DOI: 10.1002/ejic.200600385

Interactions of Insulin-Mimetic Vanadium Complexes with the Cell Constituents ATP and Glutathione

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Keywords: Insulin mimetics / Vanadium oxido complexes / Coordination modes

In order to assess the molecular form of vanadium insulin-mimetic complexes in cells, the interactions of $V^{IV}O$–maltolate and $V^{IV}O$–dipicolinate systems with adenosine 5′-triphosphate (ATP) and glutathione (GSH) in aqueous solution were studied by employing pH potentiometry, and EPR, CD and UV/Vis spectroscopy. The stoichiometries and stability constants of the complexes formed were determined at 25 °C with an ionic strength of $I = 0.2\ mol\,dm^{-3}$ (KCl). The most probable binding mode of the complexes formed in solution was determined by means of various spectral methods. The results suggest that from among the important cell constituents, GSH mostly takes part in the reduction of V^{V} to V^{IV} and helps keep V^{IV} in this oxidation state. ATP, which is a strong $V^{IV}O$ binder, chelates the metal ion, forming binary and/or ternary complexes. The results of this work strongly suggest that ATP binds relevant $V^{IV}O$ species under cellular conditions and thus might somehow be involved in the insulin-mimetic action of $V^{IV}O$ compounds.

Introduction

Since the insulin-mimetic effects of vanadium compounds were first reported, a large number of vanadium complexes in the oxidation states III to V and as peroxo complexes have been prepared, tested biologically and found to be efficient,[1–3] but only one of them, bis(maltolato)oxidovanadium(IV) ($VO(malt)_2$), has participated in clinical studies; it passed the phase I test in Canada.[2,4] In vitro, vanadium compounds may enter cells by several pathways, including passive diffusion, whereas when given orally vanadium(III,IV,V) complexes undergo parallel ligand exchange and redox reactions with the endogenous or exogenous bioligands of the human body;[1,5,6] this is mostly independent of the original form, and depends primarily on the nature of the interacting bioligand.

For this reason, the interactions of various insulin-mimetic $V^{IV}O$ compounds have been studied with the high molecular mass (h.m.m.) serum components, albumin and transferrin, and also with some of the low molecular mass (l.m.m.) components, such as lactate, oxalate, phosphate and citrate, which may be potential transporters of $V^{IV}O$ in the blood.[7–13] Detailed speciation studies led to the suggestions concerning the actual chemical forms of the vanadium compounds formed from the original carrier complex during their transport in the blood. We found that little of the vanadium remains bound to the original carrier: l.m.m. serum components (citrate) and h.m.m serum components (transferrin) displace most of the carrier molecules.[6,11–13] Accordingly, vanadium can enter cells in the oxidation state IV through the iron path, and in part in the oxidation state V through the phosphate path.

In the intracellular medium, reducing agents can interact in a redox manner with vanadium(V,IV) complexes. The most frequently discussed candidate for reduction is glutathione (GSH).[14,15] The extent to which vanadium is reduced in the redox equilibrium largely depends on the stabilisation of vanadium(IV,V) by complexation. A high intracellular excess of GSH (the usual concentration level is millimolar), i.e. about three orders of magnitude more than the biologically relevant concentration of vanadium (the usual concentration level is micromolar) increases the possibility of vanadium being present in lower oxidation states. Excess GSH and also GSSG (oxidized glutathione) readily coordinate to $V^{IV}O$.[16,17] The vanadium complexes thus formed may undergo ligand exchange with a variety of l.m.m. and h.m.m. biogenic ligands available in cells. Among the l.m.m. binders, adenosine 5′-triphosphate (ATP) may be of importance[18] as it efficiently binds $V^{IV}O$ and is present in millimolar concentrations in cells.

Sakurai and Tsuji considered the most relevant oxidation state of vanadium in the cell to be IV because of the reducing agents present in the cell (e.g. GSH or cysteine).[19] We

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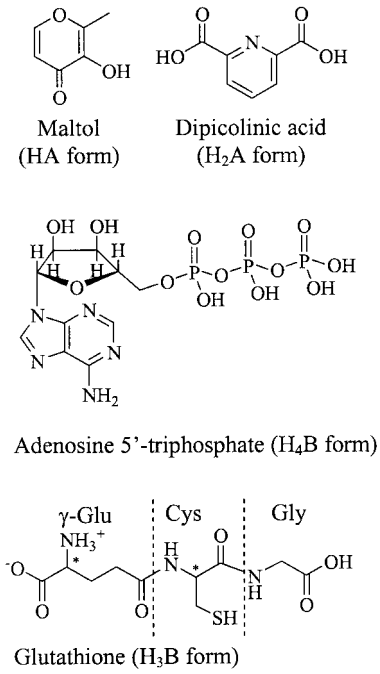
accepted this and studied vanadium only in the $V^{IV}O$ form, even though reoxidation of $V^{IV}O$ to V^{V} is an obvious assumption, which can have different importance at different compartments of the cell. Accordingly, the aim of the present work was to attain a better understanding of the behaviour of $V^{IV}O$ insulin-mimetic complexes as they enter cells and interact with l.m.m. cell constituents. Of the $V^{IV}O$ complexes, we chose the $V^{IV}O$–dipicolinate and $V^{IV}O$–maltolate systems, and as cell constituents we selected GSH and ATP (see Scheme 1). The equilibria in the systems were studied by a combination of pH potentiometry and spectroscopic methods [electron paramagnetic resonance (EPR), electronic absorption (UV/Vis) and circular dichroism (CD)].



