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VO²⁺-hydroxyapatite complexes as models for vanadyl coordination to phosphate in bone

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Abstract

We describe a 1D and 2D ESEEM investigation of VO²⁺ adsorbed on hydroxyapatite (HA) at different concentrations and compare with VO²⁺-triphosphate (TPH) complexes studied previously in detail, in an effort to provide more insight into the structure of VO²⁺ coordination in bone. Structures of this interaction are important because of the role of bone in the long-term storage of administered vanadium, and the likely role of bone in the steady-state release of vanadium leading to the chronic insulin-enhancing anti-diabetic effects of vanadyl complexes. Three similar sets of cross-peaks from phosphorus nuclei observed in the ³¹P HYSCORE spectra of VO²⁺-HA, VO²⁺-TPH, and VO²⁺-bone suggest a common tridentate binding motif for triphosphate moieties to the vanadyl ion. The similarities between the systems present the possibility that *in vivo* vanadyl coordination in bone is relatively uniform. Experiments with HA samples containing different amounts of adsorbed VO²⁺ demonstrate additional peculiarities of the ion-adsorbent interaction which can be expected *in vivo*. HYSCORE spectra of HA samples show varying relative intensities of ³¹P lines from phosphate ligands and ¹H lines, especially lines from protons of coordinated water molecules. This result suggests that the number of equatorial phosphate ligands in HA could be different depending on the water content of the sample and the VO²⁺ concentration; complexes of different structure probably contribute to the spectra of VO²⁺-HA. Similar behavior can be also expected *in vivo* during VO²⁺ accumulation in bones.

Keywords

hydroxyapatite; oxovanadium(IV); diabetes; bones; EPR; two-dimensional ESEEM

1. INTRODUCTION

Vanadium compounds have insulin-enhancing properties both *in vivo* and *in vitro* [1–6]. Their development as pharmacologically useful insulin substitutes requires a better

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understanding both of the mechanism of action, and of the factors regulating uptake and absorption. Several vanadium complexes specifically designed to improve vanadium uptake, with concomitant lower dose requirements for oral administration were synthesized and characterized and one, bis(ethylmaltolato) oxovanadium(IV) (BEOV), completed a small phase 2a clinical trial in 2008 [7]. Bis(maltolato)oxovanadium(IV) (BMOV) and analogues such as BEOV are small, neutrally-charged complexes that are quite water-soluble (up to 10 mM) [7,8]. Oral administration of BMOV results in 2–3 times greater tissue uptake (at 24 hours) in kidney, liver and bone compared to vanadyl sulfate (VOSO_4) [9], which correlates well with the 2–3 times greater pharmacological potency of BMOV vs. vanadyl sulfate in lowering blood glucose levels in streptozotocin (STZ)-induced diabetic rats [10,11].

The mechanism of vanadium's insulin enhancing effects is still unknown, although protein tyrosine phosphatase inhibition and/or protein tyrosine kinase activation by vanadium ions may enhance or substitute for insulin [12]. An important feature of vanadium's insulin mimesis *in vivo* is that glucose lowering is not accompanied by increased insulin levels [13–17]. It is not clear whether vanadate (or its complexes) are the only active species, with the *in vivo* equilibrium between the IV and V oxidation states of vanadium responsible for the observed activity with vanadyl salts and complexes, or whether the vanadium(IV) state itself (as vanadyl ion, VO^{2+}) is directly involved in insulin enhancement [18]. Studies of the process by which, and the chemical form in which, vanadium accumulates in body tissues are essential steps in understanding the insulin-like mechanism of vanadium compounds. Earlier studies conducted in the UBC laboratories using ^{48}V labeling and atomic absorption spectroscopy found that BMOV-derived vanadium is most highly accumulated in the bone ($\sim 20 \mu\text{g g}^{-1}$ tissue), at approximately twice the concentration found in the kidney and six times that determined in the liver of diabetic-induced BMOV-treated (DT) rats [2,9]. The coordinated ligands and oxidation state of the vanadium *in vivo* are important in elucidating the biological fate of exogenously added vanadium. These parameters cannot be determined by atomic spectroscopy or other previously available methods. To address these questions for the paramagnetic fraction of the vanadium, *in vivo* ESEEM spectroscopy has been exploited. Fukui *et al.* used ESEEM spectroscopy on kidney, liver and bones from rats treated with bis(picolinato)oxovanadium(IV) ($\text{VO}(\text{pic})_2$) or VOSO_4 [18,19]. Kidney and liver samples from both types of rats exhibited a signal that could be attributed to equatorially coordinated amine nitrogen. Our ESEEM studies of rat livers and kidneys from BEOV-treated animals similarly found nitrogen coupling of ~ 5 MHz consistent with direct ligation of vanadyl [20]. Applying traditional one-dimensional ESEEM approaches,

