Available online at www.sciencedirect.com
ScienceDirect
Coordination Chemistry Reviews 252 (2008) 1153–1162
COORDINATION CHEMISTRY REVIEWS
www.elsevier.com/locate/ccr

Review

Biospeciation of antidiabetic VO(IV) complexes

Tamás Kiss [a,b,*], Tamás Jakusch [b], Dominik Hollender [b], Ágnes Dörnyei [b], Éva A. Enyedy [a], João Costa Pessoa [c], Hiromu Sakurai [d], Alfredo Sanz-Medel [e]

[a] *Department of Inorganic and Analytical Chemistry, University of Szeged, P.O. Box 440, Szeged H-6701, Hungary*
[b] *Bioinorganic Chemistry Research Group of the Hungarian Academy of Sciences, University of Szeged, P.O. Box 440, Szeged H-6701, Hungary*
[c] *Centro Química Estrutural, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*
[d] *Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, 5-Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan*
[e] *Department of Physical and Analytical Chemistry, University of Oviedo, C/ Julián Clavería 8, 33006 Oviedo, Spain*

Received 27 June 2007; accepted 15 September 2007
Available online 22 September 2007

Contents

Abstract

The possible transformations of antidiabetic vanadium(IV) complexes in the organism are discussed. These reactions involve absorption processes in the gastrointestinal tract, transport in the blood stream and interactions with endogenous binding molecules in the glucose-metabolizing cells. Modeling studies were mostly used to determine the actual chemical form of VO(IV) complexes in various biological environments. The results suggest that decomposition and subsequent ternary complex formation with endogenous or exogenous ligands in the organism affects the absorption efficacy of the originally neutral VO(IV) compounds considerably. During transport in the blood stream, transferrin displaces the carrier ligands from the VO(IV) compounds and plays an important role in transporting VO(IV) to the cell. In the cell, vanadium undergoes redox interaction with glutathione and complexation with adenosine 5′-triphosphate (the two important cell components present in mM concentration). In vitro and in vivo biological results confirmed some of the basic findings obtained from the modeling.

Keywords: Insulin-like action of vanadium; Diabetes; Biospeciation; Complex formation; Transferrin

1. Introduction: the role of insulin in the glucose metabolism

The number of subjects with diabetes mellitus (DM) is steadily increasing and it was approximately 250 million worldwide in 2006.

DM is a disease that results in chronic hyperglycemia due to an absolute or relative lack of insulin and/or insulin resistance, that in turn impairs the glucose, protein and lipid metabolisms, and finally results in characteristic secondary complications [1]. DM is generally classified as either insulin-dependent (IDDM, type 1) or non-insulin-dependent (NIDDM, type 2) [2]. Various synthetic organic therapeutics have already been applied clinically for the treatment of NIDDM including sulfonylureas, sulfonamides, biguanides and the recently developed triglydazones. However, some of them have severe side-effects such as hepatotoxicity and lactic acidosis. Thus, there is an urgent

* Corresponding author at: Department of Inorganic and Analytical Chemistry, University of Szeged, P.O. Box 440, Szeged H-6701, Hungary.
E-mail address: tkiss@chem.u-szeged.hu (T. Kiss).

0010-8545/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ccr.2007.09.011

need for new types of therapeutic agents. IDDM can be controlled only by daily injections of insulin; many types of insulin injection preparations have been developed. As these impose a physical burden on the patients, huge efforts have been made to find therapeutic agents that could replace insulin injections. To date, however, no agents other than insulin have been developed for the treatment of IDDM.

Insulin is a signaling hormone which is essential for the metabolism of both carbohydrate and fat. It is secreted by the pancreas in response to elevated levels of glucose in the blood stream. The increased insulin level then normally promotes glucose uptake by the liver, gut and peripheral tissues (adipose and muscle), which results in energy production and storage, as needed by the organism [3].

The insulin receptor (IR) is an insulin-activated transmembrane protein tyrosine kinase. Following insulin binding, the IR undergoes activation by autophosphorylation and subsequently phosphorylates several endogenous proteins on tyrosine moieties. Tyrosyl phosphorylation is linked to a serine/threonine phosphorylation state of key enzymatic systems controlling the glucose and fat metabolism. When insulin is removed, termination of glucose metabolism occurs at several levels, including dephosphorylation of tyrosyl residues by endogenous protein phosphotyrosine phosphatases (PTPases) [4].

2. Insulin substitutes: vanadium compounds enhance the action of insulin

During the past 25 years, ions of elements such as Se, Cr, Mn, Mo, W, V and Zn have been reported to exhibit insulin-like effects, V proving to be one of the most efficient [5,6]. The insulin-like antidiabetic effects of vanadium compounds were reported as long ago as 1899 [7] and rediscovered about 80 years later, first in in vitro, and later in in vivo studies [8]. Even simple inorganic vanadium salts in oxidation state IV or V (e.g. vanadylsulfate or sodium vanadate) mimic most of the physiological effects of insulin, such as stimulation of the glucose uptake and metabolism in the fat cells, the enhancement of glycogenesis in the muscles and the liver, inhibition of the reformation of glucose (gluconeogenesis) from proteins, or the stimulation of fatty acid formation in the adipocytes [2]. The main advantage of these vanadium compounds relative to insulin is that they may be administered orally. Accordingly, the aim of research in this field is to make vanadium compounds which can reach the target cells with high efficacy. It is currently believed that these compounds should have low molecular mass, should be neutral and should have optimal lipophilicity in order to be mobile and cross the cell membranes with ease. Large numbers of vanadium(IV,V) complexes have been prepared and tested; one of them, VO(ethylmaltolato)$_2$, has passed clinical phase 1 test in Canada. [9].

Within a COST European research collaboration, the biological activities of numerous vanadium(IV,V) compounds were tested in a simple way, by measuring the increase in mitochondrial metabolic activity caused by the increased glucose uptake of insulin-depleted mice fibroblast cell cultures in the vanadium concentration range 0.01–1000 μM [10]. The activities of the complexes were generally about 30–70% of the activity of insulin. Interestingly, the VO(IV) complexes exhibited somewhat higher activities than those of the vanadate(V) compounds, but all the compounds displayed marked biological activity in the relevant concentration range 1–10 μM. No toxicity was exerted on the fibroblast cell cultures in this concentration range. At higher concentrations, in contrast with the general opinion in the literature, the VO(IV) compounds proved a little more toxic than the vanadium(V) complexes.

Although many details of the biological action of vanadium(IV,V) on the glucose metabolism are known, there is as yet no widely accepted mechanism.

Since vanadate is an inhibitor of phosphatases [11], it was initially believed that vanadate acts intracellularly by blocking the protein PTPases, and activating tyrosine kinase of the IR. However, this turned out not to be the case [12]. Instead of simply influencing the IR, vanadium complexes may modulate other enzymes involved in insulin signaling [13].

It is now accepted that vanadium, and probably the other metal ions too (see above) interfere with the numerous phosphorylation/dephosphorylation reactions involved in the glucose metabolism. According to Shechter et al. [14], vanadium in both oxidation states exerts antidiabetic activity, operating by separate pathways. VO(IV) is active at the level of the plasma membrane and is responsible for the facilitation of glucose uptake, and possibly for inhibiting lipolysis, while vanadium(V) operates exclusively in the cytosolic compartment, enhancing the glucose and fat metabolism.

