DOI: 10.1002/ejic.200500304

Binding of Oxovanadium(IV) to Tripeptides Containing Histidine and Cysteine Residues and Its Biological Implication in the Transport of Vanadium and Insulin-Mimetic Compounds

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Keywords: Bioinorganic chemistry / Coordination modes / EPR spectroscopy / Peptides / Vanadium

The complexation of $V^{IV}O$ ion with three tripeptides of biological importance containing L-histidine or L-cysteine (HisGlyGly, GlyGlyHis and GlyGlyCys) has been studied. This study was performed in aqueous solution by the combined application of potentiometric and spectroscopic (electronic absorption and EPR) techniques. The results indicate that these oligopeptides, if a ligand-to-metal molar ratio of 10 or 15 is used, can keep $V^{IV}O$ ion in solution until the deprotonation of the amide group with the donor set (NH_2, CO, $N_{im}{}^{ax}$) for HisGlyGly or (COO^-, CO) for GlyGlyHis and GlyGlyCys. In all the systems, at pH values around neutrality, a $VOLH_{-2}$ species is formed with an (NH_2, N^-, N^-, COO^-) donor set for HisGlyGly, (NH_2, N^-, N^-, N_{im}) for GlyGlyHis and (NH_2, N^-, N^-, S^-) for GlyGlyCys. These species, and those with one deprotonated amide group coordinated to the $V^{IV}O$ ion, can be detected by EPR spectroscopy. The N^-(amide) contribution to the hyperfine coupling constant along the z axis, A_z, depends on the total charge of the donor atoms in the equatorial plane. The participation of albumin in the transport of vanadium and insulin-mimetic $V^{IV}O$ compounds is reconsidered based on these results.

Introduction

Vanadium plays a number of roles in biological systems.[1] It is present in vanadium-dependent haloperoxidases[2] and nitrogenase enzymes,[3] and many sea squirts accumulate it in very high concentrations, although the reason for this is not completely known.[4] The most recent studies concern the insulin-mimetic activity of vanadium compounds.[5] All these observations suggest interactions between vanadium ions and proteins.

Peptides are the most closely related models for proteins, and in some cases proteins may have specific terminal metal ion binding sites, for example albumin.[6] Among the various residues, histidine and cysteine play a main role: histidine is involved in the active site of vanadium haloperoxidases[2] and in the metal ion binding to albumin,[6] and the cysteine of glutathione takes part in redox processes such as the reduction of V^V to V^{IV} inside the cell. Moreover, peptide sequences containing cysteine mimic the binding sites of thioneins.

The interaction of $V^{IV}O$ with oligopeptides needs the presence of anchoring groups. Those most effective in promoting peptide amide deprotonation and coordination are phenolate-O^- followed by alkoxide-O^- and thiolate-S^-; COO^- and NH_2 are weaker groups.[7] The first examples of amide deprotonation for vanadium involved exclusively V^V,[8] although since then examples have been characterised for $V^{IV}O$ too, both in solution and in the solid state.[9] Recent results[10] have demonstrated that a histidyl residue in the N-terminal position can act as a valid anchor because the coordination of the amino group is assisted by the imidazole nitrogen. In the absence of suitable groups in the N-terminal position, mixed complexes with the (COO^-, CO) donor set can keep vanadium in solution until amide deprotonation occurs. As a consequence, L/M molar ratios of 10 are enough to favour complexation at physiological pH values.

The speciation of $V^{IV}O$ with HisGlyGly, GlyGlyHis and GlyGlyCys is reported in this work. The ligands are displayed in Scheme 1. GlyGlyHis is a model for albumins [e.g., human serum albumin (HSA), bovine serum albumin (BSA) and rat serum albumin (RSA)], neuromedins C and K, human sperm protamine P2a and histatins.[6] This motif binds Cu^{II} and Ni^{II} specifically, although it can release the metal to appropriate ligands, by playing the role of transport site in albumins. For example, 5–10% of serum cop-

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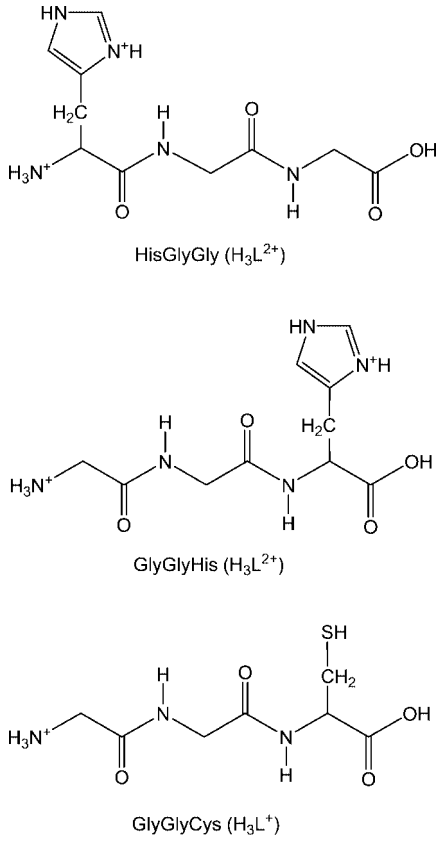
per[11] and 95% of serum nickel is bound to albumin.[12] The Cu^{II} ion can bind albumin in the form of a binary complex or ternary species with amino acids such as histidine.[13]



Scheme 1. Fully protonated forms of the ligands.

The form involved in the transport of vanadium in the human body is still unknown. However, the participation of albumin has been proposed for vanadium transport in blood.[14]

As far as $V^{IV}O$ insulin-mimetic compounds are concerned, the form that reaches target organs (bone, liver, kidney, muscles) is unknown. For many years it was suggested that it could be bound by human serum transferrin (hTF) and that the participation of albumin is negligible.[15] However the data for the $V^{IV}O$–transferrin interaction are quite uncertain, and no reliable thermodynamic data exist on the interaction with albumin. Willsky et al.[16] and Liboiron et al.[17] have reported albumin binding to a well-known insulin-mimetic compound [bis(maltolato)oxovanadium(IV)] (BMOV). The results were explained by the formation of adducts like $[VO(maltolato)_2HSA]$.[17] This suggests that adducts or ternary albumin complexes could be the pharmacologically active species or, at least, the main method of vanadium delivery to cells. However, equilibrium studies on the distribution of an insulin-mimetic complex between hTF and HSA are lacking. In particular, it is important to measure the stability constants for the interaction of albumin with $V^{IV}O$ and $V^{IV}O$ chelates to establish its participation in the transport of vanadium and insulin-mimetic vanadium compounds. GlyGlyHis represents a good model of albumin. Albumin can take part in the transport of insulin-mimetic compounds through: i) formation of binary complexes, such as those proposed for Cu^{II} and Ni^{II},[6] and studied in this work for $V^{IV}O$, and ii) formation of ternary complexes or adducts, such as [VO(maltolato)(HSA)] or $[VO(maltolato)_2(HSA)]$, as proposed by Liboiron et al.[17]

Results

L-Histidylglycylglycine (HisGlyGly)

The potentiometric titration of the ligand (H_3L^{2+}, Scheme 1 and Table 1) indicates three deprotonation steps with pK_a values of 3.10 (COOH), 5.39 ($N_{im}H^+$) and 7.34 (NH_3^+). The values are in good agreement with those reported in the literature.[18] The concentration distribution curves of the $V^{IV}O$ complexes as a function of pH are depicted in Figure 1.

