

Sarcoplasmic Reticulum Calcium ATPase Is Inhibited by Organic Vanadium Coordination Compounds: Pyridine-2,6-dicarboxylatodioxovanadium(V), BMOV, and an Amavadine Analogue

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The general affinity of the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase was examined for three different classes of vanadium coordination complexes including a vanadium(V) compound, pyridine-2,6-dicarboxylatodioxovanadium(V) (PDC–V(V)), and two vanadium(IV) compounds, bis(maltolato)oxovanadium(IV) (BMOV), and an analogue of amavadine, bis(*N*-hydroxylamidoiminodiacetato)vanadium(IV) (HAIDA–V(IV)). The ability of vanadate to act either as a phosphate analogue or as a transition-state analogue with enzymes' catalysis phosphoryl group transfer suggests that vanadium coordination compounds may reveal mechanistic preferences in these classes of enzymes. Two of these compounds investigated, PDC–V(V) and BMOV, were hydrolytically and oxidatively reactive at neutral pH, and one, HAIDA–V(IV), does not hydrolyze, oxidize, or otherwise decompose to a measurable extent during the enzyme assay. The SR Ca^{2+} -ATPase was inhibited by all three of these complexes. The relative order of inhibition was PDC–V(V) > BMOV > vanadate > HAIDA–V(IV), and the IC_{50} values were 25, 40, 80, and 325 μM , respectively. Because the observed inhibition is more potent for PDC–V(V) and BMOV than that of oxovanadates, the inhibition cannot be explained by oxovanadate formation during enzyme assays. Furthermore, the hydrolytically and redox stable amavadine analogue HAIDA–V(IV) inhibited the Ca^{2+} -ATPase less than oxovanadates. To gauge the importance of the lipid environment, studies of oxidized BMOV in microemulsions were performed and showed that this system remained in the aqueous pool even though PDC–V(V) is able to penetrate lipid interfaces. These findings suggest that the hydrolytic properties of these complexes may be important in the inhibition of the calcium pump. Our results show that two simple coordination complexes with known insulin enhancing effects can invoke a response in calcium homeostasis and the regulation of muscle contraction through the SR Ca^{2+} -ATPase.

Introduction

Vanadium oxoanions are known to inhibit or stimulate many enzymes^{1–7} such as the membrane-bound E1–E2

Ca^{2+} -ATPase.^{8,9} Because vanadate can act as a transition-state analogue as well as a phosphate analogue, the details

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of the mode of inhibition are often not clear and inhibition of organic vanadium complexes can be of mechanistic importance to sort out the different modes of action.^{1,2,6} The active site of the Ca²⁺-ATPase, a calcium pump, contains an aspartyl residue that is phosphorylated during the catalytic cycle, thereby forming an acyl phosphate anhydride.¹⁰ The mechanism of the inhibition by vanadate is attributed to the blocking by vanadate of the formation of the E2 conformation of the Ca²⁺-ATPase from the sarcoplasmic reticulum (SR).⁸ How vanadate oligomers interact with the SR calcium pump and inhibit its activity have been investigated.^{9,11} For instance, decameric vanadate species (V₁₀) in functional SR vesicles (absence of calcium ionophore) inhibit calcium accumulation coupled with ATP hydrolysis, whereas monomeric vanadate (V₁) has no effects.¹¹ Moreover, other vanadate species, for example tetrameric vanadate species (V₄), may also have an implicit effect on H⁺ ejection associated with calcium translocation.⁹ In fact, the SR calcium pump has proven to be an excellent model to study the effects of vanadium in Ca²⁺ homeostasis, particularly in instances where some oxovanadates have been found to disrupt energy coupling and enzyme turnover.⁹ Recently, decreased SR calcium pump activity was reported under specific conditions associated with diabetes that could result in impaired cardiac relaxation.¹²

Many vanadium compounds are known to have insulin-like or insulin-enhancing effects on several animal model systems.^{6,13–16} Studies have been carried out with simple salts and coordination complexes of vanadium in oxidation states III, IV,^{6,13,14,16} and V.^{6,15,16} In addition to the animal studies, a range of studies was also carried out in several cellular systems, which probed the activity of specific classes of vanadium compounds and how these compounds act.^{17–19} In such biological studies, the nature of the active vanadium compound cannot readily be identified, and consideration of chemical and metabolic processing is difficult.^{6,15,16} However, various approaches to address this problem have provided some further insight as to the biological activity

of these compounds.^{5–7,16,20} Information on how effectively specific compounds inhibit isolated enzymes is important for consideration of their mode of actions in cells and animals.⁶

A range of studies of a few vanadium compounds on isolated enzyme systems have been reported where the species exerting the effects can be more rigorously tracked.^{5,21,22} Most of these studies were carried out with phosphatases and kinases,^{21,22} but some studies with proteins in other metabolic pathways^{5,23,24} including the Ca²⁺-ATPase are available.²⁵ Vanadium complexes are generally found to be inhibitors, although the potency of the compounds varies widely from millimolar to nanomolar efficacy.⁶ The effects of oxovanadates have been reported on a range of isolated classes of enzymes such as phosphorylases and dehydrogenases, and include application of vanadate as a structural probe in X-ray diffraction studies of protein–vanadate complexes.²⁶ In this work, we consider the effects of three very different classes of vanadium compounds, two of which are known to induce insulin-enhancing effects^{16,27} and a third class that is a well-known analogue of a vanadium-containing natural product.^{28–31} The vanadium-containing natural product is very different from the other two vanadium compounds with regard to structure and properties (Figure 1).