The promotion of glucose uptake in a tissue-specific manner can be enhanced by vanadium. Vanadium compounds, however, can never substitute insulin completely. (It is thought that a complete lack of insulin precludes the effectiveness of any vanadium compound.) Nevertheless, many of the observed in vitro and in vivo effects of vanadium are insulin-like [11].

3. The importance of the solution state of antidiabetic compounds

The experimental conditions applied for the preparation of metal complexes with potential biological activity (e.g. the solvent and the pH) may differ considerably from those in the living systems where they are intended to exert their biological effects. Further differences in vivo include the presence of various molecules that may possibly have high affinity for the metal ion and therefore partly or fully displace the original metal-binding ligand(s) during the (i) absorption processes in the gastrointestinal tract (in the case of complexes administered orally), (ii) transport processes in the blood stream, and (iii) in reactions with intracellular molecules. Accordingly, the original carrier ligand(s) might be lost in these processes and the real biological/physiological activity is due to an entirely different chemical entity.

In the past few years, we have studied the potential transformations of several antidiabetic VO(IV) complexes [15] in the organism. These complexes (see Fig. 1) are all neutral bis complexes of bidentate ligands with (O, O), (O, N) or (O, S) binding mode [10].

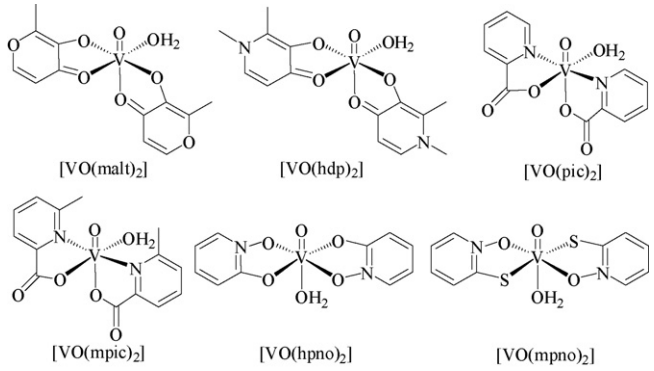

Fig. 1. Structural formulae of the studied antidiabetic VO(IV) complexes (malt = maltolate, hdp = 3-hydroxy-1,2-dimethyl-4-pyridinone, pic = picolinate, mpic = 6-methyl-picolinate, hpno = 2-hydroxy-pyridine-*N*-oxide, mpno = 2-mercapto-pyridine-*N*-oxide).

Their possible transformations during the absorption, serum transport and intracellular processes were followed. In the knowledge of the speciation description of VO(IV) complexes formed with the constituents of the various biological fluids, modeling calculations were carried out to identify the actual chemical forms existing under biological conditions. A pH-potentiometric technique was used to describe the reactions quantitatively with the low molecular mass (LMM) components, and spectroscopic methods were applied to characterize the interactions with the high molecular mass (HMM) protein components. Ultrafiltration served to separate the HMM and LMM fraction-bound metal ion contents.

4. Absorption of antidiabetic vanadium complexes in the GI tract

In consequence of the parallel processes of protonation of the metal-binding sites of the coordinating ligands, these neutral bis complexes (with good passive membrane transport behavior) will certainly partly decompose in the acidic pH range, e.g. at the pH (∼2) of the gastric juice. The species formed in this way will be charged, and will possess entirely different membrane transport properties. This is demonstrated in Fig. 2, in which the species distributions of two well-studied systems, the VO(IV)–maltolato and the VO(IV)–picolinato systems, are depicted as a function of pH.

Accordingly, all other exogenous and endogenous biomolecules present in the stomach or intestines, where the complexes are absorbed, may play a role in VO(IV) binding. Interactions with these molecules may change the charge conditions of the complex unfavorably, which will decrease their absorption efficacy. This certainly has to be taken into account in the formulation of the drug (e.g. by encapsulation techniques, whereby these problems may well be overcome). The recent results of Sakurai et al. [16] support this prediction. In their study $VOSO_4$ was administered orally in various ways: in solution, in gelatin capsules and in enteric-coated capsules. It was found that administration of the VO(IV) salt in encapsulated forms improved the metal ion absorption as compared with that associated with the simple solution form.

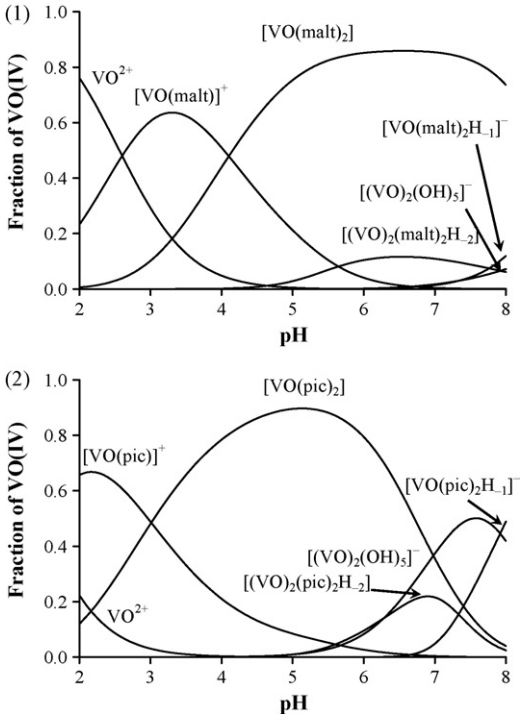

Fig. 2. Species distribution diagram of (1) VO(IV)–malt 1:2 and (2) VO(IV)–pic 1:2 systems, $[VO(IV)]_{tot}$ = 1 mM (based on data reported in Ref. [15]).

Accordingly, the encapsulated metal ion could reach the ileum, where its absorption was more efficient than at other GI sites.

5. Transport of antidiabetic vanadium complexes in the blood

During their transport in the blood stream, after absorption, complex formation with the serum components, as active VO(IV) binders, also has to be considered. The interactions of several potential antidiabetic compounds with the HMM protein constituents, e.g. albumin (HSA) and transferrin (Tf), and some of the LMM constituents (the most potent binders are the negatively charged O donor-containing ligands), e.g. lactate, phosphate, oxalate and citrate, have been studied in detail [17–25]. The actual serum concentrations of these potential VO(IV) binders are listed in Table 1. Other serum components (mostly amino acids and inorganic ligands) that are not efficient enough binders are omitted from Table 1. The results of model

Table 1
Total concentrations of components in computer model of serum [22]

Component	Concentration
High molecular mass (HMM)	
Albumin	630 μM
Transferrin	37.0 μM
Low molecular mass (LMM)	
Phosphate	1.10 mM
Citrate	99.0 μM
Lactate	1.51 mM
Oxalate	9.20 μM

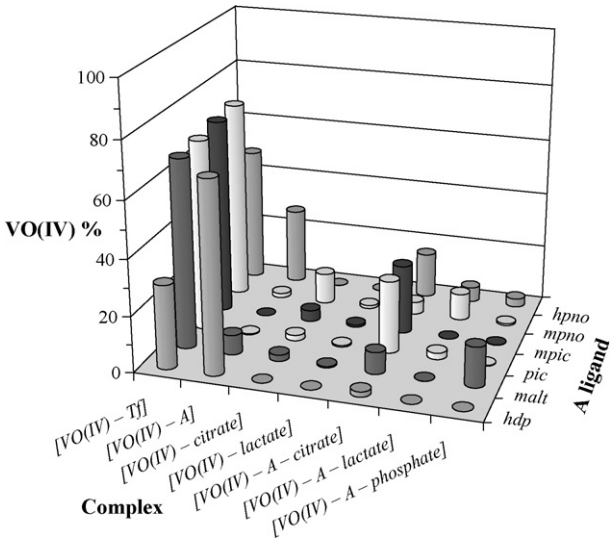

Fig. 3. Distribution of various antidiabetic VO(IV) compounds ($[VO(IV)]_{tot}$ = 100 μM, $[A\ ligand]_{tot}$ = 200 μM) in serum at pH 7.4 (based on data reported in Ref. [15]). For abbreviations of A ligands see Fig. 2.

