be replaced by a biodonor like a histidine-N prevents formation of ternary complexes.[17]

Ternary System VO^{2+}/IgG/dhp. Potentiometric data show that in the binary system VO^{2+}/dhp in the pH range 5−8 the bis-chelated complex is the main species in aqueous solution; it is present in two forms [VO(dhp)$_2$] and *cis*-[VO(dhp)$_2$(H$_2$O)].[32b,55] The anisotropic EPR spectrum recorded at pH 7.4 in the ternary system with VO^{2+}, IgG, and dhp shows the presence of a species not observable in the binary systems VO^{2+}/IgG and VO^{2+}/dhp

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Table 4. Predicted Percent Distribution of the VO^{2+} Species Formed from the Biotransformation of an Insulin-Enhancing Agent at Concentrations of 1 and 50 μM and pH 7.4[a]

	6-mepic		pic		acac		dhp	
concentration (μM)	1	50	1	50	1	50	1	50
(VO)hTf	91.9 (93.0)	10.6 (9.1)	91.8 (92.9)	10.9 (9.4)	91.9 (93.0)	10.7 (9.4)	89.1 (89.7)	14.9 (15.0)
$(VO)_2$hTf	2.7 (2.2)	79.5 (83.0)	2.7 (2.2)	77.9 (81.2)	2.7 (2.2)	78.9 (82.4)	2.6 (2.6)	4.2 (4.3)
(VO)hTf(lact)	4.4 (4.4)	0.5 (0.4)	4.4 (4.4)	0.5 (0.5)	4.4 (4.4)	0.5 (0.4)	4.2 (4.3)	0.7 (0.7)
$(VO)_2$hTf$(lact)_2$	0.0 (0.0)	0.2 (0.2)	0.0 (0.0)	0.2 (0.2)	0.0 (0.0)	0.2 (0.2)	0.0 (0.0)	0.0 (0.0)
$(VO)_2$[d]HSA	0.3 (0.3)	2.8 (4.1)	0.3 (0.3)	2.6 (3.7)	0.3 (0.3)	2.7 (3.8)	0.3 (0.3)	0.1 (0.1)
$(VO)_x$HSA	0.0 (0.0)	1.9 (2.3)	0.0 (0.0)	1.8 (2.1)	0.0 (0.0)	1.8 (2.2)	0.0 (0.0)	0.0 (0.0)
$(VO)_x$IgG	0.6	3.9	0.6	3.7	0.6	3.8	0.6	0.1
VO(6-mepic)(HSA)(OH)	0.0 (0.0)	0.0 (0.0)						
VO(6-mepic)(IgG)(OH)	0.0	0.0						
(VO)hTf(pic)			0.1 (0.1)	0.4 (0.4)				
$(VO)_2$hTf$(pic)_2$			0.0 (0.0)	0.1 (0.1)				
cis-$VO(pic)_2$(HSA)			0.0	0.0 (0.1)				
cis-$VO(pic)_2$(IgG)			0.0	0.0				
$[VO(pic)(lactH_{-1})]^-$			0.0 (0.0)	1.3 (1.6)				
$[VO(carrier)_2]$	0.0 (0.0)	0.0 (0.1)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.8 (0.9)	1.7 (1.7)	44.3 (46.2)
cis-$VO(dhp)_2$(hTf)							0.0 (0.0)	0.4 (0.4)
cis-$VO(dhp)_2$(HSA)							1.3 (1.3)	31.9 (33.3)
cis-$VO(dhp)_2$(IgG)							0.1	3.4
$[VO(lactH_{-1})_2]^{2-}$	0.1 (0.1)	0.5 (0.7)	0.1 (0.1)	0.5 (0.6)	0.1 (0.1)	0.5 (0.6)	0.1 (0.1)	0.0 (0.0)
$[VO(OH)_3]^-$	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)

[a] Between round parentheses the percent composition obtained when the complexes with IgG are not considered. For log β, the values reported in ref 16 and in this work were used. The concentrations of IgG and the other components were those reported by Hamilton in ref 11 and by Harris in ref 10: for hTf a concentration of 25.9 μM was used to take into account the fraction of transferrin involved in iron complexation.