Conversion of vanadium complexes to simple vanadate oligomers^{5,16} and salts in the biological system complicate the interpretation of the effects of the compounds and is not generally considered. Even in the micromolar range, oxovanadates can induce specific responses in enzymes such as phosphohydrolases^{5,32,33} and, therefore, effects observed in vivo^{20,34–38} may be, in part, due to the action of species such as decavanadate (V₁₀),³⁸ vanadate monomer,⁵ or vanadyl

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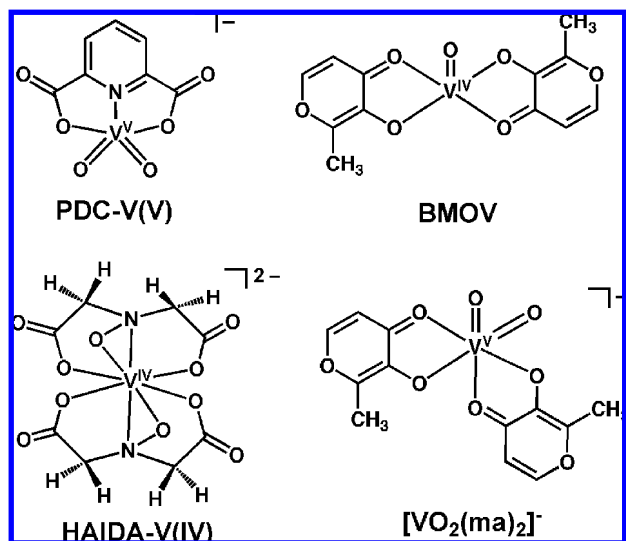


Figure 1. Molecular structures of PDC–V(V), BMOV, HAIDA–V(IV), and $[\text{VO}_2(\text{ma})_2]^-$.

cation.^{5,23,24,39} Combining biochemical kinetic studies with analysis by ^{51}V NMR spectroscopy,^{9,11} it is possible to correlate inhibition with the presence of specific vanadate species as has previously been reported.^{5,9,11,23,24,26}

In the present study, the effects of three vanadium complexes, namely pyridine-2,6-dicarboxylatodioxovanadium(V) (PDC–V(V)),¹⁶ bis(maltolato)oxovanadium(IV) (BMOV)²⁷ and an analogue of amavadine, bis(*N*-hydroxylamidoiminodiacetato)vanadium(IV) HAIDA–V(IV),²⁸ on SR Ca^{2+} -ATPase activity were evaluated (Figure 1). The two complexes known to interconvert to other species at neutral pH and in the presence of oxygen were studied to examine the extent of conversion of these systems to other vanadium(V) species and to determine whether the resulting species interact with the SR calcium pump. The most-potent inhibition observed for the PDC–V(V) complex exceeded that of vanadate solutions reported previously¹¹ and provided compelling evidence that the observed inhibition cannot simply be due to conversion of the complex to oxovanadate.⁶

Materials and Methods

Reagents. Reagent-grade chemicals were used to prepare buffers. Ammonium metavanadate was purchased from Riedel-de Haën. ATP (vanadium free) was supplied by Sigma. All other reagents were of biochemical analysis grade from BDH, Merck, or Sigma.

The synthesis of the vanadium complexes was performed as described previously for pyridine-2,6-dicarboxylatodioxovanadium(V) PDC–V(V),¹⁶ bis(maltolato)oxovanadium(V) (BMOV)⁴⁰ and the simple amavadine analogue bis(*N*-hydroxylamidoimino-

diacetato)vanadium(IV), HAIDA–V(IV).^{28,41} The chemistry of these systems is well-described and predicts that a minor fraction of the PDC–V(V) complex would retain its structural identity during the enzyme assay;¹⁶ however, a larger fraction of BMOV^{6,7,14} and all of the amavadine analogue remain in their original form.^{6,7,29,30} These predictions are based on reports that the PDC–V(V) complexes are hydrolyzed and reduced at neutral pH and under physiological conditions^{6,7} BMOV is known to oxidize in solution^{6,7,14} but amavadine is stable.^{6,7,29,30}

Stock solutions (9 mM) of vanadium complexes were prepared from pyridine-2,6-dicarboxylatodioxovanadium(V) (PDC–V(V)), bis(maltolato)oxovanadium(IV) (BMOV), and amavadine-analogue HAIDA–V(IV) in water or in media containing 25 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid), pH 7.0, and 0.1 M KCl, immediately prior to use in studies.