Scheme 1.

The complex-forming properties of $V^{IV}O$ with dipicolinate (dipic),[12] maltolate (malt),[7,8,20] ATP[18] and GSH[17] have been studied, and stability constants measured under similar experimental conditions have been reported previously.

Maltolate forms mono and bis complexes with the $V^{IV}O$ ion by the formation of five-membered (O^-, = O) chelates. The neutral bis complex $[VO(malt)_2(H_2O)]$ is the predominant species in the pH range 5–7. It occurs in two geometrical isomeric forms depending on the position of the solvent molecule; the water molecule can be either *cis* or *trans* to the oxido group of $V^{IV}O$. In aqueous solution at room temperature and neutral pH, the EPR spectra clearly indicate that the *cis* form predominates.[7,8,20] In a recent paper based on detailed EPR and ENDOR spectroscopic studies, Mustafi and Makinen concluded that in aqueous solution at room temperature maltolate coordinates to $V^{IV}O$ only in the *cis* form.[21]

Dipicolinate forms mono and bis complexes with the $V^{IV}O$ ion.[12] In the neutral mono complex, $[VO(dipic)(H_2O)_2]$, the ligand coordinates in a tridentate manner; the coordinating donor groups are two carboxylates, besides the two water molecules, and the pyridyl-N atom is in an axial position (*trans* to the oxido group). Bis complex formation in the $V^{IV}O$–dipic system is not as favoured as it is with maltolate. In $[VO(dipic)_2]^{2-}$, the first ligand coordinates in a tridentate manner, and the second ligand in a bidentate manner, having an uncoordinated carboxylate group. At the biologically relevant neutral pH values, dipicolinate is not a very efficient $V^{IV}O$ binder; mixed hydroxo and oligonuclear binary hydroxo species of $V^{IV}O$ are present in the solution, besides the neutral mono complex and the double negatively charged bis complex.[12]

Results and Discussion

$V^{IV}O$ Ternary Systems with ATP

ATP was found to coordinate to $V^{IV}O$ through the terminal phosphate donor(s) in the weakly acidic and neutral pH range to yield $[VOBH_x]^{x-2}$ (x = 2, 1, 0) and $[VOB_2]^{6-}$.[18] In the basic pH range, at low excess, ATP is not a very efficient $V^{IV}O$ binder because oligonuclear hydroxo species $\{[(VO)_2(OH)_5]^-\}_n$ are present in the solution besides the $V^{IV}O$–ATP complexes. At pH 7–8 the pH readings drift and pH equilibrium is reached within 10 min; in the pH range 8.8–10.4, pH stabilization was not reached within this time even at a 10-fold excess of ATP. Thus, the formation constants of the species formed in this pH region are only estimates. In contrast with our earlier report,[18] the formation of dinuclear species had to be assumed either to fit the pH-metric data or to explain the slight decrease in intensity of the EPR spectra. In the earlier work, formation of the binary hydroxo-bridged hydrolysed $V^{IV}O$ species $[(VO)_2(OH)_5]^-$ was not considered, though it is undoubtedly formed[22,23] and may be responsible for the decrease in the intensity of the signal rather than the dihydroxo-bridged $V^{IV}O$–ATP species: $[(VO)_2B_2H_x]^{x-4}$ (x = –2, –3, –4).

In the cells, ATP is present in high excess relative to $V^{IV}O$ and also relative to the carrier ligands. As the proton competition for the alcoholate donors decreases the ribose moiety becomes a more efficient binding site. In the slightly basic pH range, the complex $[VOB_2H_{-2}]^{8-}$ forms involving a mixed binding mode with one ATP coordinating through the phosphate chain and the other through the ribose moiety. In the species $[VOB_2H_{-4}]^{10-}$, both ATP molecules coordinate to the $V^{IV}O$ species through the ribose residue.[18] The CD spectra furnish information on the species in which the ribose moiety is coordinated to the $V^{IV}O$; these species are mostly formed above the physiological pH. In the highly basic pH range an extra deprotonation is detected by pH potentiometry and also by CD spectroscopy (see ESI-1), corresponding to the formation of $[VOB_2H_{-5}]^{11-}$. In this species the extra proton is probably lost from a coordinated water molecule.

$V^{IV}O$–dipic–ATP System

Our speciation calculations indicated that, in the ternary system with dipicolinic acid, ATP, as a stronger $V^{IV}O$

binder, will displace one of the dipicolinates from the coordination sphere of $V^{IV}O$. As a result, ternary complexes will exist, besides the binary ATP complexes, at physiological pH. The potentiometric data could be fitted by assuming the formation of the species $[VOAB]^{4-}$, $[VOABH_{-1}]^{5-}$ and $[VOABH_{-2}]^{6-}$ (see Table 1). The distribution diagram for the $V^{IV}O$–dipic–ATP system (Figure 1) reveals that the formation of the ternary complex $[VOAB]^{4-}$ is favoured in the weakly acidic pH range. Besides the carboxylate groups of dipic, the phosphate groups of ATP are expected to be coordinated in this complex while in $[VOABH_{-2}]^{6-}$, formed in the neutral pH range, the deprotonated ribose alcoholate groups are in equatorial positions.

Table 1. Stability constants of mixed ligand $V^{IV}O$ complexes of dipicolinate and maltolate (ligands A) with ATP (ligand B).