Table 1. Protonation constants (log K) and $V^{IV}O$ (log β_{pqr}) stability constants for the studied ligands and complexes at 25.0 ± 0.1 °C and I = 0.1 M (KNO_3).

log K	HisGlyGly	GlyGlyHis	GlyGlyCys
COOH	3.10	3.26	2.98
$N_{im}H^+$	5.39	6.71	–
NH_3^+	7.34	7.93	7.77
SH	–	–	9.59
log β_{pqr}			
$VOLH_2$	14.55(1)	17.44(1)	19.56(2)
VOLH	12.51(2)		
VOL	8.37(2)		
VOL_2H_4		35.01(2)	39.49(1)
VOL_2H_3		30.08(2)	34.99(2)
VOL_2H_2		25.07(1)	29.99(2)
VOL_2H	18.23(1)	18.15(1)	23.50(3)
VOL_2	12.01(1)		15.79(2)
$VOLH_{-1}$	2.22(2)	2.00(2)	
$VOLH_{-2}$	–5.40(2)	–5.01(1)	–4.50(2)

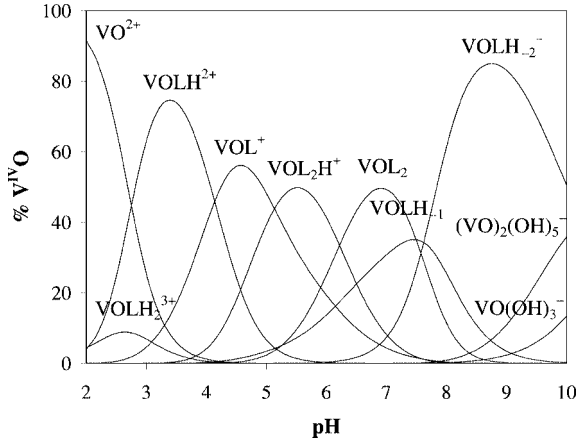


Figure 1. Species distribution for the $V^{IV}O$–HisGlyGly system at a metal-to-ligand molar ratio of 1:10 and a $V^{IV}O$ concentration of 1 mM.

A recent paper of ours[10] demonstrated that a histidyl residue in the N-terminal part of an oligopeptide is a good

anchoring group due to the binding ability of the imidazole nitrogen. $HisNH_2$, HisGly and HisHis behave as tridentate ligands by coordinating the metal ion through the amino and imidazole nitrogen atoms and the carbonyl oxygen atom.

This speciation process was substantiated by pH-potentiometric titrations (see Experimental Section). Complex formation starts with a $VOLH_2$ species which predominates at pH 3–4 (**I** in Figure 2). To evaluate the "basicity adjusted" stability constant, we assume the following reaction: $VO^{2+} + H_3L^{2+} \rightarrow VOLH_2^{3+} + H^+$. A value of –1.28 is calculated for HisGlyGly, which can be compared with those for GlyGly (–1.43), GlyGlyGly (–1.59),[19] Ala (–1.33),[20] H_2SalGly (–1.29),[9b] H_2sal-RGG (–1.56) and H_2sal-RGGG (–1.51),[9f] for which a pure carboxylate coordination has been substantiated. The EPR parameters (Table 2) support this assignment.

VOLH (**IIa** in Figure 2) is formed upon deprotonation of the amino group, with $g_z = 1.947$ and $A_z = 168 \times 10^{-4}$ cm^{-1}. We compared the EPR spectra with those obtained in the $V^{IV}O$/L-glycine methyl ester (GlyOMe) system at L/M = 500. The stepwise formation of two species was observed, with $g_z = 1.946$, $A_z = 168 \times 10^{-4}$ cm^{-1} and $g_z = 1.951$, $A_z = 163 \times 10^{-4}$ cm^{-1}, respectively.[21] They were identified as VOL and VOL_2, with (NH_2, CO) and [2 × (NH_2, CO)] coordination modes, respectively. The data are also similar to those of mono- and bis-chelated complexes of $V^{IV}O$ with L-histidine methyl ester (HisOMe),[21] $g_z = 1.946$, $A_z = 170 \times 10^{-4}$ cm^{-1} and $g_z = 1.950$, $A_z = 164 \times 10^{-4}$ cm^{-1}. The parameters reported for the mono-chelated complex formed by imidazole-4-acetic acid, with an (N_{im}, COO^-) donor set, are quite different from those observed with HisGlyGly.[22] Therefore, the spectroscopic features of VOLH are typical of an (NH_2, CO) donor set.

The deprotonation of the imidazole N takes place with $pK_a = 4.16$, and VOLH transforms into VOL (**IIb** in Fig-

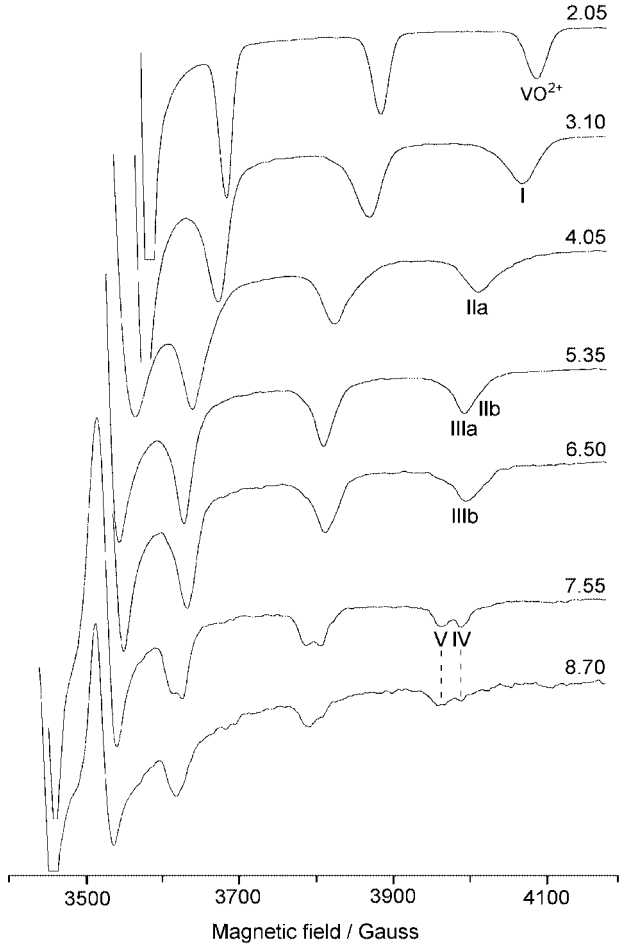


Figure 2. High-field region of the X-Band EPR spectra recorded at 140 K as a function of pH for aqueous solutions of $V^{IV}O$ and HisGlyGly at metal-to-ligand molar ratio of 1:10 and a $V^{IV}O$ concentration of 4 mM. The dotted line indicates the high-field resonance of the complexes with the deprotonated amide group.

Table 2. EPR parameters and donor sets for $V^{IV}O$ complexes.