(Figure 7b). The value of A_z (162.6×10^{-4} cm^{-1}) is intermediate between that of *cis*-$[VO(dhp)_2(H_2O)]$ (166.2×10^{-4} cm^{-1}) and $[VO(dhp)_2]$ (157.4×10^{-4} cm^{-1}),[17] indicating that the water molecule in the *cis*-octahedral species has been replaced by a stronger donor, and coincident with that of *cis*-$[VO(dhp)_2(1\text{-MeIm})]$ (163.0×10^{-4} cm^{-1}),[17] whose existence has been recently demonstrated through potentiometric and spectroscopic data.[16,17]

The behavior of the system VO^{2+}/IgG/dhp follows closely also that of VO^{2+}/hTf/dhp and VO^{2+}/HSA/dhp, where formation of compounds *cis*-$VO(dhp)_2$(hTf) and *cis*-$VO(dhp)_2$(HSA) was demonstrated; in such species, an imidazole-N of the side chain of a histidine residue, exposed on the protein surface, replaces the H_2O ligand of *cis*-$[VO(dhp)_2(H_2O)]$ and occupies the fourth equatorial position.[17] On this basis, EPR spectra can be easily interpreted by considering in the aqueous solution around the physiological pH an equimolar mixture of *cis*-$[VO(dhp)_2(H_2O)]$ and $[VO(dhp)_2]$, on one hand, and *cis*-$VO(dhp)_2$(IgG), on the other (see Figure 7d). The structure of *cis*-$VO(dhp)_2$(IgG) is represented in Scheme 2c.

DFT methods allow one to confirm these deductions; they show that for *cis*-$[VO(dhp)_2(1\text{-MeIm})]$ the expected A_z value (160.7×10^{-4} cm^{-1}) is very similar to that of *cis*-$VO(dhp)_2$(hTf), *cis*-$VO(dhp)_2$(HSA), and *cis*-$VO(dhp)_2$(IgG) (Table 3). DFT calculations suggest also that in the absence of significant interactions with other groups the plane of the aromatic ring is arranged almost parallel to the V═O direction.[17]

As pointed out recently, estimation of log β can be carried out by measuring from EPR intensities the relative ratio between the concentration of the mixed complex *cis*-$VO(dhp)_2$(IgG) and the bis-chelated species formed by dhp, $VO(dhp)_2$.[16] In the ternary system containing VO^{2+}/IgG/dhp with a 1/1/2 ratio and VO^{2+} concentration of 3.0×10^{-4} M, the amount of $VO(dhp)_2$ and $VO(dhp)_2$(IgG), measured in the $M_I = +5/2$ and $+7/2$ transitions, is 49.0% and 51.0%, respectively. Since coordination of immunoglobulin G is realized through the exposed histidines, the effective concentration of histidine residues available for metal coordination can be found by multiplying the analytical concentration of IgG by the number of His residues on the protein surface. There is not a general agreement about this number: on the one hand it has been reported that the IgG molecule has at least four surface histidines, two in the His cluster situated in the Fc region of the heavy chain,[56] on the other hand the total number of exposed residues is ca. 12.[25] These data allow for calculating the mean value of log β for formation of *cis*-$VO(dhp)_2$(IgG)

$$\log \beta(\textit{cis}\text{-}VO(dhp)_2(IgG)) = 25.6 \pm 0.6 \qquad (4)$$

It is noteworthy that this value is very close to that obtained by pH-potentiometry for the mixed complex $[VO(dhp)_2(1\text{-MeIm})]$, log β = 25.40,[16] indicating that only the number of histidine residues on the protein surface influences the formation and stability of such species.

Biotransformation of an Insulin-Enhancing Agent in the Blood. The values calculated for the thermodynamic stability constants of $(VO)_x$IgG (eq 1) and *cis*-$VO(dhp)_2$(IgG) (eq 4) and those expected for VO(6-mepic)(IgG)(OH) (eq 2) and *cis*-$VO(pic)_2$(IgG) (eq 3) allow one to predict the biodistribution of VO^{2+} ion, administrated in the solid form $VO(carrier)_2$, between the components of the blood.