Ligand A	VOAB	$VOABH_{-1}$	$VOABH_{-2}$
Dipicolinate	11.0(1)	4.3(2)	–2.5(1)
Maltolate	13.5(2)	–	–2.8(9)

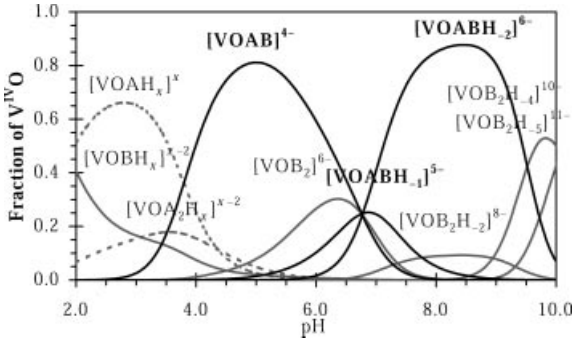


Figure 1. Concentration distribution diagram of the $V^{IV}O$–dipic(A)–ATP(B) system at 1:2:10 metal ion-to-ligand ratio, $C_{VO} \approx 0.004$ mol dm^{-3}.

The X-band EPR spectra of frozen solutions of the $V^{IV}O$–dipic binary system and the ternary system with ATP at pH < 8 could be simulated as axial spectra. At pH > 8, the species with the $(O^-,O^-)(O^-,O^-)$ donor set has a rhombic EPR spectrum. The high-field region corresponding to $A_\parallel$ or A_z and M_I = 5/2 and 7/2 yields more information on the type and number of species formed. As may be seen in Figure 2, the EPR spectra for the $V^{IV}O$–dipic–ATP system resemble those recorded for the $V^{IV}O$–ATP system; no new signals could be detected. Ternary complex formation is accompanied only by phosphate/carboxylate substitution in the equatorial plane, and does not result in any significant change in the EPR parameters. The EPR parameters of the binary complexes VOA of dipic and $[VOB_2]^{6-}$ of ATP with carboxylate or phosphate groups in the equatorial plane of $V^{IV}O$ are also expected to be the same within the uncertainties of the parameters (see Table 2). However, as dipic retards the hydrolysis of the $V^{IV}O$ complexes present in the basic pH range, an improved signal-to-noise ratio may be observed in the ternary system as compared with the binary $V^{IV}O$–ATP system; dipic helps to keep $V^{IV}O$ in solution. A similar observation may be made concerning the CD spectra; they are more intense for the ternary system.

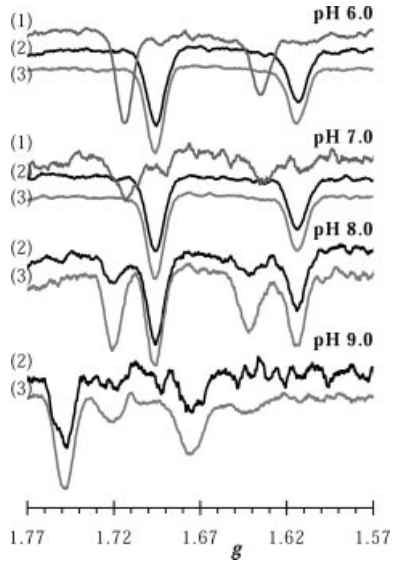


Figure 2. High field range of the first-derivative EPR spectra at 77 K of frozen solutions containing: (1) $V^{IV}O$ – dipic 1:2, (2) $2V^{IV}O$ – ATP 1:10, (3) $V^{IV}O$ – dipic – ATP 1:2:10 metal ion-to-ligand ratio, $C_{VO} \approx 0.004$ mol dm^{-3}. The pH values are indicated.

Table 2. EPR parameters ($g_\parallel$, $A_\parallel$)[a] for the binary and ternary complexes of $V^{IV}O$ formed with dipicolinate, maltolate and ATP.

Species	$g_\parallel$	$A_\parallel$ ($\times 10^4$ cm^{-1})	Chelating set
$[VO(H_2O)_5]^{2+}$	1.933	182.6	$4\times H_2O$
Dipicolinate			
VOA	1.931	178.4	$(COO^-,COO^-)\ 2\times H_2O$
$[VOA_2]^{2-}$	1.939	167.8	$(COO^-,COO^-)(COO^-,$pyridyl–N$)$
Maltolate			
$[VOA]^+$	1.937	175.1	$(O^-,=O)\ 2\times H_2O$
VOA_2	1.938	171.4	$(O^-,=O)\ (O^-,=O_{ax})\ H_2O$
$[VOA_2H_{-1}]^-$	1.942	166.8	$(O^-,=O)\ (O^-,=O_{ax})\ OH^-$
ATP			
$[VOBH_x]^{x-2}$	1.930	181.8	$(PO_3^{2-}–O–PO_2^-–O–PO_2^-–)\ 2\times H_2O$
$[VOB_2]^{6-}$	1.933	178.4	$2\times(PO_3^{2-}–O–PO_2^-–O–PO_2^-–)$
$[VOB_2H_{-2}]^{8-}$	1.946	166.8	$(PO_3^{2-}–O–PO_2^-–O–PO_2^-–)\ (O^-,O^-)$
$[VOB_2H_{-4}]^{10-}$	1.958	151.8	$(O^-,O^-)\ (O^-,O^-)$
Dipicolinate + ATP			
$[VOAB]^{4-}$	1.933	178.1	$(COO^-,COO^-)\ (PO_3^{2-}–O–PO_2^-–O–PO_2^-–)$
$[VOABH_{-1}]^{5-}$	–	–	[b]
$[VOABH_{-2}]^{6-}$	1.946	167.0	$(COO^-,COO^-)\ (O^-,O^-)$
Maltolate + ATP			
$[VOAB]^{3-}$	1.941	169.1 ?	$(O^-,=O)\ (PO_3^{2-}–O–PO_2^-–O–PO_2^-–)$
$[VOABH_{-2}]^{5-}$	1.943	166.4 ?	$(O^-,=O)\ (O^-,O^-)$