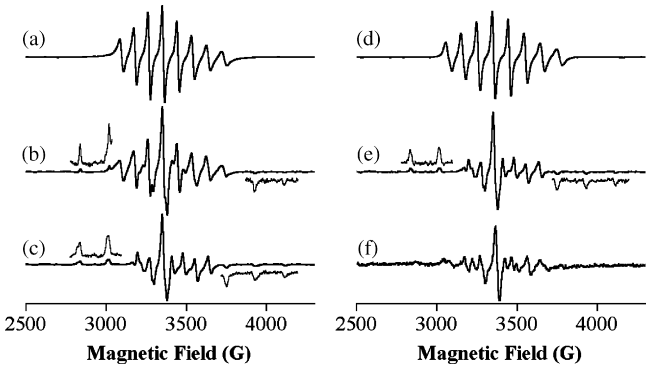

Fig. 4. Room temperature EPR spectra of (a) VO(IV)–hdp 1:2, (b) VO(IV)–hdp–apoTf 1:2:1, (c) VO(IV)–apoTf 1:1, (d) VO(IV)–pic 1:2, (e) VO(IV)–pic–apoTf 1:2:1 and (f) VO(IV)–pic–HSA 1:2:1 systems at pH 7.4, $[VO(IV)]_{tot}$ = 1 mM.

calculations for the serum conditions [15,25] are depicted in Fig. 3.

It is seen in Fig. 3 that (i) only the pyridinone derivative hdp is a strong enough carrier to preserve a significant proportion of the VO(IV) in the original complex [21,23]; in the other cases, the carrier ligands are displaced by serum components. Accordingly, at first glance, the most important role of the carrier ligand seems to be to facilitate the absorption of VO(IV). (ii) Of the two important HMM binders, Tf is much more efficient than HSA and will displace 30–70% of the original carrier from the complex. (At the biologically more relevant VO(IV) concentrations (<5 mM), practically all of the VO(IV) is bound to Tf.) Similarly, the predominant binding of vanadate(V) to Tf in the human plasma and the negligible role of HSA in transporting vanadium were studied in detailed in vitro measurements and reported by Heinemann et al. [26]. (iii) Among the LMM binders, citrate is the only efficient component able to influence the solution state of these antidiabetics. At physiological pH, VO(IV) exists mostly as the VO(IV)–citrate binary complex and as the VO(IV)–carrier ligand–citrate mixed ligand complex, but in different proportions.

The decisive role of Tf in VO(IV) binding has been demonstrated by EPR. In the presence of apoTf, all carrier complexes gave anisotropic EPR spectra even at room temperature (because of the slow tumbling motion of the VO(IV)–protein complex, the parallel and the perpendicular components of the EPR signal remain separated and could not be averaged), unambiguously indicating interactions between the carrier complex of VO(IV) and the protein [24,27,28]. As may be seen in Fig. 4, in the case of $VO(pic)_2$, the RT spectrum is practically the same as that of VO(IV)–apoTf, suggesting a complete displacement of the carrier ligand by apoTf. At the same time, in the case of the $VO(IV)(hdp)_2$ complex, the signals of the anisotropic carrier complex can also be observed, suggesting only partial displacement of the carrier ligand. A similar composite spectrum of the isotropic $VO(pic)_2$ and the anisotropic VO–HSA can be observed in the VO(IV)–pic–HSA system, indicating a partial displacement reaction between the protein and the carrier ligand. These results are in complete agreement with the species distributions depicted in Fig. 3.

The results strongly indicate that vanadium must be released from the carrier compound in order to be pharmacologically active. Perhaps the most convincing point is the different pharmacokinetics of disappearance of vanadium and the carrier ligand [29,30]. Although, in principle, the ligand should be present at roughly twice the concentration of vanadium in the blood, it is found to be present at a considerably lower concentration as compared with vanadium from the 1 h time point onward. Thus, a number of recent studies have demonstrated fairly conclusively that antidiabetic vanadium complexes dissociate rapidly once ingested or injected. In other words, the eventual in vivo metabolic fate for chelated vanadium complexes differs little, if at all, from that of non-complexed vanadium compounds, when they are administered orally [30]. Further, when the in vivo metabolic fates of different vanadium chelates were monitored in rats, different excretion kinetics were measured for the metal ion and the carrier ligand. The results indicated that, shortly after administration, the complex decomposes and the two components follow different metabolic pathways in the organism [30].

The partial carrier ligand displacement reactions of $VO(hdp)_2$ by apoTf, and $VO(pic)_2$ by HSA could be used to estimate the binding constants of the VO(IV)–protein interactions. The quantitative evaluation and simulation of the RT EPR spectra furnished the conditional binding constants. In order to keep the calculations simple, as a first approximation ternary complex formation between the carrier ligand and the protein was neglected. The conditional constant for the binding of the first VO(IV) to apoTf was found to be $\log K_1 = 14.3 \pm 0.6$ (25 °C, pH 7.4, 0.025 M HCO_3^- [25]). In the case of HSA, a value could be obtained only with higher uncertainty: $\log K = 10.0 \pm 1.0$ (25 °C, pH 7.4 [25]). This four orders of magnitude difference in the VO(IV)-binding constants of the two proteins is in accordance with the EPR results, which indicated that HSA is not an efficient VO(IV) binder in the presence of apoTf. Confirmation of

this result was achieved by membrane separation of the LMM and the HMM-bound VO(IV) using a 10 kDa membrane in $VO(L)_2$–protein samples, where L = malt, hdp or pic at 50 μM $VO(L)_2$ and 50 μM of serum proteins at physiological pH. The results showed that apoTf could completely displace the carrier ligands from the $VO(L)_2$ complexes of malt and pic, but ~20% of the VO(IV) remained in the LMM fraction in the case of hdp, due to its higher VO(IV)-binding ability. At the same time, HSA was only very slightly able to transfer the metal ion in the HMM fraction.

On the basis of similar ultrafiltration measurements, Heinemann et al. obtained the same result for vanadate(V), i.e. the binding capacity of HSA was about 1000-fold lower than that of fresh human plasma and Tf [26]. It should be mentioned that this value is very different from that reported by Chasteen et al. [31], who published a value of K(VO(IV)–Tf)/K(VO(IV)–HSA) ~6.