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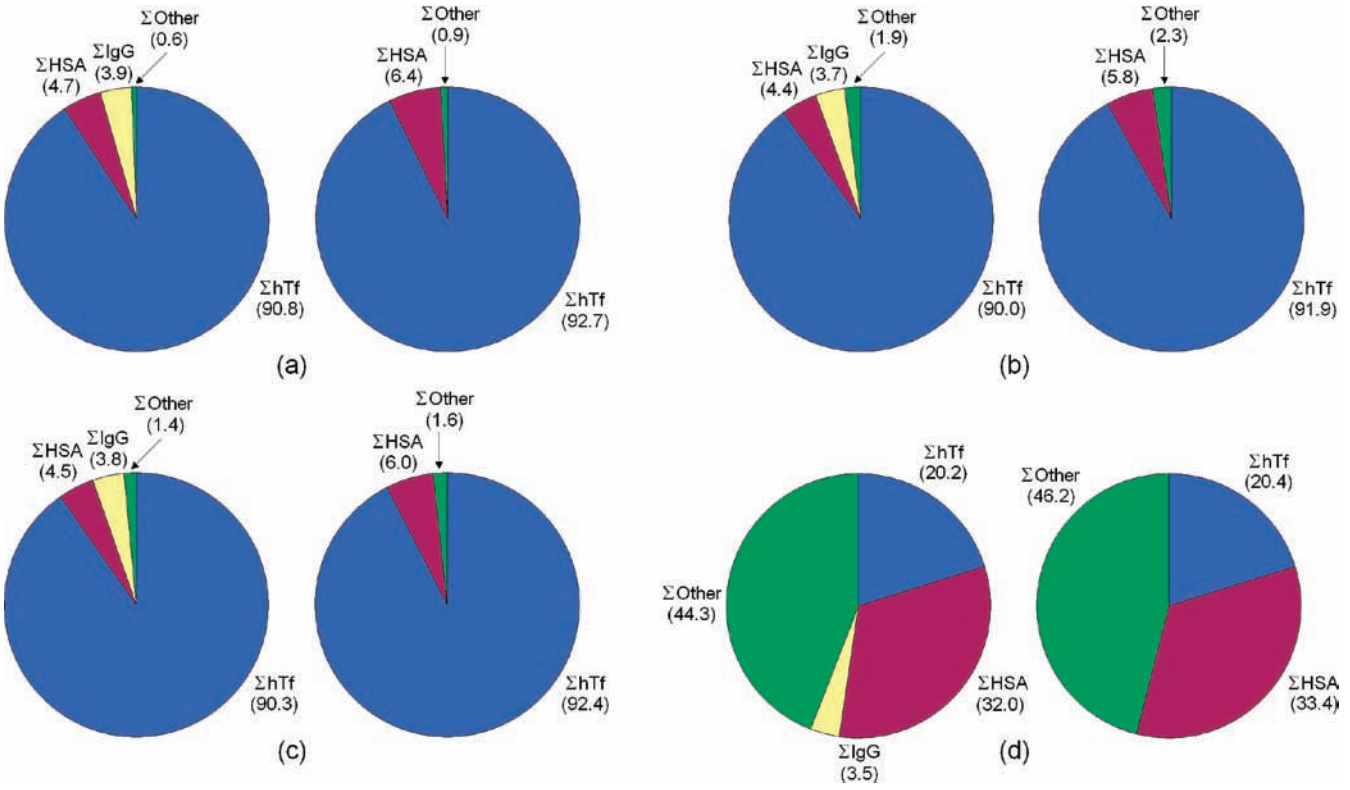

Figure 8. Percent distribution of VO^{2+} ion between hmm and other components in the blood at pH 7.4 when the metal ion concentration is 50 μM: (a) 6-mepic, (b) pic, (c) acac, and (d) dhp. For each carrier, on the left are shown the results when IgG is included and on the right when it is neglected. The sum of the VO^{2+} species bound to hTf (ΣhTf) is represented in blue, to HSA (ΣHSA) in purple, to IgG (ΣIgG) in yellow, and to other ligands such as carrier and lactate (Σother) in green.

Orvig and co-workers reported that during phases I and IIa the administration of various amounts of [bis(ethylmaltolato)-oxidovanadium(IV)] (BEOV) to volunteers resulted in different maximal systemic concentrations of vanadium.[2d,57] These concentrations depend on, besides the administered dose, the chemical form of vanadium: particularly, inorganic salts are adsorbed less effectively than chelated complexes. Smith et al. administered increasing doses of inorganic salts of vanadium, but they did not measure the vanadium concentration in the blood.[58] In rats higher concentrations of [bis(maltolato)oxidovanadium(IV)] (BMOV) resulted in higher concentrations of vanadium in the blood.[59] Since we are interested in humans, we refer to very low concentrations of vanadium in the blood, around a few micromolar, at least if we consider the concentrations effectively measured during phases I and IIa of clinical trials of BEOV. On this basis, it is reasonable to assume that the concentration of an insulin-enhancing vanadium compound in the blood serum should be not higher than some tens of micromolar. In Table 4, the results for two possible values (1 and 50 μM) are summarized. It is plausible that the latter is higher than the real value in the organism, but it may help to understand which is the trend in the biodistribution processes of an insulin-enhancing drug when its concentration in the blood reaches the maximum value. The data are compared with those obtained when the interaction of VO^{2+} ion with immunoglobulin G is not considered. It must be pointed out that since under normal conditions 30% of the binding sites of transferrin are occupied by Fe^{3+},[60] a concentration of 25.9 μM was used as really available for vanadium complexation.