[a] The relative uncertainty in the calculation of the parameters is estimated to be ca. 0.005 for $g_\parallel$ and ca. 1.0 for $A_\parallel$. In the case of rhombic distortion $g_\parallel$ and $A_\parallel$ are in fact g_z and A_z, respectively. [b] A logβ value for this stoichiometry was obtained from the pH-potentiometric data and calculations. However, its EPR signals are possibly under the complex pattern of other signals recorded and we did not find a distinct signal for this complex. Therefore we do not propose any binding mode.

In accordance with the pH-potentiometric speciation results, electronic absorption spectroscopy unambiguously revealed a new species ($[VOAB]^{4-}$) in the pH range 2.5–5.5,

with an extra band in the CT region, caused by the coordination of dipic (not shown). The difference between the CD spectra of the $V^{IV}O$–ATP and $V^{IV}O$–dipic–ATP systems (ESI-2) indicates the formation of a new type of ternary complex above the physiological pH range, where the ribose moiety becomes a more efficient binding site ($[VOABH_{-2}]^{6-}$).

$V^{IV}O$–Maltolate–ATP System

Under physiological conditions (pH ≈ 7), maltolate is a stronger $V^{IV}O$ binder than dipic. For this reason, participation of maltolate in in vivo binding (even in binary complexes) can be expected to be more significant than was found in the case of dipic. The potentiometric data were fitted with a speciation model[24] with the assumption of two mixed-ligand complexes, $[VOAB]^{3-}$ and $[VOABH_{-2}]^{5-}$ (see Table 1). The concentration distribution diagram for $V^{IV}O$–maltolate–ATP depicted in Figure 3 displays the species formed under the conditions of the spectroscopic measurements. The pH-metric measurements demonstrate that ternary complex formation is not as dominant as with dipic, where binary bis complexes of maltolate are present in significant amounts.

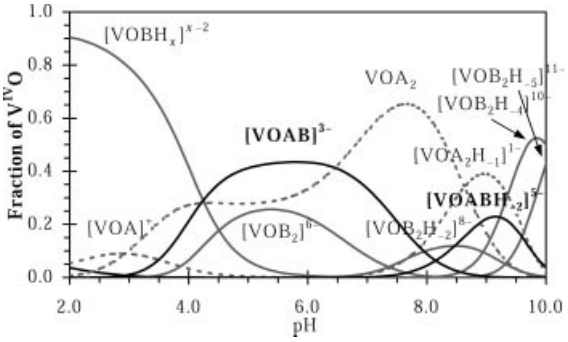


Figure 3. Concentration distribution diagram of the $V^{IV}O$ – maltol(A) – ATP(B) system at 1:2:10 metal ion-to-ligand ratio, $C_{VO} \approx 0.004$ mol dm^{-3}.

The EPR spectra of the systems $V^{IV}O$–maltolate, $V^{IV}O$–ATP and $V^{IV}O$–maltolate–ATP are presented in Figure 4, and the EPR parameters of the mixed-ligand species are listed in Table 2. Because of the difference in the type of donor groups involved in the coordination ternary complex formation is clearly indicated. Below pH 7, water and a carbonyl group are substituted by one or two phosphates, resulting in a detectable change ($A_{\parallel}$ from 171.4×10^{-4} cm^{-1} for VOA_2 to 169.1×10^{-4} cm^{-1} for $[VOAB]^{3-}$) in the EPR parameters.

A new signal from the formation of the complex $[VOABH_{-2}]^{5-}$ with a sugar-like binding mode of the ATP is not clearly seen as it is formed only from ca. 20% of the total V according to the speciation calculations. This species could be characterised by pH potentiometry only with a large uncertainty since the species distribution for the $V^{IV}O$–ATP (see above) and $V^{IV}O$–maltolate binary systems at pH > 9 is also uncertain as a result of the slight aerobic

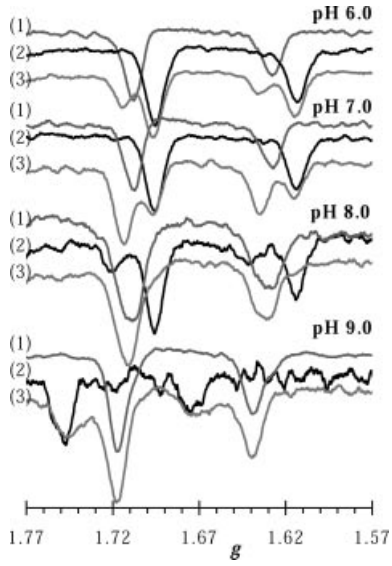


Figure 4. High field range of the first-derivative EPR spectra at 77 K of frozen solutions containing: (1) $V^{IV}O$–maltol 1:2, (2) $V^{IV}O$–ATP 1:10, (3) $V^{IV}O$–maltol–ATP 1:2:10 metal ion-to-ligand ratios; $C_{VO} \approx 0.004$ mol dm^{-3}. The pH values are indicated.

redox reaction of $V^{IV}O$–maltolate that may take place,[20] and/or the slow hydroxo complex formation processes.[13] However, in the CD spectra (ESI-3), a new type of signal can be observed at pH 8–9, unambiguously indicating coordination of the ribose residue corresponding to the formation of $[VOABH_{-2}]^{5-}$. At pH ≥ 9, the CD spectra for the binary and ternary systems become very similar, in agreement with the speciation results: both carrier molecules are displaced by the ribose-coordinated nucleotide ligands, resulting in the formation of the binary ATP complex $[VOB_2H_{-4(-5)}]^{7-(8-)}$.