Ligand	Complex	Symbol[a]	g_z	A_z[b]	Donor set
HisGlyGly	$VOLH_2$	**I**	1.935	177	(COO^-)
	VOLH	**IIa**	1.947	168	(NH_2, CO)
	VOL	**IIb**	1.947	168	(NH_2, CO, N_{im}^{ax})
	VOL_2H	**IIIa**	1.950	164	[(NH_2, CO, N_{im}^{ax}); (NH_2, CO)]
	VOL_2	**IIIb**	1.950	164	[(NH_2, CO, N_{im}^{ax}); (NH_2, CO)]
	$VOLH_{-1}$	**IV**	1.950	161	[(NH_2, N^-, CO, N_{im}^{ax}); H_2O]
	$VOLH_{-2}$	**V**	1.951	159	(NH_2, N^-, N^-, COO^-, N_{im}^{ax})
GlyGlyHis	$VOLH_2$	**I**	1.936	176	(COO^-, CO)
	VOL_2H_4	**II**	1.940	172	[2 × (COO^-, CO)]
	VOL_2H_3	**IIIa**	1.945	169	[(NH_2, CO); (COO^-, CO)]
	VOL_2H_2	**IIIb**	1.945	169	[(NH_2, CO); (COO^-, CO)]
	VOL_2H	**IIIc**	1.945	169	[(NH_2, CO); (COO^-, CO)]
	$VOLH_{-1}$	**IV**	1.950	161	[(NH_2, N^-, CO, N_{im}^{ax}); H_2O]
	$VOLH_{-2}$	**V**	1.960	153	(NH_2, N^-, N^-, N_{im})
GlyGlyCys	$VOLH_2$	**I**	1.935	176	(COO^-, CO)
	VOL_2H_4	**II**	1.940	171	[2 × (COO^-, CO)]
	VOL_2H_3	**III**	1.945	168	[(NH_2, CO); (COO^-, CO)]
	VOL_2H_2	**IV**	1.949	164	[2 × (NH_2, CO)]
	VOL_2H	**V**	1.950	160	[(NH_2, CO); (S^-, COO^-)]
	VOL_2	**VI**	1.960	152	[(NH_2, N^-, CO); (S^-, COO^{-ax})]
	$VOLH_{-2}$	**VII**	1.967	148	(NH_2, N^-, N^-, S^-)

[a] See Figure 2 for HisGlyGly, Figure 4 for GlyGlyHis and Figure 6 for GlyGlyCys. [b] A_z measured in 10^{-4} cm^{-1} units.

ure 2). The pK_a value is lower, by one order of magnitude, than in the free ligand because of the coordination of the nitrogen atom. The EPR parameters are the same as VOLH and comparable with those of complexes of dipeptides with histidine in N-terminal position, such as HisGly and HisHis.[10] In a previous work,[10] we suggested a tridentate equatorial-axial-equatorial (*eq-ax-eq*) binding mode of the ligand to explain the similar behaviour of $HisNH_2$, HisGly and HisHis. The two external donor groups (NH_2 and CO) are in the equatorial positions and N_{im} is in the axial position. The "basicity adjusted" stability constant for the reaction $VO^{2+} + HL^+ \rightarrow [VOL]^{2+} + H^+$ is –1.03. This means that the involvement of an imidazole nitrogen as the third donor increases the stability of the species with an (NH_2, CO, N_{im}^{ax}) donor set significantly compared with those of (NH_2, CO) or (COO^-, CO).

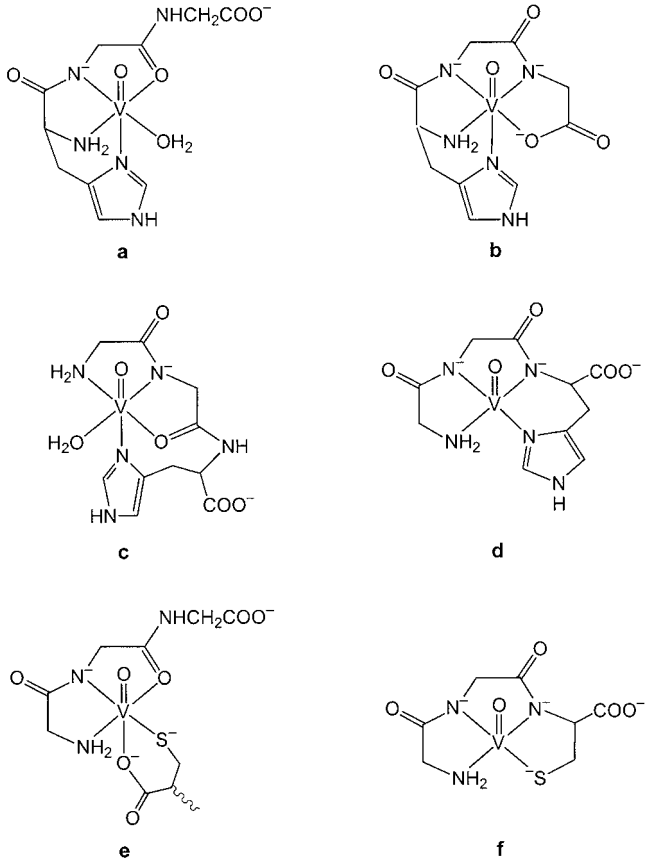
The imidazole N in the axial position of VOL does not affect the A_z value significantly according to the Chasteen "additivity rule", which assumes that only the equatorial donors contribute to the hyperfine ^{51}V constant along the *z* axis.[23]

The bis-complexes VOL_2H and VOL_2 are the major species at pH 5–7. At ligand-to-metal molar ratios as high as 10 for HisGlyGly and 15 for GlyGlyHis and GlyGlyCys, we believe that weak donor sets like (NH_2, CO) can replace monodentate water molecules in the equatorial plane of $V^{IV}O$. In VOL_2H (**IIIa** in Figure 2), only two positions of the equatorial plane are available for the second ligand, therefore the coordination mode is [(NH_2, CO, N_{im}^{ax}); (NH_2, CO)], with the imidazole nitrogen of the second ligand molecule still protonated.

The transformation of VOL_2H into VOL_2 (**IIIb** in Figure 2) takes place upon deprotonation of the $N_{im}H^+$ group. The pK_a of 5.84 is very close to that of the free ligand, suggesting that the aromatic nitrogen of the second ligand molecule does not participate in the coordination. Since the donor set is the same, the same EPR parameters are observed for VOL_2H and VOL_2 (g_z = 1.950, A_z = 164×10^{-4} cm^{-1}), comparable to those of HisGly (g_z = 1.951, A_z = 165×10^{-4} cm^{-1}) and HisHis (g_z = 1.950, A_z = 164×10^{-4} cm^{-1}).[10]

$VOLH_{-1}$ (**IV** in Figure 2 and **a** in Scheme 2) is detected both by potentiometry and EPR spectroscopy at a pH of around 7.5. Its spectral parameters are g_z = 1.950 and A_z = 161×10^{-4} cm^{-1}, and its pK_a value is 7.55. For this species we propose the deprotonation of the first amide group to give a complex with an [(NH_2, N^-, CO, N_{im}^{ax}); H_2O] coordination mode. The axial binding of an imidazole nitrogen stabilises this coordination mode differently from tripeptides lacking coordinating side-chains.