So far, any ternary interactions between the proteins and the VO(IV) carrier complexes have been neglected. However, NMR relaxation studies indicated that this was a simplified approach. In these measurements, the effect of the paramagnetic VO(IV) centers on the spin–lattice relaxation rate of the water protons was measured. The ^{1}H NMR relaxation dispersion behavior of the $VO(pic)_2$, VO(IV)–protein and $VO(pic)_2$–protein samples differed from each other, indicating some ternary interaction, independently whether there is or is not a displacement reaction between the carrier ligand and the protein.

This ternary interaction may be a primary one involving partial displacement of the binding donors of the protein by the donor groups of the carrier ligands, or it may involve secondary interactions through hydrogen-bonding formation or hydrophobic interaction(s) (see below).

A recent detailed paper by Orvig et al. [28] dealt with the interactions of $VO(malt)_2$ (BMOV) with the serum proteins. We agree with them in the main findings, but with one exception. Although they write that "Reaction of BMOV with apo-transferrin can be expected to completely dissociate the complex, however, it may not be the dominant effect in delivery of vanadium from vanadyl chelates to target tissues." They assume that "adduct or ternary albumin complexes could be the pharmacologically active species, or at the very least the main method of vanadium delivery to cells." Our NMR relaxation dispersion measurements also suggested ternary complex formation with HSA, but in the presence of apo-transferrin, the VO(IV) binding of HSA was suppressed and, accordingly, so was its ternary complex formation. At the same time, ternary complex formation with apoTf was supported by both RT EPR and CD spectral results.

The coordination of VO(IV) to apoTf could be followed by CD (see Fig. 5) and EPR (see above). On addition of VO(IV) to a solution of apoTf, a negative CD signal developed at ~635 nm up to two equivalents of VO(IV). Then, the strongest carrier ligand, the pyridinone derivative hdp, which was able to displace the protein, at least partially, was added in increasing amount to the VO(IV)–apoTf 1:1 system. Instead of a continuous decrease in the intensity of the characteristic CD signal, first a slight, but significant shift in the signal was detected, while the RT EPR spectrum changed only slightly (not

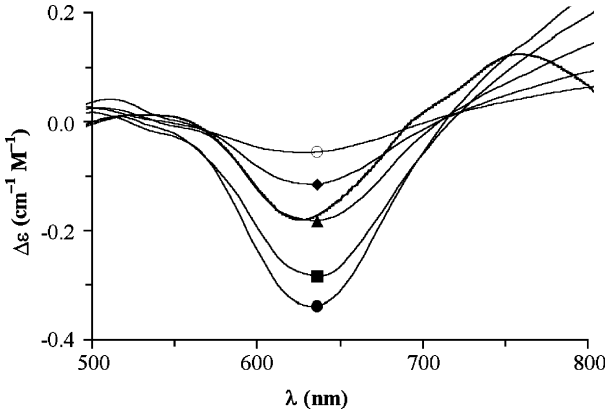

Fig. 5. The effect of increasing hdp concentration on the CD spectra of the VO(IV)–apoTf 1:1 systems at pH 7.4, $[VO(IV)]_{tot}$ = 1 mM (hdp concentration: (—) 0.0 mM, (●) 1.2 mM, (■) 2.4 mM, (▲) 4.9 mM, (♦) 10.2 mM and (○) 21.1 mM).

shown). These spectral observations indicate the formation of a new species (with different CD spectral features), obviously a ternary complex, VO(IV)–apoTf–hdp. The RT EPR spectra can provide further information: the isotropic and anisotropic components of the spectra give the exact molar ratios of the ternary VO(IV)–apoTf–hdp and the bis complex $VO(IV)(hdp)_2$.

Similar behavior observed for the corresponding malt system is demonstrated in Fig. 6. However, for the formation of the ternary VO(IV)–apoTf–malt complex, a higher excess of malt is necessary as compared with hdp.

This difference can be explained by the fact that the $VO(IV)(malt)_2$ complex is 6.5 orders of magnitude less stable than the bis complex of hdp [20,21], and thus the interaction is not as strong as in the case of hdp. The joint evaluation of a series of CD and EPR spectra afforded a possibility to determine the stability constants of all the species at equilibrium, i.e. VO(IV)–apoTf–malt; $\{VO(IV)\}_2$–apoTf–malt; $\{VO(IV)\}_2$–apoTf–$(malt)_2$, and allowed refinement of the log β value of the complex VO(IV)–apoTf [32].

The role of HSA in vanadium binding is still an issue of debate [15,28,31,32]. However, when the CD and RT EPR spectral characteristics of the VO(IV)–apoTf–malt 1:1:1 system were studied in the presence and absence of HSA, unambiguous results were obtained. As can be seen in Fig. 7, the HSA had practically no

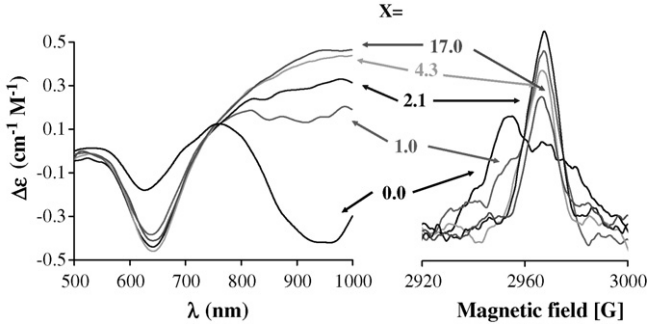

Fig. 6. The effect of increasing malt concentration on the CD and room temperature EPR spectra of the VO(IV)–apoTf 1:1 systems at pH 7.4, $[VO(IV)]_{tot}$ = 1 mM. Malt concentrations (*X* mM) are depicted in the figure.

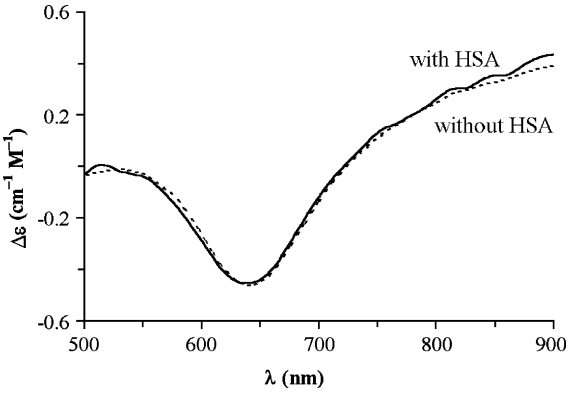

Fig. 7. Effect of equimolar HSA on the CD spectra of the VO(IV)–apoTf–malt 1:1:4 system at pH 7.4, $[VO(IV)]_{tot}$ = 1 mM.

effect on the CD spectrum of the VO(IV)–apoTf–malt ternary complex. Accordingly, the role of HSA in vanadium transport under such conditions seems to be negligible.

In the knowledge of the stability constants of all of the species formed in the antidiabetic vanadium complex–protein systems, the species distribution of the vanadium for serum conditions was calculated. It was found that, in the biologically relevant concentration range (<10 μM), VO(IV) is bound mostly in the form of the 1:1 complex VO(IV)–apoTf, and the formation of ternary complexes is not significant [32,33].

Model calculations on the distribution of VO(IV) between the carrier ligands and apoTf, one of the serum proteins, proved to be in good agreement with the results of the above-mentioned ultrafiltration measurements. A similar calculation was attempted with HSA too, but quantitative evaluation of the data seems possible only by taking into account some direct interaction (without metal ion) between the carrier ligands and HSA [32].