From the data the usual classification in weak, intermediate, and strong carriers emerges. When the concentration of insulin-enhancing compound is around 1 μM, almost all of the VO^{2+} ion ($\sim$ 99%) exists in the forms (VO)hTf (main species) and $(VO)_2$hTf (secondary species) in the case of weak (6-mepic) or intermediate (pic, acac) organic carriers; only when the carrier is strong (dhp) this percentage slightly lowers to $\sim$96%. This is agreement with the results proposed by Kiss and co-workers.[12,52]

When the concentration of insulin-enhancing drug is around 50 μM, the importance of the ternary complex (VO)hTf(lact) decreases and $(VO)_2$hTf becomes prevalent with respect to (VO)hTf. Moreover, the percentage of the species in which VO^{2+} is bound to HSA or IgG and of the undissociated form [VO(carrier)$_2$] increases; in particular, if the carrier is weak or intermediate the amount of vanadium bound in the binary and ternary species with albumin and immunoglobulin G is about 8%, whereas if it is strong this increases to 35.5%. Interestingly, for dhp, the whole of [VO(dhp)$_2$], *cis*-[VO(dhp)$_2$(H_2O)], and *cis*-VO(dhp)$_2$(Protein) (where Protein indicates hTf, HSA, or IgG bound to VO^{2+} by a surface histidine-N) reaches 80.0%.

Examination of Table 4 shows that the species $(VO)_x$IgG can be neglected only when the concentration of insulin-enhancing agent is around 1 μM, but it must be taken into account for higher concentrations; in the case of 6-mepic, pic, and acac, for a VO^{2+} concentration of 50 μM the calculated percentage is about 4%. For dhp, as mentioned above, a great importance is held by the complexes with stoichiometry *cis*-VO(carrier)$_2$(Protein); from the values obtained, the order of the relative amount,

dx.doi.org/10.1021/ic200087p |*Inorg. Chem.* 2011, 50, 3717–3728

cis-VO(carrier)$_2$(HSA) ≫ *cis*-VO(carrier)$_2$(IgG) > *cis*-VO(carrier)$_2$(hTf), follows the concentration of the hmm components in the blood.

In Figure 8 the distribution of VO^{2+} between the species formed by hmm bioligands and other components (carrier and lactate) in the blood is graphically represented; for each carrier, on the left are shown the results when IgG is included in the calculations and on the right those obtained when it is neglected. It is evident that a significant competition between hTf, HSA, and IgG for VO^{2+} complexation exists, and in all four cases the percentage of vanadium present as (VO)$_x$IgG when the concentration of the insulin-enhancing compound is 50 μM is lost by (VO)hTf and (VO)$_2$hTf on one hand and by (VO)$_x$HSA and (VO)$_2$^{d}HSA on the other. For example, with 6-methylpicolinate, 3.9% of vanadium coordinated to IgG comes from transferrin (1.9%) and albumin species (1.7%) but the sum of the species formed by hTf, HSA, and IgG remains almost unchanged (99.4% vs 99.1%).

CONCLUDING REMARKS

The results of this work demonstrate that, differently from the data in the literature which report no interaction of immunoglobulins with vanadium, IgG binds VO^{2+} ion with at least three sites with comparable affinity to form a species (VO)$_x$IgG with x = 3−4. It is probable that such sites are not specific and similar to those of albumin (histidine-N and glutamate/aspartate-O^-) with the possible participation of one serine/threonine-O^- rather than one tyrosine-O^- or cysteine-S^-. The value of the stability constant of (VO)$_x$IgG is much lower than that of hTf and comparable to that of HSA, and the slightly higher concentration of IgG with respect to transferrin (84 vs 37 μM) is not able to compensate for the difference in affinity. Therefore, the statement that in physiological conditions VO^{2+} is bound almost exclusively to hTf is still valid; however, with increasing the concentration of VO^{2+} in the blood serum the percent amount of (VO)$_2$^{d}HSA, (VO)$_x$HSA, and (VO)$_x$IgG also increases.