At higher pH values the formation of $VOLH_{-2}$ (**V** in Figure 2 and **b** in Scheme 2) starts. The EPR parameters (g_z = 1.951 and A_z = 159×10^{-4} cm^{-1}) suggest the deprotonation of the second amide bond and an (NH_2, N^-, N^-, COO^-) donor set with four donor atoms in the equatorial positions, analogously to the Cu^{II} ion;[24] hydrolysis processes occur at pH values above 9.



Scheme 2. Structures of $V^{IV}O$ complexes formed by HisGlyGly (a,b), GlyGlyHis (c,d) and GlyGlyCys (e,f) with deprotonated amide groups.

Glycylglycyl-L-histidine (GlyGlyHis)

GlyGlyHis (H_3L^{2+}, Table 1 and Scheme 1) shows three pK_a values in the titrable pH range, 3.26 (COOH), 6.71 ($N_{im}H^+$) and 7.93 (NH_3^+). These values are comparable with those in the literature.[25] Distribution curves are shown in Figure 3 and EPR spectra in Figure 4.

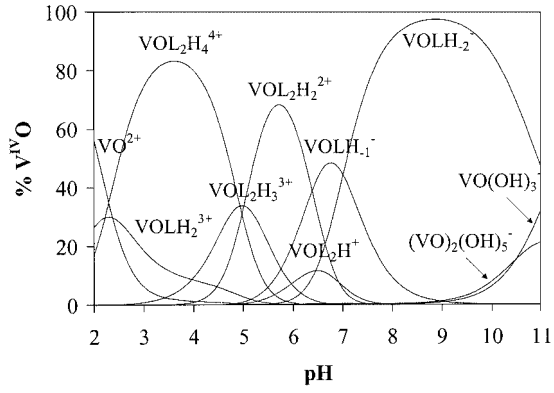


Figure 3. Species distribution for the $V^{IV}O$–GlyGlyHis system at a metal-to-ligand molar ratio of 1:15 and a $V^{IV}O$ concentration of 1 mM.

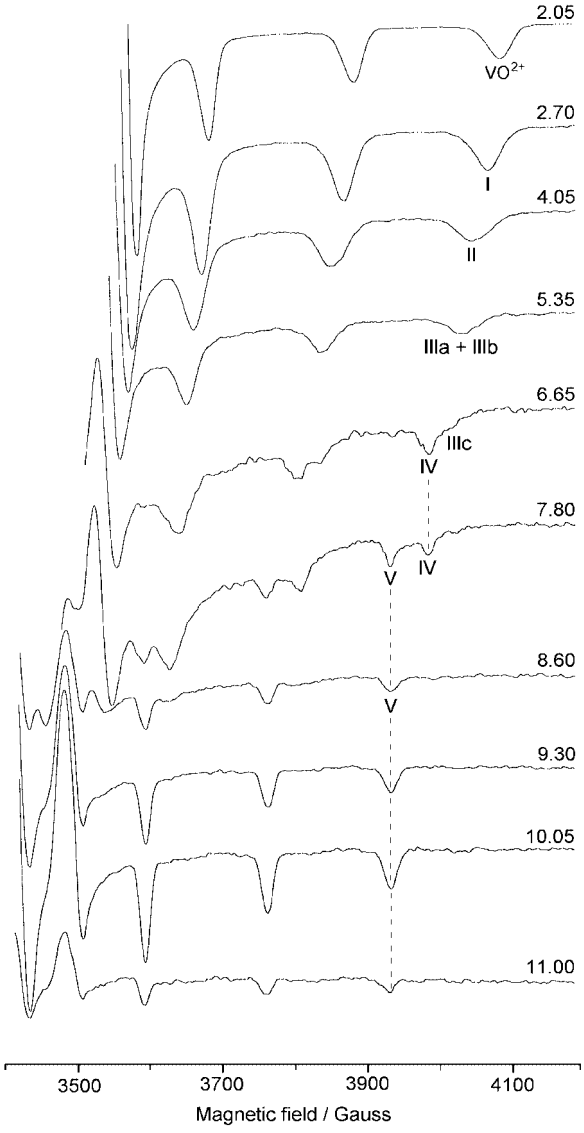
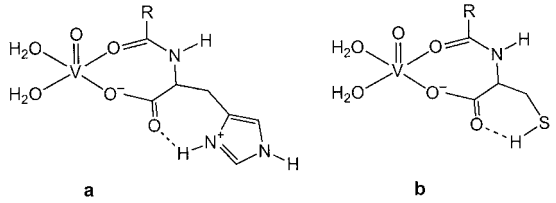


Figure 4. High-field region of the X-Band EPR spectra recorded at 140 K as a function of pH on aqueous solutions of $V^{IV}O$ and GlyGlyHis at a metal-to-ligand molar ratio of 1:15 and a $V^{IV}O$ concentration of 4 mM. The dotted line indicates the high-field resonance of the complexes with the deprotonated amide group.

If the N-terminal residue has no coordinating chain donor, the (NH_2, CO) donor set is not a good anchoring group and the (COO^-, CO) donor set is stronger.[10] This supports the spectroscopic results of previous works.[19] In this system the complexation starts with $VOLH_2$ (**I** in Figure 4), similar to the analogous complexes of GlyHis and GlyCys.[10] The "basicity adjusted" stability constant of this complex (–0.46) is somewhat higher than expected for pure carboxylate coordination[26] and indicates extra donors, presumably the amide carbonyl, which closes a seven-membered chelate ring. The EPR parameters (Table 2) are in agreement with recent reports,[9c,9h,10,27a,27b] which attribute hyperfine coupling constants in the range 175–179×10^{-4} cm^{-1} to mono-chelated species. We believe that the formation of an intramolecular hydrogen bond between the carboxylate oxygen and the NH^+ group of imidazole provides an extra stabilisation of this structure (Scheme 3). In fact, this coordination mode is not observed with dipeptides or tripeptides with glycine in the C-terminal part, for example GlyGly,[19] HisGly,[10] GlyGlyGly[19] or HisGlyGly (this work).



Scheme 3. Structures of $VOLH_2$ complexes formed by GlyGlyHis (a) and GlyGlyCys (b).

Around pH 4 a species of composition VOL_2H_4 (**II** in Figure 4) predominates. It can be described as a bis-chelated complex of the [2×(COO^-, CO)] type (Table 2). The two ligands have their amino and imidazole nitrogens still protonated. The EPR parameters are in agreement with the literature data,[9c,9h,10,27a,27b] which report hyperfine coupling constants in the range $171–174 \times 10^{-4}$ cm^{-1} for bis-chelated species.

In VOL_2H_3 (**IIIa** in Figure 4) one of the two ligands has released a proton from the amino group, and the coordination therefore becomes [(NH_2, CO); (COO^-, CO)], with EPR parameters $g_z = 1.945$ and $A_z = 169 \times 10^{-4}$ cm^{-1}, which are intermediate between those of the [2×(COO^-, CO)] and [2×(NH_2, CO)] donor sets.[10]

VOL_2H_2 and VOL_2H (**IIIb** and **IIIc**, respectively, in Figure 4) form from VOL_2H_3 upon deprotonation of the two imidazole rings (pK_a = 5.03 and 6.02, respectively). The coordination mode can be described as [(NH_2, CO); (CO, COO^-)], since the spectral parameters don't change, although an [(NH_2, CO); (N_{im}, COO^-)] donor set cannot be excluded.