The on-line coupling of a chromatographic separation such as HPLC (to ensure that the desired VO(IV) compound leaves the column adequately separated from other possible vanadium species) with ICP-MS (a specific metal detector) allows the selective and sensitive detection of both components in metallobiomolecules in complex real-life samples. The ICP-MS detector guarantees a reliable and robust determination of the metal content in the chromatographic peak of the biocompound.

Thus, such hybrid systems have proved particularly useful for the investigation of metal species produced in complex biological systems. In particular, these species are formed between metal ions (or complexes) and proteins [34].

As the serum is a complex biological fluid, such methodology is most appropriate. The effect of such a serum complex matrix on the antidiabetic vanadium complexes was therefore studied by HPLC–ICP-MS. The different antidiabetic complexes were incubated with human serum, separated on an anion-exchange column, and the VO(IV) contents of the separated fractions were specifically measured by ICP-MS. For V element measurements the isotope ^{51}V was used. At the same time, in order to follow the whole protein associated to the metal, the sulfur content of the protein was used [35] by parallel monitoring through the isotope ^{34}S in the ICP-MS measurement (multielemental character of the technique).

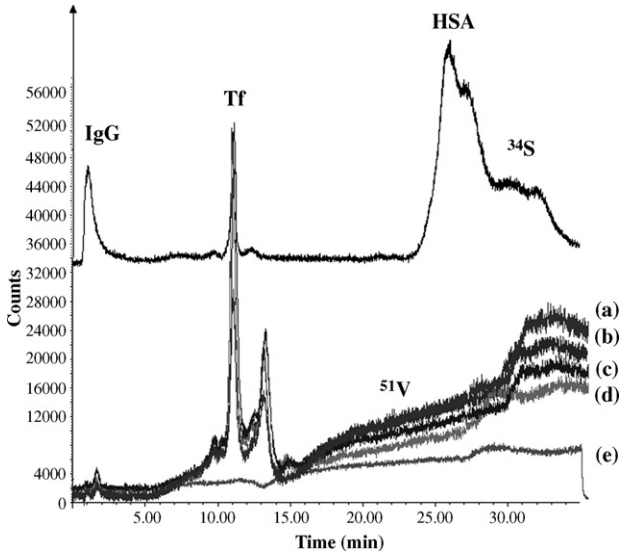

Fig. 8. Chromatographic profile for a basal human serum sample alone and VO(IV) incubated samples (50 μg ml^{-1}) and a gradient blank obtained by HPLC–ICP-MS system. (a) VO(IV)–hdp; (b) VO(IV)–pic; (c) VO(IV)–malt; (d) $VOSO_4$; (e) control (IgG: immunoglobulin; Tf: transferring; HSA: human serum albumin).

As may be seen in Fig. 8, the observed vanadium speciation results obtained for all antidiabetic complexes and with $VOSO_4$ [35] were virtually the same: curves (a)–(d) in Fig. 8 only where S and V elute together in time. Only if a retention time is ascribed to the sharp peak of a well-identified protein (e.g. for Tf in Fig. 8) we have "indication" of a possible binding of metal ion to protein (containing the S). Of course usually a further proof is needed (e.g. ESI–MS experiments or molecular spectroscopy evidence) to confirm the existing metal–protein bond. It is interesting to note that in Fig. 8 after 15 min elution time ^{51}V signal raises steadily, rather than abruptly as observed for the Tf peaks. In our experience, this increasing signal comes from V continuously "bleeding" from the column (after so much time some of the free metal is partially retained in the column and then dragged continuously by the flow of the mobile phase).

In brief, the experiments indicate that VO(IV) seems to be bound only in the Tf fraction, while HSA did not bind measurable amounts of the metal [32]. This was the same result which could be predicted by our modeling measurements and calculations.

Interestingly, Tf was able to bind VO(IV) in at least two different forms (see Fig. 8). In fact, these forms are most probably different isoforms (sialoforms) of human serum Tf. Six of those Tf sialoforms were demonstrated and characterized by integrating liquid chromatography with ICP-MS, MALDI-TOF and ESI-Q-TOF detection when iron was the metal bound to this protein [36]. Molecular MS measurements are in progress in our laboratories to investigate the chemical nature of such Tf isoforms with VO(IV).

The final confirmation of the results would be the in vivo measurement of VO(IV) in the serum. In principle, there is a possibility for this, as a new in vivo analytical technique has been developed for blood circulation monitoring-electron

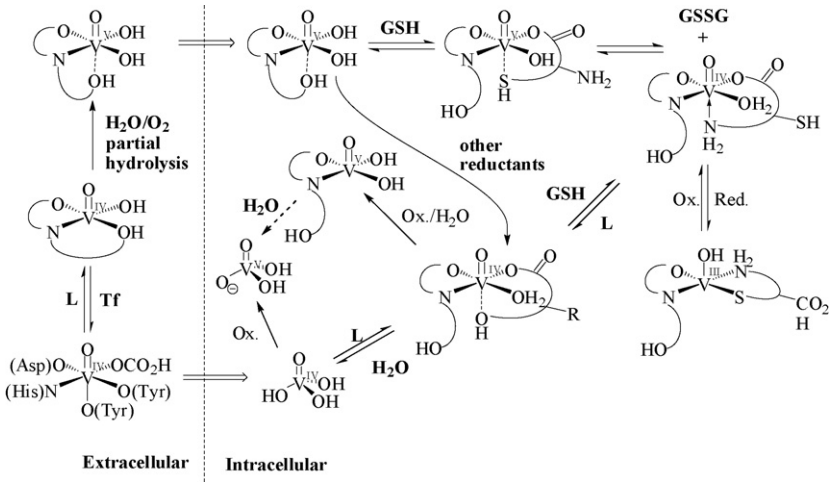

Fig. 9. Potential redox and complexation transformation reactions of an insulin-enhancing vanadium complex in the serum and in the cell. Figure was reproduced from Ref. [10], with permission of the copyright holders.

spin resonance (BCM-ESR) in order to evaluate the real-time pharmacokinetics of stable spin probes in a live animal, and the method has proved very useful for determination of the paramagnetic VO(IV) in the blood [37]. Unfortunately, the low in vivo VO(IV) speciation can as yet not be measured directly.

All the measurements reported here were carried out with metal ion-free apoTf. The results can be applied with good approximation to the interaction of VO(IV) with Tf too, as only ~30% of the metal-binding sites of the protein are saturated with Fe(III) under normal serum conditions, and thus there are enough free sites to bind other, mostly hard metal ions (e.g. Al(III) or VO(IV)) and to transport them in the blood stream. This partial saturation of Tf was taken into account in the speciation calculations.