Examination of the ternary systems formed by IgG with human serum transferrin (hTf) and human serum albumin (HSA) allows one to find that the order of complexing strength is hTf ≫ IgG ≈ HSA. The mean value for the association constant (log β) of (VO)$_x$IgG is 10.3 ± 1.0. The behavior of the systems with IgG and four potent insulin-enhancing agents, like [VO(6-mepic)$_2$], *cis*-[VO(pic)$_2$(H$_2$O)], [VO(acac)$_2$], and [VO(dhp)$_2$], is very similar to that of the corresponding systems with albumin. In particular, at the physiological pH value, complexes with identical stoichiometry VO(6-mepic)(IgG)(OH), *cis*-VO(pic)$_2$(IgG), and *cis*-VO(dhp)$_2$(IgG) are formed. In such species, IgG coordinates nonspecifically VO^{2+} ion through an imidazole-N belonging to a histidine residue exposed on the protein surface. For *cis*-VO(dhp)$_2$(IgG), log β = 25.6 ± 0.6, comparable with that of the analogous species formed by transferrin and albumin, *cis*-VO(dhp)$_2$(hTf) and *cis*-VO(dhp)$_2$(HSA).

With the stability constants of the compounds formed by IgG, the predicted percent distribution of the main species formed after biotransformation of the insulin-enhancing agents at physiological conditions can be calculated. When their concentration is about some tens of micromolar, the percentage of binary species formed by IgG, (VO)$_x$IgG, is small but cannot be neglected; moreover, for dhp, the mixed species *cis*-VO(carrier)$_2$(IgG), *cis*-VO(carrier)$_2$(HSA), and *cis*-VO(carrier)$_2$(hTf) have a particular importance.

On the basis of the results discussed in this work the following conclusions can be drawn: (i) in principle, any protein provided with histidine and glutamic/aspartic acid residues exposed on its surface may bind VO^{2+} ion forming binary species with stoichiometry (VO)$_x$(Protein), with x variable in dependence on the number of such residues; (ii) imidazole-N belonging to surface histidines can replace the water molecules coordinated in the equatorial plane of an insulin-enhancing agent or of a species derived from its biotransformation at physiological pH, yielding the corresponding mixed species; in particular, when the geometry of the insulin-enhancing compound in aqueous solution is *cis*-octahedral, histidine-N replace the equatorial water molecule of *cis*-[VO(carrier)$_2$(H$_2$O)] (where carrier is, for example, picolinic acid or 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone) to form species with *cis*-VO(carrier)$_2$(Protein) composition that, as noticed by Orvig and co-workers,[21] can contribute significantly to the transport processes toward the target organs. We believe that these conclusions are generalizable to whatever protein. Of course, it is desirable that other studies, for example, UV−vis, CD, and ultrafiltration measurements, could confirm these results.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Costa Pessoa, J.; Tomaz, I. *Curr. Med. Chem.* **2010**, *17*, 3701–3738 and references therein.

(2) (a) Thompson, K. H.; McNeill, J. H.; Orvig, C. *Chem. Rev.* **1999**, *99*, 2561–2571 and references therein. (b) Thompson, K. H.; Orvig, C. *Coord. Chem. Rev.* **2001**, *219−221*, 1033–1053 and references therein. (c) Shechter, Y.; Goldwaser, I.; Mironchik, M.; Fridkin, M.; Gefel, D. *Coord. Chem. Rev.* **2003**, *237*, 3–11. (d) Thompson, K. H.; Orvig, C. *J. Inorg. Biochem.* **2006**, *100*, 1925–1935. (e) Sakurai, H.; Yoshikawa, Y.; Yasui, H. *Chem. Soc. Rev.* **2008**, *37*, 2383–2392.

(3) Rehder, D. *Bioinorganic Vanadium Chemistry*; Wiley: Chichester, 2008.

(4) (a) Tolman, E. L.; Barris, E.; Burns, M.; Pansini, A.; Partridge, R. *Life Sci.* **1979**, *25*, 1159–1164. (b) Shechter, Y.; Karlish, S. J. D. *Nature* **1980**, *286*, 556–558.

(5) (a) Posner, B. I.; Faure, R.; Burgess, J. W.; Bevan, A. P.; Lachance, D.; Zhang-Sun, G.; Fantus, I. G.; Ng, J. B.; Hall, D. A.; Soo Lum, B.; Shaver, A. *J. Biol. Chem.* **1994**, *269*, 4596–4606.(b) Posner, B. I.; Yang, C. R.; Shaver, A. In *Vanadium Compounds: Chemistry, Biochemistry, and Therapeutic Applications*; Tracey, A. S., Crans, D. C., Eds.; ACS Symposium Series 711; American Chemical Society: Washington DC, 1998; pp 316−328.