The deprotonation of the first amide group leads to $VOLH_{-1}$, which has an [(NH_2, N^-, CO, $N_{im}{}^{ax}$); H_2O] donor set (**IV** in Figure 4 and **c** in Scheme 2). The weak coordination of the imidazole nitrogen in the axial position could stabilise this species.

The deprotonation of the second amide group gives $VOLH_{-2}$ (**V** in Figure 4 and **d** in Scheme 2), with an (NH_2, N^-, N^-, N_{im}) donor set and g_z = 1.960, A_z = 153×10^{-4} cm^{-1}. The difference between this and the $VOLH_{-2}$ complex formed by HisGlyGly is the presence of an imidazole nitrogen instead of a carboxylate group in the fourth equatorial position, which lowers the hyperfine coupling constant. For an (NH_2, N^-, N^-, COO^-) coordination we expect the same parameters observed for $VOLH_{-2}$ of HisGlyGly.

A special comment must be devoted to the different behaviour of GlyGlyHis and GlyGlyGly. We studied the system with GlyGlyGly up to an L/M molar ratio of 20.[21] The complexation process stops at acid pH values with the formation of species with only COO^- coordination. The higher stability of the (COO^-, CO) donor set for GlyGlyHis is responsible for the stabilisation of the mixed species

VOL_2H_3, VOL_2H_2 and VOL_2H, which avoids hydroxide precipitation and promotes the amide deprotonation.

Glycylglycyl-L-cysteine (GlyGlyCys)

With oligopeptides containing L-cysteine the complexation of Ni^{II}, Pd^{II}, Co^{II}, Zn^{II} and Cd^{II} depends strongly on the position of the residue in the peptide and on the "hardness" of the metal ion.[28] $V^{IV}O$ follows the same trend: with CysGly a stable cysteine-like (NH_2, S^-) coordination is favoured and mono [VOA] and bis $[VOA_2]^{2-}$ complexes are formed. On the other hand, with a C-terminal cysteine dipeptide like GlyCys, $V^{IV}O$ promotes the coordination of the deprotonated amide group as with Ni^{II} and Pd^{II}.[24a,24b]

GlyGlyCys (H_3L^+, Table 1 and Scheme 1) shows three pK_a values: at 2.98 (COOH), 7.77 (NH_3^+) and 9.59 (SH), in good agreement with those in the literature.[24a] The species distribution for this system as a function of pH is displayed in Figure 5.

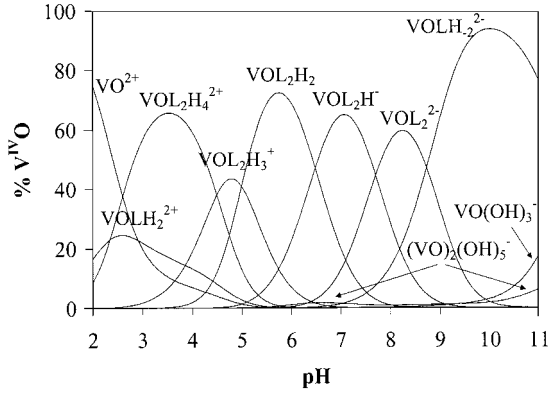


Figure 5. Species distribution for the $V^{IV}O$–GlyGlyCys system at a metal-to-ligand molar ratio of 1:15 and a $V^{IV}O$ concentration of 1 mM.

As with GlyGlyHis, the complexation scheme starts with the formation of $VOLH_2$ (**I** in Figure 6) and VOL_2H_4 (**II** in Figure 6) at pH around 3 and 4, respectively, in strongly overlapping processes. The donor sets are (COO^-, CO) and [2×(COO^-, CO)], respectively, and the EPR parameters (g_z = 1.935, A_z = 176×10^{-4} cm^{-1} and g_z = 1.940, A_z = 171×10^{-4} cm^{-1}) are in good agreement with those of the species formed by 2-mercaptopropionylglycine,[27a] GlyHis and GlyCys,[10] and GlyGlyHis (see above). In both complexes each ligand maintains protonated amino and thiol groups. As for GlyGlyHis, the (COO^-, CO) coordination is stabilised by an intramolecular hydrogen bond between the carboxylate oxygen atom and the SH group of cysteine (Scheme 3).

The consecutive deprotonation of the amino groups gives rise to VOL_2H_3 and VOL_2H_2 (**III** and **IV** in Figure 6) in the pH range 5–7. A mixed [(NH_2, CO); (COO^-, CO)] coordination can be proposed for the first complex and a [2×(NH_2, CO)] coordination for the second. The EPR parameters support this assignment (Table 2). With increasing pH the first SH group undergoes deprotonation and VOL_2H_2 is replaced by VOL_2H (**V** in Figure 6), which is

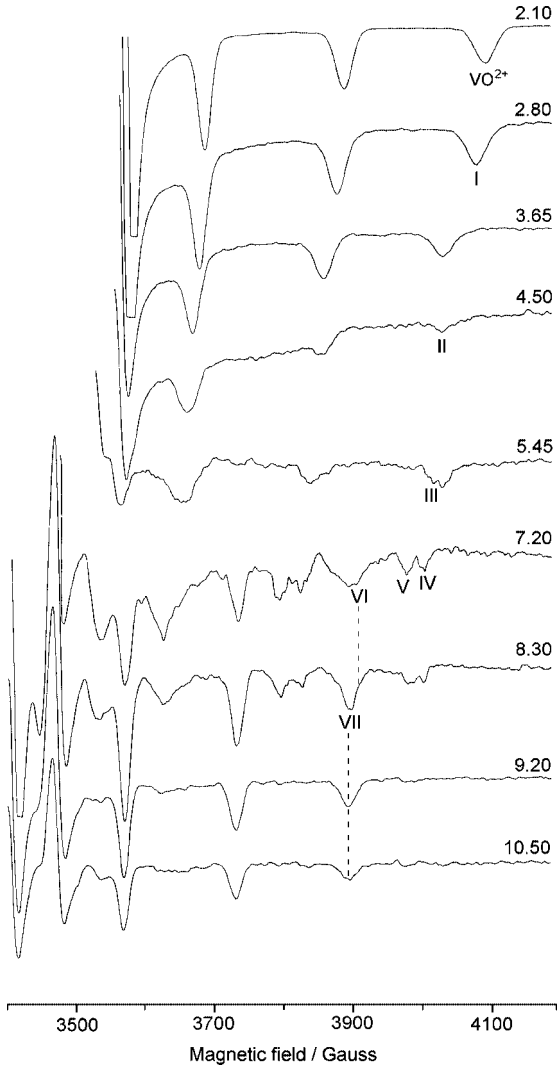


Figure 6. High-field region of the X-Band EPR spectra recorded at 140 K as a function of pH on aqueous solutions of $V^{IV}O$ and GlyGlyCys at a metal-to-ligand molar ratio of 1:15 and a $V^{IV}O$ concentration of 4 mM. The dotted line indicates the high-field resonance of the complexes with the deprotonated amide group.

presumably a complex with a mixed donor set [(S^-, COO^-); (NH_2, CO)]. This conclusion is supported by the EPR parameters (g_z = 1.950, A_z = 160×10^{-4} cm^{-1}), which are intermediate between those of [2×(NH_2, CO)] and [2×(S^-, COO^-)] and similar to those of the analogous species with GlyCys.[10]

Upon deprotonation of the first amide group the coordination of the first ligand molecule switches from (NH_2, CO) to (NH_2, N^-, CO) and VOL_2, with the donor set [(NH_2, N^-, CO); (S^-, COO^{-ax})], is formed (**VI** in Figure 6 and **e** in Scheme 2). Its spectral parameters (Table 2) are consistent with our expectations, as will be discussed later.