$V^{IV}O$ Ternary Systems with GSH

GSH is a cysteine-containing tripeptide (γ-Glu-Cys-Gly) with eight potential donor atoms. As mentioned above, it is possible that GSH plays an important role in relation to the biochemistry of vanadium. It is present in high excess in cells, but GSH is a much weaker $V^{IV}O$ binder than ATP. For $V^{IV}O$ concentrations above ca. 1 mM and more than a 10-fold excess of the ligand, the bis amino acid type binding mode ($[VOB_2H_2]^{2-}$) with the donor set $2\times(COO^-, NH_2)_{eq}$ is relevant in the pH range 5.0–6.5.[15,17,25,26] Participation of the thiolate donor may occur above pH ca. 7, forming the complex $[VOBH_{-1}]^{2-}$. This $[VOBH_{-1}]^{2-}$ stoichiometry corresponds to two different EPR components, and coordination of the deprotonated N_{amide} group can be assumed in the minor isomer.[17] At a 10-fold excess of GSH, ternary complex formation could be detected only with rather high uncertainty in the pH-potentiometric titrations (see Table 3), and this excess of GSH was not enough to prevent the hydrolysis of the metal ion at pH > 6.5 for the dipic system and at pH > 8.0 for the maltolate system. Higher GSH concentrations could not be used in the pH potent-

iometry because of the high buffer capacity of the ligand, and thus there was even higher uncertainty in the pH-potentiometric calculations. Most of the measured pH effect resulted from deprotonation of the excess ligand, and only a very small amount of it related to the metal-induced proton displacement reaction. Hence, complex formation between $V^{IV}O$ and GSH could be monitored by pH potentiometry only with great uncertainty. Accordingly, mostly spectroscopic measurements (EPR, UV/Vis and CD) were used to detect the formation of ternary complexes.

Table 3. Stability constants of mixed ligand $V^{IV}O$ complexes of dipicolinate and maltolate (ligands A) with GSH (ligand B).

Ligand A	$VOABH_2$	VOA_2BH_2	VOA_2BH
Dipicolinate	25.9(8)	–	–
Maltolate	28.6(3)	35.5(8)	27.7(5)

$V^{IV}O$–dipic–GSH System

A concentration distribution diagram for the $V^{IV}O$–dipic–GSH system with the metal ion-to-ligand ratio and $V^{IV}O$ concentration used in the spectroscopic measurements is depicted in Figure 5 (the formation constants of the binary complexes used in the speciation calculation were taken from ref.[12] and ref.[17]).

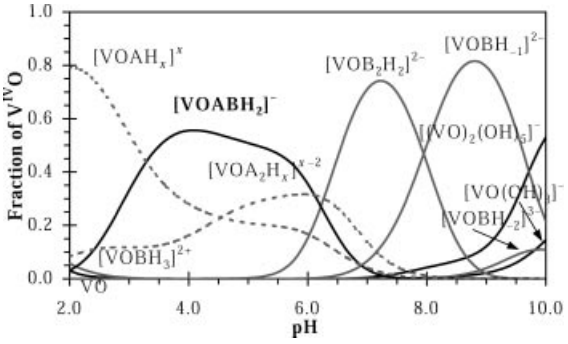


Figure 5. Concentration distribution diagram of the $V^{IV}O$–dipic(A)–GSH(B) system at a 1:2:50 metal ion-to-ligand ratio, $C_{VO} \approx 0.004$ mol dm^{-3}.

The speciation calculations suggested that $V^{IV}O$ starts to coordinate to dipic at pH < 2. At a 10-fold or higher excess of GSH, besides the binary species, a ternary complex $[VOABH_2]^-$ could be detected in which GSH most probably coordinates in a monodentate way through the C-terminal COO^- or with the possibility of chelation together with the peptide carbonyl-O. Neither CD nor EPR spectroscopy can distinguish between these two different binding modes. This complex can be determined potentiometrically only with high uncertainty because the coordinating carboxylate donor is in the deprotonated form over most of the pH range of formation (pK(COOH) ca. 3.5),[17] and thus its coordination does not result in a direct pH change. At pH > 6, binary complexes of GSH are formed. Subsequently, because of the significantly weaker $V^{IV}O$-binding ability of GSH, even a 10-fold excess of GSH was not sufficient to prevent hydrolysis of the metal ion and formation of the oligonuclear hydroxo species $\{[(VO)_2(OH)_5]^-\}_n$.

By EPR spectroscopy (Figure 6, Table 4) a new species could be detected in the pH range 3–6; this is probably the ternary species $[VOABH_2]^-$. However, at pH ca. 7, the EPR spectrum of the $V^{IV}O$–dipic–GSH system coincides fully with that of $V^{IV}O$–GSH, indicating that GSH completely displaces the carrier ligands from the coordination sphere of the metal ion. Although GSH is a weaker vanadium binder than dipic, it is present at a 50–100-fold excess relative to dipic.