(6) Thompson, K. H.; Orvig, C. *Dalton Trans.* **2000**, 2885–2892.

(7) (a) Josephson, L.; Cantley, L. C., Jr. *Biochemistry* **1977**, *16*, 4572–4578. (b) Cantley, L. C., Jr.; Josephson, L.; Warner, R.; Yanagisawa, M.; Lechene, C.; Guidotti, G. *J. Biol. Chem.* **1977**, *252*, 7421–7423.(c) Gresser, M. J.; Tracey, A. S. In *Vanadium in Biological Systems*; Chasteen, N. D., Ed.; Kluwer Academic Publishers: Dordrecht, 1990; pp 63−79. (d) Stankiewicz, P. J.; Tracey, A. S.; Crans, D. C. In *Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 1995; Vol. 31, pp 287−324 and references therein.

(e) Ou, H.; Yan, L.; Mustafi, D.; Makinen, M. W.; Brady, M. J. *J. Biol. Inorg. Chem.* **2005**, *10*, 874–886.
(8) (a) Yasui, H.; Adachi, Y.; Katoh, A.; Sakurai, H. *J. Biol. Inorg. Chem.* **2007**, *12*, 843–853. (b) Sakurai, H.; Fujii, K.; Watanabe, H.; Tamura, H. *Biochem. Biophys. Res. Commun.* **1995**, *214*, 1095–1101.(c) Shechter, Y.; Eldberg, G.; Shisheva, A.; Gefel, D.; Sekar, N.; Qian, S.; Bruck, R.; Gershonov, E.; Crans, D. C.; Goldwasser, Y.; Fridkin, M.; Li, J. In *Vanadium Compounds: Chemistry, Biochemistry, and Therapeutic Applications*; Tracey, A. S., Crans, D. C., Eds.; ACS Symposium Series 711; American Chemical Society: Washington DC, 1998; pp 308−315. (d) Marzban, L.; McNeill, J. H. *J. Trace Elem. Exp. Med.* **2003**, *16*, 253–267.
(9) Yasui, H.; Takechi, K.; Sakurai, H. *J. Inorg. Biochem.* **2000**, *78*, 185–196.
(10) Harris, W. R. *Clin. Chem.* **1992**, *38*, 1809–1818.
(11) Hamilton, R. G. *Clin. Chem.* **1987**, *33*, 1707–1725.
(12) Kiss, T.; Jakusch, T.; Hollender, D.; Dörnyei, A.; Enyedy, E. A.; Costa Pessoa, J.; Sakurai, H.; Sanz-Medel, A. *Coord. Chem. Rev.* **2008**, *252*, 1153–1162 and references therein.
(13) Willsky, G. R.; Goldfine, A. B.; Kostyniak, P. J.; McNeill, J. H.; Yang, L. Q.; Khan, H. R.; Crans, D. C. *J. Inorg. Biochem.* **2001**, *85*, 33–42.
(14) Kiss, T.; Jakusch, T.; Bouhsina, S.; Sakurai, H.; Enyedy, E. A. *Eur. J. Inorg. Chem.* **2006**, 3607–3613.
(15) Jakusch, T.; Hollender, D.; Enyedy, E. A.; Sánchez Gonzáles, C.; Montes-Bayón, M.; Sanz-Medel, A.; Costa Pessoa, J.; Tomaz, I.; Kiss, T. *Dalton Trans.* **2009**, 2428–2437.
(16) Sanna, D.; Micera, G.; Garribba, E. *J. Biol. Inorg. Chem.* **2010**, *15*, 825–839.
(17) Sanna, D.; Micera, G.; Garribba, E. *Inorg. Chem.* **2010**, *49*, 174–187.
(18) Sanna, D.; Garribba, E.; Micera, G. *J. Inorg. Biochem.* **2009**, *103*, 648–655.
(19) (a) Campbell, R. F.; Chasteen, N. D. *J. Biol. Chem.* **1977**, *252*, 5996–6001. (b) Sun, H.; Cox, M. C.; Li, H.; Sadler, P. J. *Struct. Bonding (Berlin)* **1997**, *88*, 71–102.
(20) Sanna, D.; Micera, G.; Garribba, E. *Inorg. Chem.* **2009**, *48*, 5747–5757.
(21) Liboiron, B. D.; Thompson, K. H.; Hanson, G. R.; Lam, E.; Aebischer, N.; Orvig, C. *J. Am. Chem. Soc.* **2005**, *127*, 5104–5115.
(22) De Cremer, K.; Van Hulle, M.; Chery, C.; Cornelis, R.; Strijckmans, K.; Dams, R.; Lameire, N.; Vanholder, R. *J. Biol. Inorg. Chem.* **2002**, *7*, 884–890.
(23) Fernandes, K. G.; Montes-Bayon, M.; Gonzalez, E. B.; Del Castillo-Busto, E.; Nobrega, J. A.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **2005**, *20*, 210–215.
(24) Baker, B.; Hultquist, D. H. *J. Biol. Chem.* **1978**, *253*, 1195–1200.
(25) Nishihara, S.; Shimizu, A.; Asata, Y. *Mol. Immun.* **1986**, *23*, 285–290.
(26) Dower, S. K.; Dwek, R. A.; McLaughlin, A. C.; Mole, L. E.; Press, E. M.; Sunderland, C. A. *Biochem. J.* **1975**, *149*, 73–82.
(27) Zamora, P. O.; Mercer-Smith, J. A.; Marek, M. J.; Schulte, L. D.; Rhodes, B. A. *Nucl. Med. Biol.* **1992**, *19*, 797–802.
(28) Groessl, M.; Hartinger, C. G.; Polec-Pawlak, K.; Jarosz, M.; Keppler, B. K. *Electrophoresis* **2008**, *29*, 2224–2232.
(29) (a) Fujisawa, Y.; Fujimoto, S.; Sakurai, H. *J. Inorg. Biochem.* **1997**, *67*, 396–396. (b) Sakurai, H.; Fujisawa, Y.; Fujimoto, S.; Yasui, H.; Takino, T. *J. Trace Elem. Exp. Med.* **1999**, *12*, 393–401.