Preparation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum vesicles (SRV) were prepared from skeletal rabbit muscles as described elsewhere.^{11,42} For activity measurements, the SR were isolated from rabbits that had been subjected to a 48 h starvation diet⁴³ to decrease the amount of glycogen phosphorylase associated with the SR membranes. Isolated SR vesicles suspended in 0.1 M KCl, 10 mM HEPES (pH 7.0) were diluted 1:1 with 2.0 M sucrose and frozen in liquid nitrogen prior to storage at -80°C . For SR-vesicle preparations used in NMR studies, sucrose was omitted from sample preparations because sucrose is known to form complexes with vanadate.^{9,11} Protein concentration was determined spectrophotometrically at 280 nm by the Biuret method, using bovine serum albumin as a standard⁴⁴ and in the presence of 1% of sodium dodecyl sulfate.⁴⁵ The percentage of each protein present in the SR-vesicle preparations was determined through densitometry analysis of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (7.5% acrylamide). The SR Ca^{2+} -ATPase analyzed by SDS-polyacrylamide gel electrophoresis was at least 70% of the total protein in the SR-vesicles. The SERCA 1, sarcoplasmic, or endoplasmic reticulum Ca^{2+} -ATPase 1 was found as the predominant isoform in our SR preparations.⁴⁵

NMR Measurements. Vanadium-51 (^{51}V) nuclear magnetic resonance spectroscopy measurements on vanadium complex solutions were recorded on a Bruker AM-400 MHz or a Varian Inova 400 MHz spectrometer using a 90° pulse angle. Chemical shift values are referenced to a VOCl_3 (0.00 ppm) standard. The relative areas of vanadium(V) resonances were integrated, and the line widths were obtained by subtracting the 20 Hz line broadening used in the data processing. The concentrations of vanadium(V) species were calculated from the fractions of the total integrated areas observed in the recorded spectra as described elsewhere.^{5,11}

The ^{51}V NMR spectra of solutions of vanadium complexes (PDC–V(V) and oxidized BMOV) were obtained at several concentrations in the reaction medium, containing 0.1 M KCl, 5.0 mM MgCl_2 , 50 μM CaCl_2 , and 500 μM ATP in 25 mM (pH 7.0) HEPES. Spectra of the complexes were obtained in the reaction medium at ambient temperature, in the absence or presence of SR preparations (up to 10 mg/mL). The spectral parameters for these

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solutions were spectral width 40 355 Hz, accumulation time 0.05 s, number of transients between 12 000 and 25 000, and a relaxation delay of 0.01 s. These parameters were held constant for all quantitative measurements.¹¹

EPR Measurements. EPR spectra were obtained with a conventional X-band Bruker EMX 6/1 spectrometer equipped with a Bruker ER4116DM dual mode cavity. An Oxford ESR900 continuous-flow cryostat was used. The spectra of frozen solutions were acquired at 77 K using 0.2 mL of samples. Typical measurement conditions for CW-EPR were field modulation frequency, 100 kHz; modulation amplitude, 10 G; receiver gain, 5.02×10^4 ; time constant, 40.96 ms; sweep time 167.77 s; microwave power, 0.2 mW; and the number of scans, 4.

ATP Hydrolysis By Calcium Pump. Steady-state assays of the sarcoplasmic reticulum Ca^{2+} -ATPase were measured spectrophotometrically at 25 °C, using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay⁴⁵ under the following conditions: 25 mM HEPES (pH 7.0), 100 mM KCl, 5 mM MgCl_2 , 50 μM CaCl_2 , 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 18 IU lactate dehydrogenase, and 7.5 IU pyruvate kinase. The experiments were initiated after the addition of 10 $\mu\text{g}/\text{ml}$ protein in the presence of 4% (w/w) of calcium ionophore A23187 and followed for 10 min. Vanadium complex solutions were added to the medium prior to protein addition. The absorbance of the vanadium complexes had no effect on the observation of the rapid decrease in absorbance at 340 nm upon addition of 40 μM ADP after the assay.

Model Microemulsion Preparation. The microemulsion model system (reverse micelles, RMs, AOT/isooctane/ H_2O) were prepared by pipetting the solution of $[\text{VO}_2(\text{ma})_2]^-$ (the oxidized form of BMOV, Figure 1)⁴⁰ at pH 4.4, 5.9, and 7.6, into aliquots of the 0.500 M AOT (sodium bis(2-ethylhexyl) sulfosuccinate) stock solution in isooctane. $[\text{VO}_2(\text{ma})_2]^-$ was prepared from 60 mM NaVO_3 and 120 mM maltol (3-hydroxy-2-methyl-4-pyrone) to form the 1:2 complex in D_2O as described previously.⁴⁰ All samples were mixed by shaking prior to spectroscopic measurements, and the resulting solutions were transparent. Dynamic light scattering experiments were performed to demonstrate that RMs form in solution and to measure their size and polydispersity (DynaPro-MSTC) as reported previously.⁴⁶