At pH values above 8 potentiometry suggests a $VOLH_{-2}$ species, which is explainable by the deprotonation of the second amide group and the coordination of an S^- donor (**VII** in Figure 6 and **f** in Scheme 2). The EPR parameters, g_z = 1.967, A_z = 148×10^{-4} cm^{-1}, are consistent with an

(NH_2, N^-, N^-, S^-) coordination mode, which is also observed with Ni^{II}.[28a]

Discussion

The Chasteen "additivity rule" is a powerful tool to estimate the ^{51}V hyperfine constant along the z axis of $V^{IV}O$ complexes and is based on the individual contribution to A_z from the four equatorial donors.[23] This rule provides a valid criterion for the identification of the equatorial donor atoms. Data for the contribution of amino and imidazole nitrogen atoms and for thiolate group were taken from Chasteen's publication.[23] For carboxylate, the value of 42.1×10^{-4} cm^{-1} reported by Kiss and Costa-Pessoa was chosen.[27a] The mean value for a neutral oxygen atom belonging to an amide CO group is 43.5×10^{-4} cm^{-1}.[29]

The amide contribution, A_z(amide), is in the range 29–43×10^{-4} cm^{-1},[30] and is sensitive to the charge of the coordination sphere. We have demonstrated previously that this contribution decreases with an increased donation of electron density from the ligands to the $V^{IV}O$ ion.[10] In particular, we found that if the total charge of the donor atoms in the equatorial plane (TEC) is –2, –3 or –4 (including the –1 charge of the deprotonated peptide nitrogen), the mean A_z(amide) values are 35.3, 38.6 and 40.9×10^{-4} cm^{-1}, respectively, in good agreement with those calculated by Kabanos and Deligiannakis.[9d] We were able to extrapolate the contribution of the amide group when TEC is –1 (32.7×10^{-4} cm^{-1}).[10]

Table 3 is based on these contributions to the value of A_z. If we examine the three fully deprotonated $VOLH_{-2}$ species no ambiguity exists in the coordination mode: two amide groups are deprotonated and three of the four equatorial positions are occupied by amino and two deprotonated amide groups. The fourth position is occupied by three different donors: the carboxylate group in HisGlyGly and the side-chain of the C-terminal amino acid in GlyGlyHis and GlyGlyCys (i.e. the imidazole nitrogen of histidine and the thiolate group of cysteine, respectively; Table 3). This is substantiated by the A_z values of 158.6×10^{-4} cm^{-1} for HisGlyGly, 152.9×10^{-4} cm^{-1} for GlyGlyHis and 147.9×10^{-4} cm^{-1} for GlyGlyCys. The experimental values are very close to the calculated ones, with differences of less than 1%.

A substantially good agreement is also found for the other species involving a deprotonated amide group (Table 3), according to our previous analysis.[10] The structures of the complexes formed by deprotonation of the amide groups are displayed in Scheme 2.

We used the thermodynamic data to evaluate in which form the $V^{IV}O$ ion and $V^{IV}O$ insulin-mimetic compounds are transported in the human body. Given the chemical composition of serum,[31] the two h.m.m. (high molecular mass) metal-ion binders in the serum are albumin and transferrin,[16,17] whilst among the l.m.m. (low molecular mass) constituents, oxalic acid, lactic acid, citric acid and phosphate are the most likely.[15] Detailed studies have been reported on the interaction of $V^{IV}O$ ion with transferrin and albumin.[32]

Human serum transferrin (hTF), a bilobal, single-chain protein, is the main Fe^{III} transport protein in serum. It is responsible for tight, reversible binding of two equivalents of Fe^{III} and binds a variety of other metal ions such as $V^{IV}O$.[33] It has two non-equivalent vanadium binding sites, with different binding constants.[32f,34a,34b] This requires a synergistic anion binding.[33] These sites should correspond to the usual N-lobe and C-lobe binding sites of the protein. An approximate value for the two stability constants ($\log K_1^* = 13.2$ and $\log K_2^* = 12.2$) was reported recently by some of us.[15]

Human serum albumin (HSA), bovine serum albumin (BSA) and rat serum albumin (RSA) share the same structural motif in the N-terminal part of the protein, namely two amino acids with non-coordinating or weakly coordinating side-chains in the first two positions and a histidine residue in the third position (AspAlaHis for HSA, AspThrHis for BSA and GluAlaHis for RSA). This results in an identical chemical and spectroscopic behaviour; for instance, Cu^{II} shows the same electronic[35] and EPR parameters.[36] It has been proposed that this is the site preferred by $V^{IV}O$ too, although there are four or five additional weaker binding sites in the side-chain, namely Asp and Glu carboxylates.[14,32c] The substitution in other mammalian species of the histidine residue in the third position, e.g. Tyr in dog serum albumin (DSA) and Glu in chicken serum albumin (CSA), produces a lack of specific binding to metal ions.[37]

These features, as well as the geometry of the ligand array in the native protein and the nature of the donor groups, were considered in the design of the first model of this structural motif, GlyGlyHis-*N*-methylamide. Moreover, theoretical calculations using energy minimisation show similar conformations for AspAlaHis and GlyGlyHis bound to Cu^{II}.[38] For these reasons, we propose that

Table 3. Coordination modes of $V^{IV}O$ complexes with deprotonated amide groups on the basis of the EPR data.

Ligand	Complex	g_z	A_z exp.[a]	A_z calcd.[a]	Donor atoms
HisGlyGly	$VOLH_{-1}$	1.950	161.1	161.9	[(NH_2, N^-, CO, $N_{im}{}^{ax}$); H_2O]
HisGlyGly	$VOLH_{-2}$	1.951	158.6	159.4	(NH_2, N^-, N^-, COO^-, $N_{im}{}^{ax}$)
GlyGlyHis	$VOLH_{-1}$	1.950	161.4	161.9	[(NH_2, N^-, CO, $N_{im}{}^{ax}$); H_2O]
GlyGlyHis	$VOLH_{-2}$	1.960	152.9	151.4	(NH_2, N^-, N^-, N_{im})
GlyGlyCys	VOL_2	1.960	151.9	150.8	[(NH_2, N^-, CO); (S^-, COO^{-ax})]
GlyGlyCys	$VOLH_{-2}$	1.967	147.9	149.3	(NH_2, N^-, N^-, S^-)

[a] A_z measured in 10^{-4} cm^{-1} units.