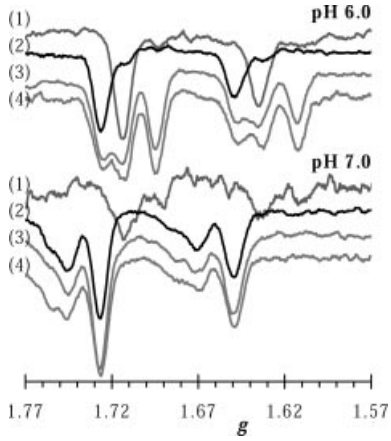


Figure 6. High-field range of the first-derivative EPR spectra at 77 K of frozen solutions containing: (1) $V^{IV}O$–dipic 1:2, (2) $V^{IV}O$–GSH 1:50, (3) $V^{IV}O$–dipic–GSH 1:2:50 and (4) 1:2:100 metal ion-to-ligand ratios, $C_{VO} \approx 0.004$ mol dm^{-3}. The pH values are indicated.

Table 4. EPR parameters ($g_\parallel$, $A_\parallel$)[a] for the binary and ternary complexes of $V^{IV}O$ formed with dipicolinate, maltolate and GSH.

Species	$g_\parallel$	$A_\parallel$ ($\times 10^4$ cm^{-1})	Chelating set
GSH			
$[VOBH_2]^+$	1.937	174.2	
VOBH	1.941	170.0	
$[VOB_2H_2]^{2-}$	1.948	163.8	2×(COO^-, NH_2)
$[VOBH_{-1}]^{2-}$ (I)	1.956	154.5	(COO^-/amide-O, NH_2, S^-) OH^-
$[VOBH_{-1}]^{2-}$ (II)	1.959	147.0	
Dipicolinate + GSH			
$[VOABH_2]^-$	1.930	177.2	(COO^-,COO^-) (COO^-, amide-O/H_2O)
Maltolate + GSH			
$VOABH_2$	1.936	174.2	(O^-, =O) (COO^-, amide-O/H_2O)
$[VOA_2BH_2]^-$	1.941	169.0	(O^-, =O) (O^-, $=O_{ax}$) COO^-
$[VOA_2BH]^{2-}$	1.949	159.1	(O^-, =O) (O^-, $=O_{ax}$) S^-

[a] The relative uncertainty in the calculation of the parameters is estimated to be ca. 0.005 for $g_\parallel$ and ca. 1.0 for $A_\parallel$. In the case of rhombic distortion $g_\parallel$ and $A_\parallel$ are in fact g_z and A_z, respectively.

$V^{IV}O$–Maltolate–GSH System

A concentration distribution diagram of the $V^{IV}O$–maltolate–GSH system under the experimental conditions of the spectral measurements is depicted in Figure 7. In accord-

ance with the species distribution, new EPR signals could be detected that can be assigned to the ternary species (see Table 4).

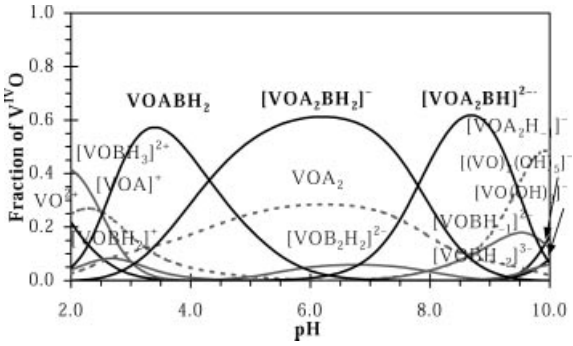


Figure 7. Concentration distribution diagram of the $V^{IV}O$–maltolate(A)–GSH(B) system at a 1:2:50 metal ion-to-ligand ratio, $C_{VO} \approx 0.004$ mol dm^{-3}.

In the pH range 2.5–4.0, besides the signals from the species $[VOBH_x]^{x-1}$ (x = 2, 3), a new complex ($g_{\|}$ = 1.941 and $A_{\|} = 169.5 \times 10^{-4}$ cm^{-1}) was detected that was not seen in the EPR spectra of the binary systems. In the pH range 6–7, this signal was slightly shifted ($g_{\|}$ = 1.941 and $A_{\|} = 168.6 \times 10^{-4}$ cm^{-1}), but remained in the uncertainty range of the EPR parameters. Above pH 7, another new signal appeared at $g_{\|}$ = 1.949 and $A_{\|} = 159.1 \times 10^{-4}$ cm^{-1}. Some of the EPR spectra recorded by varying the B ligand concentration at pH 7.0 are shown in Figure 8.

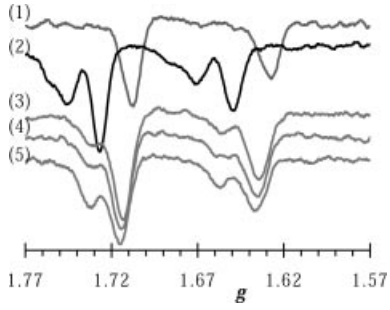


Figure 8. High field range of the first-derivative EPR spectra at 77 K of frozen solutions containing: (1) $V^{IV}O$–maltolate 1:2, (2) $V^{IV}O$–GSH 1:50, (3) $V^{IV}O$–maltolate–GSH 1:2:25,(4) 1:2:50 and (5) 1:2:100 metal ion-to-ligand ratios, $C_{VO} \approx 0.004$ mol dm^{-3} at pH 7.0.