Results and Discussion

We find that the addition of the three different vanadium complexes, PDC-V(V), BMOV, and HAIDA-V(IV), has markedly different effects from oxovanadate on the activity of the calcium ATPase from the sarcoplasmic reticulum. The ATPase activity is measured by coupling pyruvate dehydrogenase/lactate dehydrogenase oxidation of NADH and plotted as a percentage activity compared to the observed activity in the absence of test compound. All three vanadium compounds inhibit the ATP hydrolysis, with the PDC-V(V) inhibiting the hydrolysis at lower concentrations. The 50% inhibition level was observed for PDC-V(V) and BMOV at 25 μM and 40 μM , respectively (part A of Figure 2). The amavadine analogue HAIDA-V(IV) also inhibits the enzymatic activity but to a lower extent, with an IC_{50} value of 325 μM (part B of Figure 2). The inhibition is observed at compound levels higher than typically administered in animal studies,^{6,15,20,40} however, it is not uncommon that the

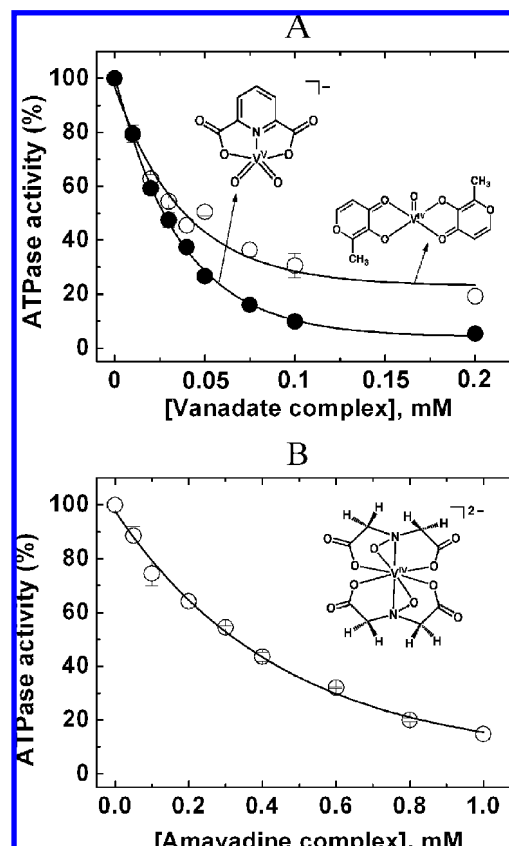


Figure 2. Effects of (A) BMOV (○) and PDC-V(V) (●), (B) HAIDA-V(IV) on Ca^{2+} -ATPase activity by the SR calcium pump. Sarcoplasmic reticulum vesicles, 10 $\mu\text{g}/\text{ml}$, were incubated during 5 min at 25 °C in a medium containing 25 mM HEPES (pH 7.0), 100 mM KCl, 5.0 mM MgCl_2 , 50 μM CaCl_2 , 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, and the concentration of the vanadium complexes in (A) (PDC-V(V) (●) and BMOV (○)) and (B) (HAIDA-V(IV)) was varied as indicated on the abscissa. After the incubation, 18 IU lactate dehydrogenase plus 7.5 IU pyruvate kinase, 0.4 $\mu\text{g}/\text{ml}$ A23187, and 2.5 mM ATP were added to the medium. The ATPase activity was measured as indicated in the Materials and Methods section. Experimental values are means of at least 3 different experiments with 3 different sarcoplasmic reticulum vesicles preparations. In most experiments, the SD values are covered by the symbol.

inhibition level of isolated enzymes does not correspond with inhibition observed in cells.

No effects were observed on the basal activity recorded just before the addition of the calcium ionophore. This observation suggests that, at the concentration of compounds studied, the complexes do not uncouple the calcium accumulation from the ATP hydrolysis of the native vesicles from the sarcoplasmic reticulum calcium pump. Recent studies demonstrated that vanadium citrate complexes are weak inhibitors of the calcium ATPase with an IC_{50} of 500 μM .²⁵ By comparison, under identical experimental conditions as used here, a metavanadate solution results in an IC_{50} value of 80 μM (data not shown). Therefore, we conclude that the BMOV and PDC-V(V) complexes are stronger inhibitors of the calcium pump than the most-potent oxovanadates. Given these observations, we investigated the interaction of the vanadium species that existed under the assay conditions in the presence of the SR preparation.

The ^{51}V -NMR spectra of the PDC-V(V) complex in the medium shows a single resonance at -534 ppm (Figure 3) with a half-line width of 120 ± 10 Hz. This chemical shift

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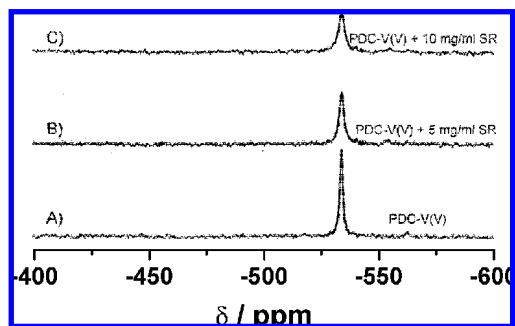