GlyGlyHis is also a good model for the interaction between $V^{IV}O$ and albumin. The structure of Cu^{II}-GlyGlyHis-*N*-methylamide has been solved by X-ray crystallography.[39] Cu^{II} ion is chelated by the NH_2-terminal nitrogen, the next two deprotonated amide nitrogens and a histidyl imidazole nitrogen of GlyGlyHis through an (NH_2, N^-, N^-, N_{im}) donor set in a slightly distorted square-planar arrangement similar to that which we propose for $VOLH_{-2}$ (Table 2), the most relevant form under physiological conditions.

An association constant of 2.6×10^6 M^{-1} has been measured for the N-terminal site of BSA at pH 5, although at physiological pH the corresponding value is thought to be several orders of magnitude higher.[14] No data exist for the interaction with a model system like GlyGlyHis.

Chasteen et al.[32b] have found that transferrin binds $V^{IV}O$ about six times more strongly than albumin in vitro. If we accept Chasteen's assumption that albumin is an efficient binder and apply his estimation [K(hTF)/K(alb) ≈ 6], the calculated biodistribution indicates that only the h.m.m. fraction of the serum (80% albumin and 20% transferrin) binds $V^{IV}O$.[15]

By taking into account the data for the association constants for transferrin,[15] the stability constants of the model ligand for albumin (GlyGlyHis), and the binary systems with oxalate,[40] lactate,[41] citrate[42] and phosphate,[43] the complete distribution of $V^{IV}O$ ion can be calculated for the conditions in the serum. The results are reported in Table 4.

Table 4. Species distribution [%] of the $V^{IV}O$ ion between the components of the blood serum at pH 7.4.[a]

$c_{V^{IV}O}$ [μM]	l.m.m. components	Transferrin	GlyGlyHis
1	0.1	99.7	0.2
10	0.1	99.7	0.2
100	22.0	70.8	7.2

[a] Oxalate = 9.20 μM; lactate = 1.51 mM; citrate = 99.0 μM; phosphate = 1.10 mM; transferrin = 37 μM; GlyGlyHis = 630 μM.

Table 4 reveals that at a $V^{IV}O$ concentration of 1 μM and 10 μM the $V^{IV}O$ ion is almost totally bound to transferrin, with less than 1% bound to albumin. However, at a concentration of 100 μM both albumin and l.m.m binders participate in the transport of $V^{IV}O$ ion, with approximately 7% bound to albumin and about 22% bound to l.m.m. ligands, especially citrate.

This discussion can be extended to the transport of insulin-mimetic compounds. We examined the bis-chelate compounds formed by $V^{IV}O$ ion with maltolato (ma),[44] picolinato (pic) and 6-methylpicolinato (6-Mepic),[45] 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridinone (HDP),[46] 5-methoxycarbonylpicolinic acid (5-MeOpic),[47] 2-hydroxypyridine *N*-oxide (HPO) and 2-mercaptopyridine *N*-oxide (MPO).[48] For the calculations we used the association constants for transferrin;[15] the stability constants of the binary complexes with GlyGlyHis, oxalate,[40] lactate,[41] citrate,[42] and phosphate;[43] and the thermodynamic data for the $V^{IV}O$ species formed by the seven carriers[43,47,48b,49] and for the $V^{IV}O$ ternary complexes formed by the carriers and the l.m.m. binders of the serum.[43,47,48b,49b,49c] If a 10 μM level of the insulin-mimetic compounds in the serum is used, $V^{IV}O$ is totally bound to transferrin, with a minor amount bound to albumin, whereas the binding to the l.m.m. components is negligible. Only in the system with HDP, due to the high value of the stability constant of the bis chelated complex, is about 30% of $V^{IV}O$ bound to the carrier.

If the concentration of the $V^{IV}O$ complex is increased to 100 μM, albumin and l.m.m. binders also take part in the metal binding. The results of the biodistribution of insulin mimetics in serum are presented in Table 5.

Table 5. Species distribution [%] of $V^{IV}O$ insulin-mimetic compounds between the components of blood serum at pH 7.4.[a]

Complex[b]	VO^{IV} carrier	l.m.m components		h.m.m. components	
		Binary species	Ternary species	Transferrin	GlyGlyHis
$[VO(ma)_2]$	10.4	4.6	15.4	66.7	2.9
$[VO(pic)_2]$	0.3	2.7	28.8	65.7	2.5
$[VO(6\text{-}Mepic)_2]$	0.1	4.6	24.2	67.7	3.4
$[VO(HDP)_2]$	79.6	0.0	3.4	16.9	0.1
$[VO(5\text{-}MeOpic)_2]$	0.1	2.8	29.3	65.4	2.4
$[VO(HPO)_2]$	30.2	0.1	29.0	40.4	0.3
$[VO(MPO)_2]$	1.5	10.9	13.7	69.2	4.7

[a] $V^{IV}O$ = 100 μM; oxalate = 9.20 μM; lactate = 1.51 mM; citrate = 99.0 μM; phosphate = 1.10 mM; transferrin = 37 μM; GlyGlyHis = 630 μM. [b] ma = maltolato; pic = picolinato; 6-Mepic = 6-methylpicolinato; HDP = 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridinone; 5-MeOpic = 5-(methoxycarbonyl)picolinic acid; HPO = 2-hydroxypyridine *N*-oxide; MPO = 2-mercaptopyridine *N*-oxide.

The amount of $V^{IV}O$ bound to transferrin and albumin depends on the strength of the $V^{IV}O$–carrier interaction. With the two stronger binders (HDP and HPO) a high fraction of $V^{IV}O$ is found in the native form of $[VO(carrier)_2]$, whereas in the other systems most of the $V^{IV}O$ (65–70%) is bound to transferrin and the rest is distributed among the binary and ternary compounds of the l.m.m. binders and the complexes with albumin. In particular, with the five weaker binders about 2–5% is associated with albumin. Of this amount, the highest part is present as $VOLH_{-2}$ and the rest as $VOLH_{-1}$. These data confirm the results of previous studies substantiating that the amount of $V^{IV}O$ bound to albumin is lower than that bound to transferrin.[15] However, it is worth noting that albumin can play a secondary role comparable to that of the l.m.m. components.

The distribution of $V^{IV}O$ between h.m.m. and l.m.m. fractions is very sensitive to the binding constants of $V^{IV}O$–hTF.[15] As a consequence of the relatively high uncertainties in the transferrin binding constants (±0.8 log units),[50] the results could change considerably if the association constants are overestimated. For example, if the values of $\log K_1^*$ and $\log K_2^*$ were 12.4 and 11.4, the amount of $V^{IV}O$ transported by albumin, at a vanadium concentration of 100 μM, rises to 9.1%. In the systems with the insulin-mimetic compounds, the fraction increases to 4.4% with ma, 4.1% with pic, 5.3% with 6-Mepic, 0.2% with HDP, 3.7% with 5-MeOPic, 0.5% with HPO and 6.3% with MPO.

However, in light of the recent results of Liboiron et al.,[17] the equilibrium data concerning the formation of ter-

nary complexes between the insulin-mimetic $V^{IV}O$ compounds and albumin could be important.