6. Antidiabetic VO(IV) complexes in the cell

However, the above-mentioned in vivo BCM-ESR studies on rats [37] 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. The binding of VO(IV) to ligands, and mostly to Tf (see above), prevents its oxidation to vanadate(V), which would otherwise take place rapidly at intracellular pH. Nevertheless, oxidation may occur, resulting in the formation of a limited amount of vanadate(V). Accordingly, vanadium may be assumed to enter the cell, either in oxidation state IV through the Tf receptor following the iron pathway, or in oxidation state V, through the phosphate or sulfate pathway. In the intracellular medium, reducing agents can redox-interact with vanadate(V). A frequently discussed candidate for the reduction is glutathione (GSH) [38], although it is a rather ineffectual reducing agent [39,40]. To what extent such redox interactions take place largely depends on the stabilization of vanadium in oxidation state V or IV through the complexation of cell constituents such as GSH, oxidized GSH (GSSG), adenosine 5′-triphosphate (ATP), etc. A high intracellular excess of GSH increases the possibility of formation of VO(IV) and its complexation with either GSH or GSSG. Both have been shown to be reasonably potent binders for VO(IV) [38,41,42]. Other effective reducing agents, such as NADH or ascorbate, may cause the formation of V(III) species [43,44]. Further hydrolytic degradation of VO(IV) may be responsible for the reoxidation to vanadate(V). As seen in Fig. 9, via the redox and complexation reactions, the finely tuned speciation of vanadium may lead to the efficiency of the metal to enhance the effects of insulin.

The results discussed above revealed that the original complexes may remain partially intact, i.e. they keep the original carrier ligand bound to vanadium(IV,V), although the endogenous binders of the biological fluids displace them partially, e.g. through the formation of ternary complexes. Accordingly, if the carrier ligands find a way to reach the cell, some of the cell constituents will compete with them for the central metal ion.

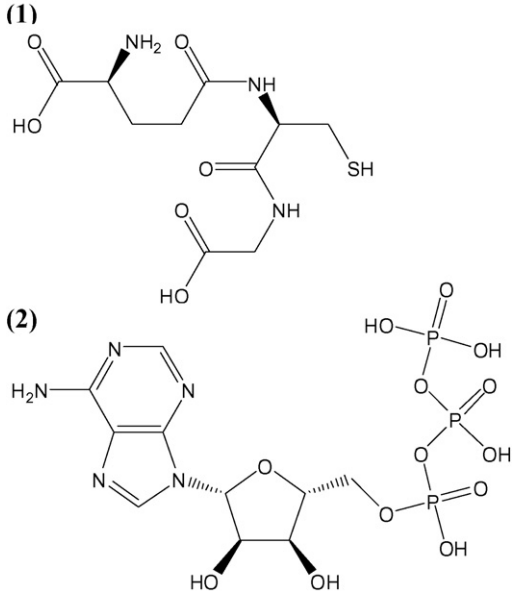

Fig. 10. Structural formulae of the studied cell constituents (1) GSH and (2) ATP.

In order to assess the molecular form of vanadium antidiabetic complexes in cells, the interactions in the model systems of VO(IV)–malt and VO(IV)–dipicolinate with various cell components were studied by employing pH-potentiometric and spectroscopic (EPR, CD and UV–vis) techniques [45]. Two LMM molecules (see Fig. 10), GSH and ATP, are present in rather high concentration in the cell, in high excess relative to the antidiabetic compounds, and thus their interactions with the insulin-enhancing VO(IV) complexes were studied.

In cells, GSH is present in high excess as compared with vanadium; its intracellular concentration level is millimolar, about three orders of magnitude more than the biologically relevant concentration of vanadium. According to speciation studies, for VO(IV) concentrations above ~1 mM and more than a 10-fold excess of the ligand, the bis amino acid type binding mode ($[VO(GSH)_2H_2]^{2-}$) with the donor set 2× (COO^-, NH_2)$_{eq}$ is relevant in the pH range 5.0–6.5 [38,42,46,47]. Participation of the thiolate donor occurs above pH ~7, with formation of the complex $[VO(GSH)H_{-1}]^{2-}$.

As concerns the competition between the carrier ligand malt and GSH, at a 25-fold excess of GSH and a twofold excess of carrier ligand, ternary complex formation (see Fig. 11) could be detected by pH-potentiometry only with rather high uncertainty; this excess of GSH was not sufficient to prevent hydrolysis of the metal ion and formation of the oligonuclear hydroxo species $\{[(VO)_2(OH)_5]^-\}_n$ at pH > 8.0. At the same time, the high buffer capacity of the ligand excess strongly limited the applicability of pH-potentiometry in speciation at such high ligand concentrations. Spectroscopic measurements (EPR, UV–vis and CD), however, unambiguously indicated the formation of ternary complexes.

In accordance with the pH-potentiometric speciation calculations, new EPR signals could be detected [45], which can be assigned to the ternary species formed ($[VO(malt)(GSH)H_2]$, $[VO(malt)_2(GSH)H_2]^-$ and $[VO(malt)_2(GSH)H]^{2-}$). In view of the EPR parameters (the most probable binding set of the complexes formed was estimated on the basis of the additivity rule of the EPR parameters developed by Chasteen [48]) and the low CD signals recorded, we concluded [46] that GSH most probably coordinates at the Gly end via (COO^-, H_2O/O-amide) donors, while participation of the thiolate donor occurs only at pH > 7 (see Fig. 10).

Among the LMM binders, the widely distributed ATP may also be of importance [49], as it efficiently binds VO(IV) and is also present in millimolar concentrations in cells. From a comparison of the VO(IV) complex-forming properties of ATP and GSH, it can be concluded that in the whole pH range ATP is a more efficient VO(IV) binder. ATP coordinates to VO(IV) through the terminal phosphate donor(s) in the weakly acidic and neutral pH range to yield $[VO(ATP)H_x]^{x-2}$ ($x = 2, 1, 0$) and $[VO(ATP)_2]^{6-}$ [49]. On increase of the pH, 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 $[VO(ATP)_2H_{-2}]^{8-}$ is formed, involving a mixed binding mode, one ATP coordinating through the phosphate chain and the other through the ribose moiety. In the species $[VO(ATP)_2H_{-4}]^{10-}$, both ATP molecules coordinate to the VO(IV) via the ribose residue [49]. The CD spectra furnish information on the species in which the ribose moiety is coordinated to the VO(IV); these species are mostly formed above the physiological pH [45].

In cells, ATP is present in high excess relative to VO(IV) and also to the carrier ligands. Our speciation calculations indicated that, in the ternary system with ATP as a strong VO(IV) binder, ATP may displace one of the malt from the bis complex and/or a water in the coordination sphere of VO(IV) from the mono complex. As a result, ternary complexes will exist besides binary ATP complexes at physiological pH too (see Fig. 12). The potentiometric data could be fitted by assuming the formation of the species $[VOAB]^{3-}$ and $[VOABH_{-2}]^{5-}$ in the system [17]. Because of the significant difference in the types of donor groups involved in the coordination, ternary complex formation is clearly indicated by EPR spectroscopy. Below pH 7, a water and a carbonyl group of the bis maltolato complex are substituted by one or two phosphates of the ATP, resulting in a detectable change ($A_{||}$ from 171.4×10^{-4} cm^{-1} for VOA_2 to 169.1×10^{-4} cm^{-1} for $[VOAB]^{3-}$) in the EPR parameters. At pH 8–9, a significant change in the CD spectra (not shown) unambiguously indicated the coordination of the ribose residue, corresponding to the formation of $[VOABH_{-2}]^{5-}$. At pH ≥ 9,

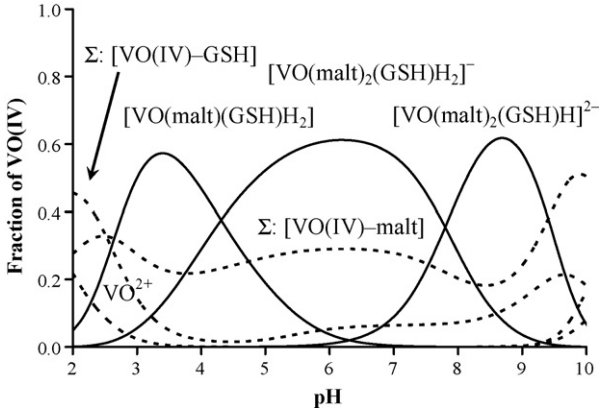

Fig. 11. Species distribution diagram of the VO(IV)–malt–GSH 1:2:50 system, $[VO(IV)]_{tot} = 4$ mM.