Figure 3. ^{51}V -NMR spectra (105.2 MHz) of 4 mM PDC–V(V) complex in a medium containing 25 mM HEPES pH 7.0, 0.1 M KCl, 5 mM MgCl_2 , in the absence (A) or in the presence of sarcoplasmic reticulum vesicles (5 mg/mL total protein (B) or 10 mg/mL (C)).

is close to the -531 ppm reported for the PDC–V(V) complex in aqueous solution¹⁶ and demonstrates that this is the species present under assay conditions. Addition of sarcoplasmic reticulum vesicles (up to 10 mg/mL) to the PDC–V(V) solution in the reaction medium results in significant line broadening (up to 300 Hz) and decrease in intensity of the ^{51}V -NMR signal with increasing protein concentration (parts B and C of Figure 3). Signal broadening has previously been observed when vanadium derivatives interact with proteins.^{47–49} There was no significant change in chemical shift in the spectra as protein concentration was increased. The solution was very sensitive to temperature and vanadate oligomers formed when slight-heating was used during solution preparation. In this case, besides the resonance at -534 ppm, signals were observed at -560 , -574 , and -578 ppm due to monomeric (V_1), dimeric (V_2), and to cyclic tetrameric (V_4) vanadate species (data not shown), respectively. Because sucrose is often present in the SR preparations, spectra were acquired in the presence of sucrose. No broadening of the NMR signal was detected by the addition of the equivalent amounts of sucrose to those present in the SR preparations (data not shown). These results indicate that the observed line broadening is due to vanadium complex interaction with the SR preparation.

The possibility that signal broadening may be due to components in the SR-vesicles other than the calcium pump was considered. Significant amounts of glycogen phosphorylase (16%) and caldesquestrin (10%) besides others proteins (as observed by SDS-PAGE gel electrophoreses) may be present in these preparations. Previous studies with glycogen phosphorylase indicate that this enzyme interacts with vanadate; however, the interaction is not extensive (unpublished data⁵⁰). Although vanadate does interact with other proteins, significant amounts of protein must generally be present to result in the observed line broadening.^{5,23,24,51,52} Therefore, although these studies do not rule out that these

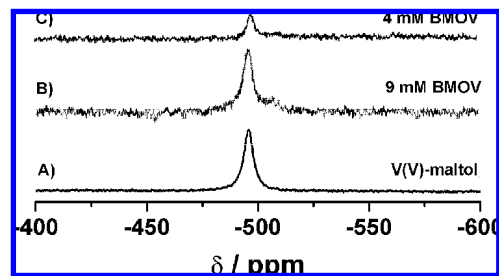


Figure 4. ^{51}V -NMR spectra (105.2 MHz), at 22 °C, of 9.0 mM $[\text{VO}_2(\text{ma})_2]^-$ (V(V)-maltol) (A) in aqueous solution, 9.0 mM (B), and 4.0 mM (C) BMOV complex solution prepared in the reaction medium; the spectra observed in (B) and (C) represent the oxidized forms of BMOV.

interactions with the vanadium complexes exist, the magnitude of the observed response would require a protein with an exceedingly strong affinity for vanadate to exhibit the observed effect.

The possibility that the line broadening is caused by association of the compounds with the lipid arrangement (assembly) in the SR-vesicle was also considered. We have previously observed that PDC–V(V) is able to penetrate a model lipid surface.⁵¹ In the model system, line broadening of the PDC–V(V) signal was attributed to immobilization of the compound because proton 2D NOESY spectroscopy studies demonstrated that the protons on the aromatic ring were near the lipid tail group, and thus the complex penetrates the nonpolar layer.^{51,52} In the studies with the SR-vesicle, line broadening may also occur because of this type of interaction. However, because the concentration of lipid in the assay is small and the vanadium complex is in large excess, the lipid interaction in these preparations is not likely able to support the 4 mM PDC–V(V) complex used in these studies. These considerations are consistent with attributing line broadening of the complex signal primarily to interaction with the Ca^{2+} -ATPase.

The BMOV solutions contain vanadium(IV) species observable by EPR spectroscopy (data not shown) consistent with reports describing the solution properties of this compound.⁴⁰ In addition, BMOV is known to oxidize in the presence of oxygen and form the corresponding V(V)–maltol, $[\text{VO}_2(\text{ma})_2]^-$ complex (part A of Figure 4).^{40,53} The oxidation of BMOV under the assay conditions described in this work immediately result in the formation of $[\text{VO}_2(\text{ma})_2]^-$ (parts B and C of Figure 4); however, even after 24 h, less than 50% of BMOV oxidized in cell-free samples. It is known that such oxidation is much less after administration of BMOV to cells and animals.^{40,54} Studies, using ^{51}V NMR spectroscopy, were conducted to monitor how much of the BMOV oxidized to form $[\text{VO}_2(\text{ma})_2]^-$ complex and oxovanadates and to demonstrate that placement in the assay medium did not impact the complex otherwise. The spectrum presented in Figure 4 has a signal at -495 ppm that is