The EPR spectra clearly indicate ternary complex formation in the $V^{IV}O$–maltolate–GSH system at a 25-fold excess of GSH. Considering the EPR parameters (Table 4) and the low CD signals recorded (not shown), we conclude that GSH most probably coordinates at the Gly end through (COO^-, O-amide/H_2O) donors, while participation of the thiolate donor occurs only at pH > 7. The most likely binding modes of the complexes formed in the $V^{IV}O$–maltolate–GSH system are shown in Table 4. In the complex $VOABH_2$ formed in the slightly acidic pH range, GSH coordinates bidentately or monodentately in the equatorial plane, while in $[VOA_2BH_2]^-$ formed in the neutral pH range, GSH coordinates in a monodentate manner through the C-terminal COO^- function to the fourth equatorial position of $V^{IV}O$. In the case of VOA_2, the *cis* complex, with one empty equatorial coordination site, is the predominant or the exclusive isomeric form. In the species $[VOA_2BH]^{2-}$, when Equation (1) is applied to the equatorial donor set, (2 × (O^-, =O), S^-) may be assumed. The estimated value $A_{\|}^{est}$ = 157.6×10^{-4} cm^{-1} agrees fairly well with the experimental value $A_{\|} = 159.1 \times 10^{-4}$ cm^{-1}. This suggests equatorial thiolate coordination.

$V^{IV}O$–Carrier Ligand–ATP–GSH Systems

When ATP and GSH are simultaneously considered as potential $V^{IV}O$ binders, GSH is not expected to be able to compete with ATP for binding to $V^{IV}O$ since ATP is a much stronger ligand. The competition reaction was studied by EPR spectroscopy (see Figure 9) in the absence and in the presence of the insulin-mimetic complexes. In the case of dipic, no spectral changes could be detected between the spectra measured in the presence or absence of GSH, in agreement with what was found in the $V^{IV}O$–dipic–ATP system. Therefore, EPR spectroscopy does not give any information on the changes that might have occurred in the coordination sphere because of the high similarity of the partial contributions of the coordinating donors to the $A_{\|}$ value (see above).

For the $V^{IV}O$–ATP–GSH ternary system, when ATP was in a 10-fold excess relative to $V^{IV}O$, only the EPR signals of the $V^{IV}O$–ATP complexes were detected. New signals appeared only at a 2.5-fold excess (EPR parameters: $A_{\|}$ = 172.1×10^{-4} cm^{-1} and 165.7×10^{-4} cm^{-1}). Comparison of the EPR spectra of solutions containing $V^{IV}O$, maltolate, ATP and GSH at pH ca. 7 (see Figure 9) revealed significant changes only when ATP was not present in at least a 10-

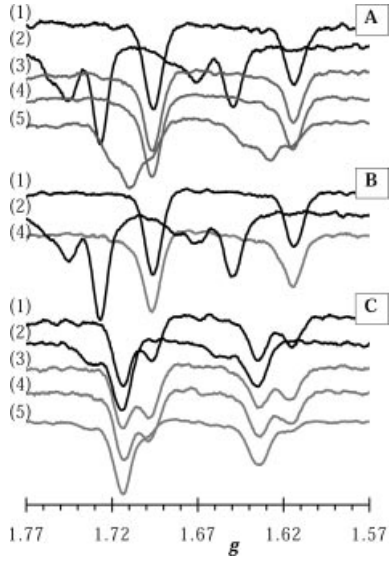


Figure 9. High field range of the first-derivative EPR spectra at 77 K of frozen solutions: (A) without carrier ligand, (B) with 8 mM dipicolinate, (C) with 8 mM maltolate, (1) $V^{IV}O$–ATP 1:10, (2) $V^{IV}O$–GSH 1:50, (3) $V^{IV}O$–ATP–GSH 1:10:25,(4) 1:10:50 and (5) 1:2.5:50 metal ion-to-ligand ratios; $C_{VO} \approx 0.004$ mol dm^{-3} at pH 7.0.

fold excess (see above). Below such an excess of ATP, the changes in the ratio of the predominating signals seem to indicate the participation of GSH in complex formation.

Conclusions

With respect to the two important cell constituents GSH and ATP, our results indicate that if the carrier ligands can somehow find a way to enter the cell strong $V^{IV}O$-binder cell constituents will partly displace the carrier ligands and ternary complexes with relevant biomolecules of the cell will be formed. Some of the ternary species are highly anionic, which are partly neutralised and probably undergo ion paring with cations, K^+ and Mg^{2+}, of the cell.

From among the important cell constituents, GSH will possibly take part in the reduction of V^V to V^{IV} and will help keep V^{IV} in this oxidation state. As a strong $V^{IV}O$ binder, ATP will chelate the metal ion, forming binary and/or ternary complexes. The results of this work strongly suggest that ATP binds relevant $V^{IV}O$ species under cell conditions, and thus might somehow be involved in the insulin-mimetic action of $V^{IV}O$ compounds. However, the time courses of these parallel redox and complexation reactions require further investigations.