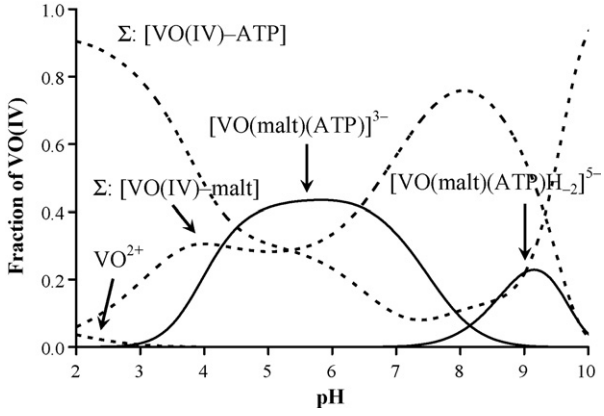

Fig. 12. Species distribution diagram of the VO(IV)–malt–ATP 1:2:10 system, $[VO(IV)]_{tot} = 4$ mM.

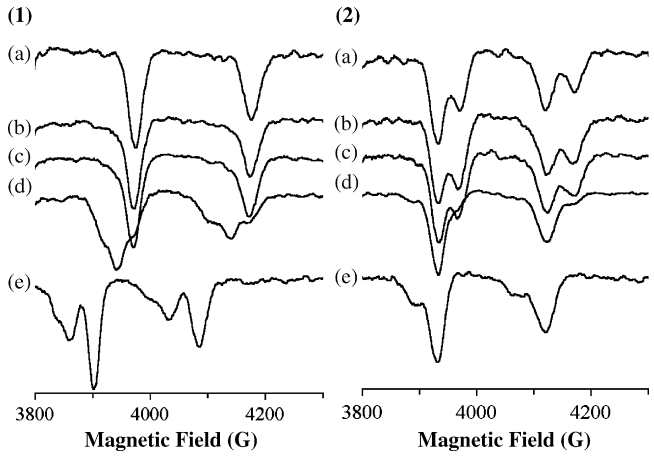

Fig. 13. High field range of the EPR spectra at 77 K of frozen solutions—(1) without carrier ligand and (2) with 8 mM malt: (a) VO(IV)–ATP 1:10; VO(IV)–ATP–GSH (b) 1:10:25, (c)1:10:50 and (d) 1:2.5:50; (e) VO(IV)–GSH 1:50; $[VO(IV)]_{tot} = 4$ mM at pH 7.0.

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 formation of the binary ATP complex, $[VOB_2H_{-4(-5)}]^{7-(8-)}$.

When ATP and GSH are simultaneously considered as potential VO(IV) binders, GSH is not expected to be able to compete with ATP for binding to VO(IV) since ATP is a much stronger ligand. The competition reaction was studied by EPR spectroscopy in the absence and in the presence of the antidiabetic complexes. For the VO(IV)–ATP–GSH ternary system, when ATP was in a 10-fold excess relative to VO(IV), only the EPR signals of the VO(IV)–ATP complexes were detected. New signals appeared only at a 2.5-fold excess of ATP (EPR parameters: $A|| = 172.1 \times 10^{-4}\,cm^{-1}$ and $165.7 \times 10^{-4}\,cm^{-1}$). Comparison of the EPR spectra of solutions containing VO(IV), malt, ATP and GSH at pH ∼7 (see Fig. 13) revealed significant changes only when ATP was not present in at least a 10-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.

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 VO(IV)-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, and are therefore partly neutralized probably in ion-pairing with cations such as K^+ and Mg^{2+} in the cell.

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

7. Conclusions

Several examples have been given to illustrate how modeling calculations can help in characterizing the solution state of antidiabetic VO(IV) complexes in the body in order acquire information on the biologically important/active form of the metal ion in the different biofluids and tissues. We found that:

1. At the acidic pH of the gastric juice, the neutral bis complexes of VO(IV) will dissociate to mono complexes and also free VO^{2+} ions, and thus ternary complex formation with the endogenous or exogenous bioligands of the stomach or intestine may unfavorably affect the absorption efficacy of these molecules. However, as proved by in vitro studies, different encapsulation techniques may overcome this problem and improve the biological effect of the original molecules by defending them from decomposition.
2. After absorption, during their transport in the blood stream, the strong VO(IV) binders of the serum will mostly displace the original VO(IV)-binding ligands and they will be transported by Tf. Some of the original carrier ligand may reach the cell in the form of a VO(IV)–Tf–carrier ternary complex. Even if its concentration is about 20 times higher than that of Tf, the other protein, HSA, seems to be unable to compete with Tf in carrying VO(IV) to the cell. In vitro studies on human serum confirm these results obtained from modeling calculations.
3. In the cell, VO(IV) has a high probability of interacting with GSH and ATP, two LMM components of the cell that are present in high excess, in mM concentration. The modeling calculations suggest that GSH will behave primarily as a redox partner, its role being to keep vanadium in oxidation state IV, while ATP, as a strong VO(IV) binder, will complex the metal ion in binary and ternary complexes formed with the original carrier ligands.

We are aware that these modeling calculations are oversimplifications of the real systems, primarily because the thermodynamic models relate to equilibrium conditions, which do not exist in living systems. Thus, all these models are strongly affected by kinetic factors. For these reasons, the models always need in vitro or even in vivo confirmation. Such measurements, however, are not always easy to perform, e.g. because of the insensitivity of the method or for technical reasons. In such cases, modeling still provides an estimated description of the biological system, although with limited validity.

Acknowledgments

The authors thank Prof. I. Bertini and C. Luchinat for the possibility to carry out the NMRD measurements and for the valuable discussions on the evaluation of the results. This work was performed in the frame of the COST D21/009/01 project (Insulin-Mimetic Vanadium Compounds) and the

Hungarian–Portuguese Intergovernmental S&T Co-operation Program for 2004–2005 and supported by the National Science Research Fund (OTKA Nos. T49417 and NI 61786).