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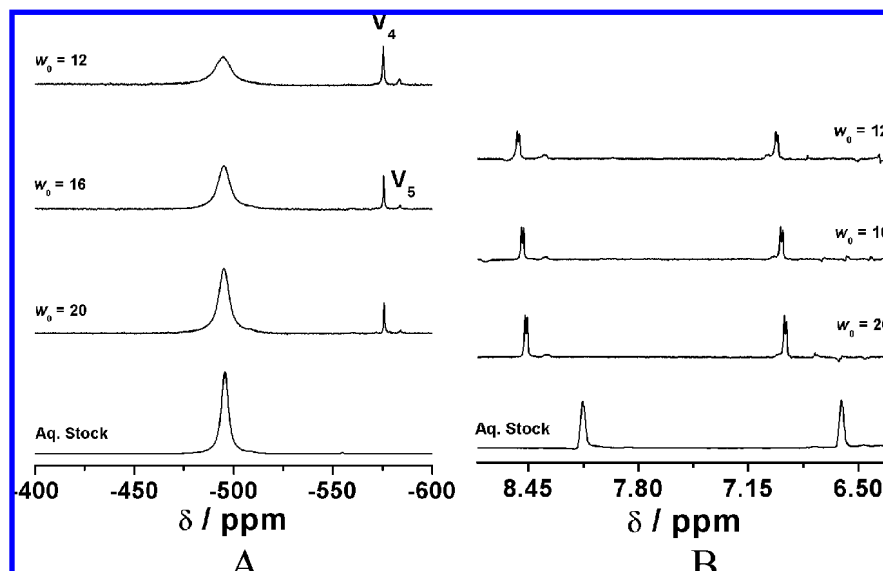


Figure 5. ^{51}V (panel A) and ^1H (panel B) NMR spectra of $[\text{VO}_2(\text{ma})_2]^-$ in $\text{D}_2\text{O}/\text{AOT}/\text{isooctane}$ microemulsions with $w_0 = 12, 16,$ and 20 . Stock $[\text{VO}_2(\text{ma})_2]^-$ solution was prepared from 60 mM of NaVO_3 and 120 mM of maltol at $\text{pH } 7.6$. Reverse micelles were prepared by adding a calculated volume of pH -adjusted aqueous stock solutions to a calculated volume of 0.2 M AOT stock solution in isooctane, followed by vortexing to yield an optically transparent homogeneous solution.

Table 1. ^{51}V NMR Parameters for $[\text{VO}_2(\text{ma})_2]^-$ in Aqueous Solution Prepared from 60 mM NaVO_3 and 120 mM maltol at $\text{pH } 7.6$ and Microemulsions size ($\text{D}_2\text{O}/\text{AOT}/\text{Isooctane}$ RMs) Prepared from 0.2 M AOT in Isooctane as Described in Figure 5

w_0^a	chemical shift (ppm)	linewidth (Hz)	r_h (nm) ^b
stock	-495.6	420 ± 3	
20	-495.2	630 ± 6	12.5 ± 0.3
16	-495.2	732 ± 7	6.4 ± 0.4
12	-494.6	960 ± 12	5.7 ± 0.1

^a $w_0 = [\text{H}_2\text{O}]/[\text{surfactant}]$; this is a term used to describe the microemulsions.⁴⁶ ^b r_h is the diameter of the reverse micelle measured using DLS.⁴⁶

consistent with formation of the most stable $\text{V(V)}-\text{maltol}$ complex near neutral pH .⁴⁰

In addition to the major oxidation product of BMOV, $[\text{VO}_2(\text{ma})_2]^-$, (at -495 ppm , a second signal near -509 ppm is observed and attributed to $[\text{VO}_2(\text{ma})(\text{H}_2\text{O})_2]$ (Figure 4). Equilibration of these two $\text{V(V)}-\text{maltol}$ will cause some line broadening between the signals, and the presence of the paramagnetic BMOV in these solutions can further increase the relaxation rates, which contributes to line broadening. For comparison, the spectrum is shown of an aqueous solution of $[\text{VO}_2(\text{ma})_2]^-$ (part A of Figure 4), demonstrating that less line broadening is in fact observed under the conditions of the enzyme assay.

When the solution of $[\text{VO}_2(\text{ma})_2]^-$ is placed in a microemulsion (Figure 5), the signal is broadened. In addition to the $[\text{VO}_2(\text{ma})_2]^-$ signal, a signal for oxovanadates ($\sim -555\text{ ppm}$) appeared. This observation suggests that the stability of $[\text{VO}_2(\text{ma})_2]^-$ is lower in the microemulsions, and the complex hydrolyzed to form both V_4 and V_5 oxovanadates. Whether the $\text{V(V)}-\text{maltol}$ complexes are similar to the $\text{PDC}-\text{V(V)}$ complex⁵¹ in ability to penetrate the lipid interface can now be considered. As shown in Figure 5, both the ^{51}V (A) and the ^1H (B) NMR signals shifted and broadened in the microemulsion environment. In Table 1, the line widths of the $[\text{VO}_2(\text{ma})_2]^-$ signal for the corresponding sizes of the RM are shown. The line width of the