Experimental Section

Materials: The ligands investigated were Sigma products of the highest analytical purity. They were used without further purification, but their purities were checked and the exact concentrations of their solutions was determined by the Gran method.[27] The $V^{IV}O$ stock solution, prepared as described earlier,[28] was standardized for metal ion concentration by permanganate titration and for hydrogen ion concentration by potentiometry, using the appropriate Gran function. The ionic strength was adjusted to 0.20 mol dm^{-3} KCl. The temperature was 25.0 ± 0.1 °C for potentiometric measurements, and 25.0 ± 0.3 °C for CD and Vis spectra.

Potentiometric Measurements: For potentiometric titrations, an automatic titration set, including a Dosimat 665 autoburette, an Orion 710A precision digital pH meter and an IBM-compatible personal computer was used. A Metrohm 6.0234.100 semimicro combined glass electrode was calibrated for hydrogen ion concentration according to the method of Irving et al.[29] The ionic product of water was taken as $pK_w = 13.76 \pm 0.01$.

When we refer to the stoichiometries of complexes present in solution, the notation $M_pA_qB_rH_s$ is used, where M = $V^{IV}O^{2+}$, H_2A or HA refers to the fully protonated form of dipicolinate acid or maltolate, respectively, and H_5B^+ denotes totally protonated ATP. However two of the four protons of the triphosphate chain dissociate in very acidic media (pH < 1). H_4B^+ refers to totally protonated GSH. The concentration stability constants, $\beta_{pqrs} = [M_pA_qB_rH_s]/([M]^p[A]^q[B]^r[H]^s)$, were calculated by using the computer programme PSEQUAD.[30] The equilibria corresponding to the formation of hydroxo complexes of $V^{IV}O$ were taken into account in the calculation of the stability constants of the complexes. The following stability constants ($\log\beta$) were assumed for these complexes: $[VO(OH)]^+$ ($\log\beta_{100-1} = -5.94$); $[(VO)_2(OH)_2]^{2+}$ ($\log\beta_{200-2} = -6.95$), calculated from the data published by Henry et al.,[31] using the Davies equation to take into account the different ionic strengths; $[VO(OH)_3]^-$ ($\log\beta_{100-3} = -18.00$);[22] and $[(VO)_2(OH)_5]^-$ ($\log\beta_{200-5} = -22.50$),[22,23] were taken from the literature.

Spectroscopic Measurements: The CD spectra were recorded with a JASCO 720 spectropolarimeter with a red-sensitive photomultiplier (EXWL-308). Visible spectra were recorded with a Perkin–Elmer Lambda 9 spectrophotometer. The spectral range covered was normally 400–900 nm (Vis) and 400–1000 nm (CD). By Vis and CD spectra, we mean representations of ε_m or $\Delta\varepsilon_m$ values vs. λ [ε_m = absorption/(bC_{VO}) and $\Delta\varepsilon_m$ = differential absorption/(bC_{VO}), where b = optical path and C_{VO} = total $V^{IV}O$ concentration].

The EPR spectra were recorded at 77 K with a Bruker ESR-ER 200D X-band spectrometer. The spin-Hamiltonian parameters were obtained by simulation of the spectra with the aid of the modified computer programme of Rockenbauer and Korecz.[32] The EPR spectra of the $V^{IV}O$ systems help to elucidate the binding mode of the complexes formed in solution; Chasteen developed an additivity rule to estimate the hyperfine coupling constant A_z^{est} [Equation (1)], based on the contributions $A_{z,i}$ of each of the four equatorial donor groups.[33] The estimated accuracy of A_z^{est} is ca. 3×10^{-4} cm^{-1}.

$$A_z^{est} = \sum_{i=1}^{4} A_{z,i} \quad (1)$$

Most of the $A_{z,i}$ data relevant for this work were presented by Chasteen.[33] For the carboxylate (COO^-) donor, Chasteen estimated its contribution in Equation (1) to be 42.7×10^{-4} cm^{-1}, based on the A_z of the VO(oxalato)$_2^{2-}$ complex, assuming that the four COO^- groups coordinate equatorially. However, the solution structure presumably involves the set ($3 \times COO^-$, $H_2O)_{eq}$,[34] so this contribution should be 42.1×10^{-4} cm^{-1}, the value used in the present work. The contribution of each O donor of maltolate in Equation (1) was taken to be $41–42 \times 10^{-4}$ cm^{-1}. This value was calculated from the A_z parameters of *cis* and *trans* complexes of VO(malt)$_2$, determined by assuming the [(O^-, =O)(O^-, $=O_{ax}$) H_2O] and [(O^-, =O)(O^-, =O)] sets, respectively.[8] These data can be used to establish the most probable binding mode of the complexes formed, but care must be taken as the contributions of the donor groups to the hyperfine coupling may depend, for instance on their orientation,[35] or the charge of the ligand.[36] The influence of the axial donor groups (if any) is not taken into account. In the absence of ethylene glycol, a relatively broad background was present in most of the frozen solution EPR spectra, and therefore most spectra were obtained from aqueous solutions containing 5% of ethylene glycol.

Acknowledgments

This work was carried out in the frame of the COST D21/009/01 project (Insulin-Mimetic Vanadium Compounds). The authors are grateful to the National Research Fund (OTKA NI61786 and OTKA T49417), the Hungarian Academy of Sciences, the Fundo Europeu para o Desenvolvimento Regional, Fundação para a Ciência e Tecnologia, the POCI Programme (project POCI/56949/QUI/2000), and the Hungarian-Portuguese Intergovernmental S & T Co-operation Programme for 2004–2005.

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Received: April 27, 2006
Published Online: August 7, 2006