References

[1] M.A. Atkinson, N.K. Maclaren, Sci. Am. 260 (1990) 42.
[2] Diabetes mellitus: Reports of a WHO Study Group, WHO Technical Report Series, 727, 1985, p. 10.
[3] K.H. Thompson, J.H. McNeill, C. Orvig, Chem. Rev. 99 (1999) 2885.
[4] I. Goldwaser, D. Gefel, E. Gershonov, M. Fridkin, Y. Shechter, J. Inorg. Biochem. 80 (2000) 21.
[5] H. Sakurai, Y. Kojima, Y. Yoshikawa, K. Kawabe, H. Yasui, Coord. Chem. Rev. 226 (2002) 187.
[6] K.H. Thompson, J. Chiles, V.G. Yuen, J. Tse, J.H. McNeill, C. Orvig, J. Inorg. Biochem. 98 (2004) 683.
[7] B. Lyonnet, X. Martz, E. Martin, La Presse Med. 32 (1899) 191.
[8] Y. Shechter, S.J.D. Karlish, Nature 284 (1980) 556.
[9] K.H. Thompson, C. Orvig, J. Inorg. Biochem. 100 (2006) 1925.
[10] D. Rehder, J. Costa Pessoa, C.F.G.C. Geraldes, M.M.C.A. Castro, T. Kabanos, T. Kiss, B. Meier, G. Micera, L. Pettersson, M. Rangel, A. Salifoglou, I. Turel, D. Wang, J. Biol. Inorg. Chem. 7 (2002) 384.
[11] M.C. Cam, R.W. Brownsey, J.H. McNeill, Can. Physiol. Pharmacol. 78 (2000) 829.
[12] N. Venkatesan, A. Avidan, M.B Davidson, Diabetes 40 (1991) 492.
[13] A.K. Srivastava, M.Z. Mehdi, Diabet. Med. 22 (2005) 2.
[14] Y. Shechter, I. Goldwaser, M. Mironchik, M. Fridkin, D. Gefel, Coord. Chem. Rev. 237 (2003) 3.
[15] T. Kiss, T. Jakusch, in: M. Gielen, E.R.T. Tiekink (Eds.), Metallotherapeutic Drugs and Metal-based Diagnostic Agents, Wiley, Chichester, 2005, p. 143 (and references therein).
[16] H. Sakurai, J. Fugono, H. Yasui, Mini-Rev. Med. Chem. 4 (2004) 41.
[17] T. Kiss, E. Kiss, G. Micera, D. Sanna, Inorg. Chim. Acta 283 (1998) 202.
[18] P. Buglyó, E. Kiss, I. Fábián, T. Kiss, D. Sanna, E. Garribba, G. Micera, Inorg. Chim. Acta 306 (2000) 174.
[19] E. Kiss, K. Petrohán, D. Sanna, E. Garribba, G. Micera, T. Kiss, J. Inorg. Biochem. 78 (2000) 97.
[20] T. Kiss, E. Kiss, E. Garribba, H. Sakurai, J. Inorg. Biochem. 80 (2000) 65.
[21] P. Buglyó, T. Kiss, E. Kiss, D. Sanna, E. Garribba, G. Micera, J. Chem. Soc., Dalton Trans. (2002) 2275.
[22] W.R. Harris, Clin. Chem. 38 (1992) 1809.
[23] H. Sakurai, A. Tamura, J. Fugano, H. Yasui, T. Kiss, Coord. Chem. Rev. 245 (2003) 31.
[24] H. Yasui, Y. Kunori, H. Sakurai, Chem. Lett. 32 (2003) 1032.
[25] T. Kiss, T. Jakusch, S. Bouhsina, H. Sakurai, É.A. Enyedy, Eur. J. Inorg. Chem. 18 (2006) 3607.
[26] G. Heinemann, B. Fichtl, M. Mentler, W. Vogt, J. Inorg. Biochem. 90 (2002) 38.
[27] K.H. Thompson, B.D. Liboiron, G.R. Hanson, C. Orvig, Medicinal inorganic chemistry, in: J.L. Sessier, S.R. Doctrow, T.J. McMurry, S.J. Lippard (Eds.), ACS Symposium Series 903, ACS, Washington, DC, 2005, p. 384.
[28] B.D. Liborion, K.H. Thompson, G.R. Hanson, E. Lam, N. Aebischer, C. Orvig, J. Am. Chem. Soc. 127 (2005) 5104.
[29] K.H. Thompson, Y. Tsukuda, Z. Xu, M. Bartell, J.H. McNeill, C. Orvig, Biol. Trace Elem. Res. 86 (2002) 31.
[30] K.H. Thompson, B.D. Liboiron, Y. Sun, K.D.D. Bellman, I.A. Setyawati, B.O. Patrick, V. Karunarante, G. Rawji, J. Wheeler, K. Sutton, S. Bhanot, C. Cassidy, J.H. McNeill, V.G. Nguen, C. Orvig, J. Biol. Inorg. Chem. 8 (2003) 66.
[31] N.D. Chasteen, J.K. Grady, C.E. Holloway, Inorg. Chem. 25 (1986) 2754.
[32] M. Montes-Bayón, T. Jakusch, D. Hollender, J. Costa Pessoa, A. Sanz-Medel, T. Kiss, in preparation.
[33] H.Z. Sun, M.C. Cox, H.Y. Li, P.J. Sadler, Struct. Bond. 88 (1997) 71.
[34] A. Sanz-Medel, M. Montes-Bayón, M.L. Fernández Sánchez, Anal. Bioanal. Chem. 377 (2003) 236.
[35] K.G. Fernandes, M. Montes-Bayón, E.B. González, E.D. Castillo-Busto, J.A. Nóbrega, A. Sanz-Medel, J. Anal. At. Spectrom. 20 (2005) 210.
[36] M.E. del Castillo Busto, M. Montes-Bayón, E. Blanco-González, J. Meija, A. Sanz-Medel, Anal. Chem. 77 (2005) 5615.
[37] H. Sakurai, S. Shimomura, K. Fukazawa, K. Ishizu, Biochem. Biophys. Res. Commun. 96 (1980) 293.
[38] T. Goda, H. Sakurai, T. Yashimura, Nippon Kagaku Kaishi (1998) 654.
[39] J. Li, G. Elberg, D.C. Crans, Y. Shechter, Biochemistry 35 (1996) 8314.
[40] I.G. Macara, K. Kustin, L.C. Cantley, Biochim. Biophys. Acta 629 (1980) 95.
[41] J. Costa Pessoa, I. Tomaz, T. Kiss, P. Buglyo, J. Inorg. Biochem. 84 (2001) 259.
[42] J. Costa Pessoa, I. Tomaz, T. Kiss, E. Kiss, P. Buglyo, J. Biol. Inorg. Chem. 7 (2002) 225.
[43] A. Stern, A.J. Davison, Q. Wu, J. Moon, Arch. Biochem. Biophys. 299 (1992) 125.
[44] K. Kanamori, Y. Kinebuchi, H. Michibata, Chem. Lett. (1997) 423.
[45] Á. Dörnyei, S. Marcão, J. Costa Pessoa, T. Jakusch, T. Kiss, Eur. J. Inorg. Chem. 18 (2006) 3614.
[46] A. Dessi, G. Micera, D. Sanna, J. Inorg. Biochem. 52 (1993) 275.
[47] A.J. Tasiopoulos, A.N. Troganis, A. Evangelou, C.P. Raptopoulou, A. Terzis, Y.G. Deligiannakis, T.A. Kabanos, Chem. Eur. J. 5 (1999) 910.
[48] N.D. Chasteen, in: J. Lawrence, L.J. Berliner, J. Reuben (Eds.), Biological Magnetic Resonance, vol. 3, Plenum, New York, NY, 1981, p. 53.
[49] E. Alberico, D. Dewaele, T. Kiss, G. Micera, J. Chem. Soc., Dalton Trans. 3 (1995) 425.