$[\text{VO}_2(\text{ma})_2]^-$ signal increases from 420 Hz in aqueous solution at $\text{pH } 7.6$ to more than double in small RMs, and, importantly, as the water pool of the RM increases the line width of the complex approaches that in aqueous solution. The ^1H NMR spectra (part B of Figure 5) also show a significant shift upon placement of $[\text{VO}_2(\text{ma})_2]^-$ in the RM and a small but continuous shift as the RM size decreases, consistent with the increased interaction with the lipid interface. The change in the line width is indicative of the $[\text{VO}_2(\text{ma})_2]^-$ complex being located in the water pool possible closely associated with the lipid interface in these structures and not as $\text{PDC}-\text{V(V)}$, which penetrated into the lipid portion of the interface. The large downfield changes in the ^1H NMR chemical shifts are in contrast to the small upfield changes in the ^{51}V chemical shifts. However, as previously reported, the downfield ^1H NMR shift is consistent with interaction with the lipid interface,^{58,59} although we have recently found some inconsistencies with these empirical correlations.⁵¹ We conclude that our experiments in microemulsions are consistent with $\text{PDC}-\text{V(V)}$ penetrating the lipid interface, whereas $[\text{VO}_2(\text{ma})_2]^-$ is more associated with the aqueous pool interface.

We conclude that the observed inhibition of the ATPase is caused by solutions containing BMOV and two $\text{V(V)}-\text{maltol}$ complexes, and either of these complexes or a combination thereof could be the inhibiting species of the ATPase. Regardless of the differential affinity of these complexes for lipids, because the $\text{PDC}-\text{V(V)}$ and BMOV systems have very similar affinity for the Ca^{2+} -ATPase, and $\text{PDC}-\text{V(V)}$ and $[\text{VO}_2(\text{ma})_2]^-$ have differential level of penetration of the lipid interface in microemulsions, it follows that the inhibiting species in the BMOV system for the Ca^{2+} -ATPase is not likely to be the $[\text{VO}_2(\text{ma})_2]^-$ complex, the BMOV oxidation product.

In contrast to the other two complexes investigated in this study, the HAIDA- V(IV) solutions only contains the V(IV)

compound added to solutions, as confirmed by EPR spectroscopy (data not shown).^{28,41} No ⁵¹V NMR signal was detected, consistent with all the vanadium remaining in the form of the original V(IV) complex. This spectrum is consistent with no observable oxidation to a vanadium(V) complex under these assay conditions and is in agreement with previous studies.^{29,30}

Combined, these studies show that the calcium pump has a great affinity for PDC–V(V) and BMOV. These compounds are the most effective insulin-enhancing compounds examined, and we point to the possibility that there is a correlation between insulin-enhancing properties and inhibition of Ca²⁺-ATPase activity. However, more studies are needed to substantiate such a hypothesis further. Because these complexes are known to undergo hydrolytic and redox chemistry under physiological conditions, such conversions are anticipated under our experimental conditions. The affinity of Ca²⁺-ATPase for the PDC–V(V) and BMOV systems is similar to that reported previously for decavanadate (V₁₀), an oxovanadate species recently found to be stabilized the calcium pump and also by actin.⁵⁵ V₁₀ also is found to affect mitochondria at nanomolar concentrations and induce changes in oxidative stress markers after in vivo administration.^{11,55–57} The calcium pump has previously been found to be inhibited by the vanadium citrate complex;²⁵ however, the studies shown here demonstrate more potent interactions with the PDC–V(V) and BMOV systems. Recent studies demonstrated that the redox properties of the vanadium complexes are important to their insulin-enhancing action.¹⁶ The studies presented show that the greatest inhibition is observed for vanadium compounds with greater insulin-enhancing effects. Although a trend should be based on investigation of more compounds, these observations are consistent with the proposed modes of inhibition of the Ca²⁺-ATPase by BMOV and PDC–V(V) involving the compounds' coordination chemistry.

Whether the vanadates act by forming a transition-state complex or binding as a substrate analogue depends on the specific enzyme.⁶ Many phosphorylases are inhibited by vanadate, and the mode of inhibition is the formation of a stable transition-state complex.²⁶ ATPases that catalyze ATP hydrolysis while translocating specific ions are referred to as pumps. For these enzymes that are inhibited by vanadate, the mechanism is through trapping the vanadate at the phosphate site accompanied by slow release, assuring reduced catalytic turnover and inhibition of the enzyme activity.^{1–9,11} The SR Ca²⁺-ATPase catalysis proceeds through a noncovalent phosphoenzyme intermediate as has

been observed for other ATPases and phosphorylases and subsequent formation of a phosphorylenzyme intermediate during ATP hydrolysis.¹⁰ In the absence of ATP and calcium, for this ATPase vanadate is proposed to bind in the vicinity of the aspartyl phosphate acceptor so as to block the active site and thus prevent phosphoenzyme intermediate formation, ultimately trapping the enzyme in the E2 conformation.^{9,11} Phosphorylation of the SR calcium pump by ATP, and conversion to the E1-P conformation, decreases the affinity of specific oxovanadates for the enzyme. This is consistent with the vanadate binding sites being in the region of the phosphorylation site.^{9,11} Other modes of action have been reported such as vanadate binding in a manner similar to an ATP analogue.⁶ Vanadium-containing complexes have the potential to act through mechanisms similar to the simple oxovanadates if they can hydrolyze to form oxovanadate while in the presence of the enzyme.