(30) Kawabe, K.; Yoshikawa, Y.; Adachi, Y.; Sakurai, H. *Life Sci.* **2006**, *78*, 2860–2866.
(31) (a) Reul, B. A.; Amin, S. S.; Buchet, J. P.; Ongemba, L. N.; Crans, D. C.; Brichard, S. M. *Br. J. Pharmacol.* **1999**, *126*, 467–477. (b) Amin, S. S.; Cryer, K.; Zhang, B.; Dutta, S. K.; Eaton, S. S.; Anderson, O. P.; Miller, S. M.; Reul, B. A.; Brichard, S. M.; Crans, D. C. *Inorg. Chem.* **2000**, *39*, 406–416.
(32) (a) Rangel, M.; Tamura, A.; Fukushima, C.; Sakurai, H. *J. Biol. Inorg. Chem.* **2001**, *6*, 128–132. (b) Buglyó, P.; Kiss, T.; Kiss, E.; Sanna, D.; Garribba, E.; Micera, G. *J. Chem. Soc., Dalton Trans.* **2002**, 2275–2282.
(33) Nagypál, I.; Fábián, I. *Inorg. Chim. Acta* **1982**, *61*, 109–113.
(34) Kilár, F.; Simon, I.; Lakatos, S.; Vonderviszt, F.; Medgyesi, G. A.; Závodszky, P. *Eur. J. Biochem.* **1985**, *147*, 17–25.
(35) Chasteen, N. D.; Grady, J. K.; Holloway, C. E. *Inorg. Chem.* **1986**, *25*, 2754–2760.
(36) *The Plasma Proteins*, 2nd ed.; Putman, F. W., Ed.; Academic Press: New York, 1975; Vol. I, pp 133−181.
(37) Kiss, T.; Jakusch, T.; Costa Pessoa, J.; Tomaz, I. *Coord. Chem. Rev.* **2003**, *237*, 123–133 and references therein.
(38) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, revision C.02; Gaussian, Inc.: Wallingford, CT, 2004.
(39) Parr, R. G.; Yang, W. *Density-Functional Theory of Atoms and Molecules*; Oxford University Press: Oxford, 1989.
(40) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648–5652.
(41) Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785–789.
(42) (a) Munzarová, M. L.; Kaupp, M. *J. Phys. Chem. B* **2001**, *105*, 12644–12652 and references therein. (b) Saladino, A. C.; Larsen, S. C. *J. Phys. Chem. A* **2003**, *107*, 1872–1878. (c) Neese, F. *J. Chem. Phys.* **2003**, *118*, 3939–3948. (d) Aznar, C. P.; Deligiannakis, Y.; Tolis, E. J.; Kabanos, T. A.; Brynda, M.; Britt, R. D. *J. Phys. Chem. A* **2004**, *108*, 4310–4321. (e) Saladino, A. C.; Larsen, S. C. *Catal. Today* **2005**, *105*, 122–133 and references therein.
(43) Micera, G.; Garribba, E. *Dalton Trans.* **2009**, 1914–1918.
(44) (a) Saul, F. A.; Amzel, L. M.; Poljak, R. J. *J. Biol. Chem.* **1978**, *253*, 585–597. (b) Silverton, E. W.; Navia, M. A.; Davies, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5140–5144. (c) Marquart, M.; Deisenhofer, J.; Huber, R.; Palm, W. *J. Mol. Biol.* **1980**, *141*, 369–391. (d) Deisenhofer, J. *Biochemistry* **1981**, *20*, 2361–2370.
(45) (a) Chasteen, N. D. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1981; Vol. 3, pp 53−119. (b) Smith, T. S., II; LoBrutto, R.; Pecoraro, V. L. *Coord. Chem. Rev.* **2002**, *228*, 1–18.
(46) Smith, T. S.II; Roof, C. A.; Kampf, J. W.; Rasmussen, P. G.; Pecoraro, V. L. *J. Am. Chem. Soc.* **2000**, *122*, 767–775.
(47) Jakusch, T.; Buglyó, P.; Tomaz, A. I.; Costa, Pessoa, J.; Kiss, T. *Inorg. Chim. Acta* **2002**, *339*, 119–128.
(48) Gevondyan, N. M.; Volynskaia, A. M.; Gevondyan, V. S. *Biochemistry (Moscow)* **2006**, *71*, 279–284.
(49) (a) In *Calculation of NMR and EPR Parameters. Theory and Applications*; Kaupp, M., Buhl, M., Malkin, V. G., Eds.; Wiley-VCH: Weinheim, 2004. (b) Remenyi, C.; Reviakine, R.; Arbuznikov, A. V.; Vaara, J.; Kaupp, M. *J. Phys. Chem. A* **2004**, *108*, 5026–5033 and references therein. (c) Neese, F. *Coord. Chem. Rev.* **2009**, *253*, 526–573 and references therein.
(50) Chasteen, N. D. *Coord. Chem. Rev.* **1977**, *22*, 1–36.
(51) Chasteen, N. D.; Francavilla, J. *J. Phys. Chem.* **1976**, *80*, 867–871.
(52) Kiss, T.; Jakusch, T.; Hollender, D.; Dörnyei, A. In *Vanadium: the Versatile Metal*; Kustin, K., Costa Pessoa, J., Crans, D. C., Eds.; ACS Symposium Series 974; American Chemical Society: Washington, DC, 2007; pp 323−339.
(53) Kiss, E.; Garribba, E.; Micera, G.; Kiss, T.; Sakurai, H. *J. Inorg. Biochem.* **2000**, *78*, 97–108.