Finally, we would like to note that we have neglected the possibility of reduction and oxidation of $V^{IV}O$ species, although $V^{IV}O$ compounds in aqueous solution at varying pH, ligand-to-metal molar ratios and concentration and in the presence of biogenic ligands are subject to several processes, including redox activity.[51] However, in the conditions of transport (acid pH in the stomach and pH 7.4 in the blood serum), a complex of $V^{IV}O$ is relatively stable. Only after its translocation across the cell membrane into the intracellular medium can reducing agents such as glutathione (GSH), nicotinamide adenine dinucleotide (NADH) or ascorbate reduce $V^{IV}O$ to V^{III} [52a–52c] or potential oxidizing agents such as oxygen, peroxide, superoxide[53] or $OH^{\cdot}$ radicals[54] oxidise $V^{IV}O$ to V^{V}.

Conclusions

Simple HisGlyGly, GlyGlyHis and GlyGlyCys tripeptides coordinate the $V^{IV}O$ ion at acidic pH values and keep it in solution until the deprotonation of the amide group. Two main differences distinguish HisGlyGly, GlyGlyHis and GlyGlyCys. With HisGlyGly, only the weak coordination of carboxylate is observed at very acidic pH values, and this is easily replaced by a stronger (NH_2, CO, N_{im}^{ax}) donor set. With GlyGlyHis and GlyGlyCys an intramolecular hydrogen bond between the carboxylate oxygen and NH or SH bond of GlyGlyHis and GlyGlyCys stabilises the (COO^-, CO) donor set, so that $VOLH_2$ and VOL_2H_4 species with (COO^-, CO) and [2×(COO^-, CO)] coordination are formed. This coordination mode is indicated by a higher value of the "basicity adjusted" stability constant than expected for pure carboxylate coordination and by an accurate measurement of the A_z value, which is around 175–176×10^{-4} cm^{-1} for mono-chelated complexes and 171–172×10^{-4} cm^{-1} for bis-chelated species.

With GlyGlyHis and GlyGlyCys the final complexes (NH_2, N^-, N^-, N_{im}) and (NH_2, N^-, N^-, S^-) are more stable than (NH_2, N^-, N^-, COO^-) because the imidazole nitrogen and thiolate sulfur are more effective donors than carboxylate oxygen. Therefore, at constant L/M molar ratios (15), $VOLH_{-2}$ is the main species in solution until pH 11.6 with GlyGlyCys, pH 11.1 with GlyGlyHis and pH 10.8 with HisGlyGly.

The final species with both the amide groups deprotonated − (NH_2, N^-, N^-, COO^-), (NH_2, N^-, N^-, N_{im}) and (NH_2, N^-, N^-, S^-) − are easily detected by EPR spectroscopy. A contribution of 38.6, 35.3 and 38.6×10^{-4} cm^{-1} to the A_z value is attributed to the N^- donor according to a total charge in the equatorial plane (TEC) of –3, –2 and –3, respectively.[10]

A direct application of these results is of biological interest. In fact, the binding to GlyGlyHis can be considered a good model for the interaction of the $V^{IV}O$ ion with albumin.[6] At physiological pH, GlyGlyHis binds mainly $V^{IV}O$ with an (NH_2, N^-, N^-, N_{im}) donor set, as with Cu^{II} and Ni^{II},[6] to form a very stable $VOLH_{-2}$ species.

Albumin can also take part in the transport of vanadium and insulin-mimetic $V^{IV}O$ compounds such as those formed by maltolato, picolinato, 6-methylpicolinato, 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridinone, 5-methoxycarbonylpicolinato, 2-hydroxypyridine *N*-oxide and 2-mercaptopyridine *N*-oxide. Its contribution is less important than that of transferrin, but cannot be neglected completely as proposed by previous studies.[15,47,48b,49c] Accordingly, the formation of adducts between the insulin-mimetic compounds [VO(maltolato)$_2$] and albumin has been reported.[17] Thus, the role of albumin in these processes must be re-evaluated.

Potentiometric and spectroscopic studies on the biodistribution of $V^{IV}O$ ion between GlyGlyHis and an organic carrier will be useful to establish the importance and role of the ternary complexes in the transport of the insulin-mimetic compounds in vivo.

Experimental Section

Chemicals: The ligands were Aldrich products of puriss. quality. Their purity and concentration were determined by Gran's method.[55] $V^{IV}O$ solutions were prepared following literature methods.[56] The concentration of the metal ion was determined by $KMnO_4$ titration and that of H^+ by a potentiometric titration using the appropriate Gran function. The ionic strength was adjusted to 0.1 M with KNO_3. The temperature was 25.0±0.1 °C.

Potentiometric Measurements: The stability constants of proton and $V^{IV}O$ complexes were determined by pH-potentiometric titrations on 1.2–2 mL of the samples. The ligand-to-metal molar ratio was 1:1, 5:1, 10:1 and 15:1 and $V^{IV}O$ concentration was 0.001–0.004 M. Titrations were performed from pH 2.0 until precipitation or very extensive hydrolysis by adding carbonate-free NaOH of known concentration (ca. 0.1 M NaOH). The pH was measured with a Russel CMAWL/S7 semi-micro combined electrode, calibrated for hydrogen concentration by the method of Irving et al.[57] Measurements were carried out at 25.0±0.1 °C and at a constant ionic strength of 0.1 M KNO_3 with a MOLSPIN pH-meter equipped with a digitally operated syringe (the Molspin DSI 0.250 mL) controlled by a computer. Purified argon was bubbled through the samples to ensure the absence of oxygen. The number of experimental points was 100–150 for each titration curve and the reproducibility of the points included in the evaluation was within 0.005 pH units in the whole pH range examined. As usual, the stability of the complexes is reported as the logarithm of the overall formation constant $\beta_{pqr} = [VO_pL_qH_r]/[VO]^p[L]^q[H]^r$, where L is the deprotonated form of the ligand and H is the proton, calculated with the aid of the SUPERQUAD program.[58] Standard deviations were calculated by assuming random errors. The conventional notation has been used. Negative indices for protons indicate either the dissociation of groups that do not deprotonate in the absence of $V^{IV}O$ coordination, or hydroxo ligands. Hydroxo complexes of $V^{IV}O$ were taken into account. The following species were assumed: $[VO(OH)]^+$ ($\log\beta_{10-1} = -5.94$), $[(VO)_2(OH)_2]^{2+}$ ($\log\beta_{20-2} = -6.95$), with stability constants calculated from the data of Henry et al.[59] and corrected for the different ionic strengths by use of the Davies equation,[60] $[VO(OH)_3]^-$ ($\log\beta_{10-3} = -18.0$) and $[(VO)_2(OH)_5]^-$ ($\log\beta_{20-5} = -22.0$).[61]

Spectroscopic Measurements: Anisotropic EPR spectra were recorded on aqueous solutions with an X-band (9.15 GHz) Varian E-9 spectrometer in the temperature range 120–140 K. As usual for

low-temperature measurements, a few drops of DMSO were added to the samples to ensure good glass formation. The spectra were simulated with the computer program Bruker WinEPR SimFonia. Electronic spectra were recorded with a Perkin-Elmer Lamda 9 spectrometer. All operations were performed under a purified argon atmosphere in order to avoid oxidation of $V^{IV}O$ ion.

Supporting Information (see footnote on the first page of this article): Figures S1–S3 present the titration curves of the free ligands and of the systems with $V^{IV}O$ ion. Figures S4–S6 show the X-Band EPR spectra of $VOLH_{-2}$ complexes formed by the three ligands.

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Received: April 13, 2005
Published Online: October 27, 2005