Monomeric vanadate is not the only form of oxovanadates that can interact with these enzymes.⁶ The interaction of decavanadate with the Ca²⁺-ATPase has been reported, and unlike vanadate, such inhibition is not affected by ATP or Ca²⁺.¹¹ Therefore, SR ATPase conformational changes in the presence of vanadium species can explain why the different affinities for the protein are observed. For monomeric vanadate, the E2 conformation of the calcium pump is favorable; however, V₁₀ appears to interact with either the E1 or E1-P conformation.¹¹ Moreover, it is known that oxovanadates bind to enzymes surfaces with positive charges,^{9,32} namely lysine and arginine residues, locking the enzyme in a specific conformation and in such a manner inhibiting the enzyme activity.^{32,56} In this work, we report the inhibition of Ca²⁺-ATPase by three vanadium coordination complexes. Because all three complexes cause inhibition of the calcium pump different from that of the simple oxovanadates, these results suggest that their mode of action is different than the uncomplexed vanadium.

Conclusions

A combination of inhibition studies, ⁵¹V NMR, and EPR spectroscopy was used to determine the interaction of vanadium complexes with the calcium pump. The effects of three well-characterized complexes, the pyridine-2,6-dicarboxylatodioxovanadium(V) (PDC–V(V)), bis(maltolato)oxovanadium(IV) (BMOV), and bis(*N*-hydroxylamidoimino-diacetato)vanadium(IV) (HAIDA–V(IV)), on SR preparations of the calcium pump from sarcoplasmic reticulum were determined. Because BMOV partially oxidizes into vanadium(V) species, and the PDC–V(V) complex in part hydrolyzes into different amounts of monomeric (V₁); dimeric (V₂) and tetrameric (V₄) species, the complexes were monitored in the enzyme assay medium. Because of the chemistry of the chosen insulin-enhancing compounds under physiological conditions, a third complex that is expected to be more resistant to the reaction conditions, HAIDA–V(IV), was also investigated. The order of the inhibition of the calcium ATPase activity by the complexes is PDC–V(V) ≥ BMOV > HAIDA–V(IV), with IC₅₀ values of 25 μM, 40 μM, and 325 μM, respectively. These affinities are much greater than

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the effects of the vanadium citrate complex that inhibits the calcium ATPase activity with an IC_{50} of 0.5 mM.²⁵ For comparison, a vanadate solution (prepared from metavanadate) has an IC_{50} of 80 μ M. It is important to note that the most potent inhibition was observed for the complexes that can undergo oxidative or hydrolytic reactions under the conditions of the assay, whereas the HAIDA–V(IV) complex is a much less effective inhibitor. These observations are significant because these compounds and their differential inhibitory activities may serve as useful probes for unravelling complex catalytic details for the Ca^{2+} -ATPases.

Because the PDC–V(V) and BMOV complexes convert into other species under the assay conditions, the presence of the complexes in the assay medium was monitored. The target compounds and other decomposition compounds were observed in the medium. Because the complexes convert into other species in the reaction medium and none of these species are more potent than these coordination complexes, the simple oxovanadate decomposition products are not causing most of the inhibition. The well-known inhibition of phosphorylases by vanadate through formation of a transition-state analogue generally involves a covalent complex. Thus, in many cases, such observations can readily be attributed to the hydrolysis of such complexes to simple vanadate. The studies presented here deviate from this description in that two of the coordination complexes, PDC–V(V) and BMOV, are more potent than the simple oxovanadate, monomeric vanadate. Because no decomposition products were observed for the HAIDA–V(IV) complex, the observed inhibition can be attributed exclusively to this complex. Importantly, this coordination complex was less effective than oxovanadate in inhibiting the calcium pump.

Because Ca^{2+} -ATPase can adopt several conformations, the specific nature of the interactions of each of the vanadium species with the sarcoplasmic reticulum Ca^{2+} -ATPase is beyond the scope of this investigation. However, inhibition by BMOV and PDC–V(V) was unchanged by ATP or Ca^{2+} , two effectors of enzyme in the E1 and E1-P conformation, indicating a very different mode of action from that of vanadate. These insulin-enhancing complexes that readily undergo hydrolytic reactions were found to be more potent inhibitors than the simple salt (vanadate). In contrast, the HAIDA–V(IV) complex is a less-potent inhibitor. These facts are consistent with the inhibition of the Ca^{2+} -ATPase by the vanadium compounds and involve adduct formation between protein and the vanadium compound. These findings are also consistent with the mechanism of the inhibition by vanadate being through complexation of the vanadium atom to the catalytic site of Ca^{2+} -ATPase.

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