(54) (a) Crans, D. C.; Khan, A. R.; Mahroof-Tahir, M.; Mondal, S.; Miller, S. M.; la Cour, A.; Anderson, O. P.; Jakusch, T.; Kiss, T. *Dalton Trans.* **2001**, 3337–3345. (b) Garribba, E.; Micera, G.; Sanna, D. *Inorg. Chim. Acta* **2006**, *359*, 4470–4476.

(55) Rangel, M.; Leite, A.; Amorim, M. J.; Garribba, E.; Micera, G.; Lodyga-Chruscinska, E. *Inorg. Chem.* **2006**, *45*, 8086–8097.

(56) (a) Todorova-Balvay, D.; Pitiot, O.; Bourhim, M.; Srikrishnan, T.; Vijayalakshmi, M. *J. Chromatogr. B* **2004**, *808*, 57–62. (b) Bayramoglu, G.; Celik, G.; Arica, M. Y. *Colloids Surf., A* **2006**, *287*, 75–85.

(57) Thompson, K. H.; Lichter, J.; LeBel, C.; Scaife, M. C.; McNeill, J. H.; Orvig, C. *J. Inorg. Biochem.* **2009**, *103*, 554–558.

(58) Smith, D. M.; Pickering, R. M.; Lewith, G. T. *Q. J. Med.* **2008**, *101*, 351–358.

(59) Zhang, S.-Q.; Zhong, X.-Y.; Chen, G.-H.; Lu, W.-L.; Zhang, Q. *J. Pharm. Pharmacol.* **2008**, *60*, 99–105.

(60) Taylor, D. M. In *Perspectives on Bioinorganic Chemistry*; Hay, R. W., Dilworth, J. R., Nolan, K. B., Eds.; JAI Press: London, 1993; Vol. 2, pp 139−159.