however, Fukui *et al.* were unable to provide any details of VO^{2+} coordination in bones except reporting the line on the Zeeman frequency of ^{31}P indicating the presence of weakly coupled phosphorus nuclei in the surroundings of the VO^{2+} ions. In contrast, utilizing a more advanced two-dimensional ESEEM approach, we were able, besides the matrix contribution, to detect three distinct ^{31}P hyperfine couplings indicating three identifiable V-O-P(phosphate) binding modes, or several structurally distinct vanadyl-phosphate complexes in the bone mineral [21]. This result represented the first structural description of an important biological vanadium sink. It seems likely that vanadyl-phosphate complexes serve as a long-term storage form for accumulated vanadium. It is conceivable the action of vanadium pharmaceuticals that extend beyond cessation of treatment may be a result of a steady-state release of accumulated vanadium bound in the bone mineral.

Without additional studies, it is impossible to discern between the two structural possibilities indicated above. Model studies are frequently used to provide analogous data applicable to an unknown system through comparison of coupling constants obtained for structurally-characterized species. Continuing this study we also found the tridentate coordination of vanadium with TPH in frozen water solutions at pH 5.0 characterized by three significantly different hyperfine couplings of the order 15, 9 and 1 MHz, consistent with two phosphate groups coordinated equatorially and one axially [22].

The interaction of vanadium ions (vanadates and vanadyl) with hydroxyapatite has also been studied in some detail [23–26]. Concern over vanadium accumulation due to increased environmental exposure has led researchers to examine the effects of vanadium species on bone mineral [27]. Hydroxyapatite (HA) is a synthetic calcium phosphate polymer that is considered to be an excellent model for the inorganic phase of bone [28,29]. Due to its similarity to bone mineral, vanadyl binding to HA should prove a suitable reproduction of the *in vivo* coordination state. Ideally, the model systems should accurately reproduce the ^{31}P coupling constants observed in the *in vivo* sample, so that through knowledge of the known system, insight into the *in vivo* coordination state can be obtained.

This study describes the ESEEM investigation of VO^{2+} adsorbed on HA in different concentrations and a detailed comparison with the similar data for VO^{2+} -bone and VO^{2+} -TPH with the aim to provide deeper insight into the structure of VO^{2+} coordination in bones. The structure of this complex is important because of the role of bone in the long-term storage of administered vanadium, and its likely role in the steady-state release of vanadium that could lead to the extended anti-diabetic effect.

2. EXPERIMENTAL

All water was doubly distilled and deionized before use. Pentasodium triphosphate, calcium chloride and sodium hydrogen phosphate were obtained from Sigma-Aldrich and used without modification. Vanadium atomic absorption standard solution (in 5% HCl) was obtained from Aldrich and carefully neutralized to pH 1.0 with 10% NaOH solution. Vanadyl concentrations were confirmed by titration against potassium permanganate. The synthesis of VO^{2+} -HA was based on established methods for the isolation of microcrystalline hydroxyapatite [30–34] (see also Supplementary Material).

Hydroxyapatite (HA) with VO²⁺ (Sample VO-HA1)

CaCl₂·2H₂O (2.20 g, 14.9 mmol) was dissolved in 100 mL 0.15 M TRIZMA buffer, pH 7.4, and in separate solution Na₂HPO₄ (1.42 g, 10.0 mmol) was dissolved in 100 mL of TRIZMA buffer, pH 7.4. To this solution, 2.5 mL (0.045 mmol) of vanadyl stock solution ([VO²⁺] = 0.0181 M) was added with vigorous stirring. These two solutions were mixed and stirred under Ar for 20 hours. The solution was filtered over a glass frit, and the white pasty solid washed with 2 portions of pH 10 aqueous NH₄OH, and acetone. The solid was ground and dried under vacuum overnight. A room temperature powder EPR spectrum of the solid showed that vanadyl ions were present in the sample.

HA with VO²⁺ (Sample VO-HA2)

All procedures as for sample VO-HA1 were followed, except that no buffer solutions were used. The reaction solution was prepared, but for this synthesis a large aliquot of vanadyl stock solution was added ([VO²⁺] = 0.0181 M, 11.00 mL) to give a reaction solution 1 mM in vanadyl. The pH was adjusted to ~8 with concentrated NaOH. The pH of the solution was readjusted to ~8 every hour for 4 hours, and then allowed to stir overnight. Upon filtering, a light blue solid was obtained. This solid was dried on a vacuum line and ground to a fine powder.

HA with VO²⁺ (Sample VO-HA3)

CaCl₂·2H₂O (2.20 g, 14.9 mmol) was dissolved in 100 mL H₂O, previously degassed by Ar sparge. In a separate solution, Na₂HPO₄ (1.42 g, 10.0 mmol) was dissolved in 100 mL H₂O. The two solutions were mixed (under Ar); a white solid precipitated immediately. An aliquot of a vanadyl stock solution ([VO²⁺] = 0.0181 M, 11.0 mL, 0.199 mmol) was added with vigorous stirring. The initial pH was ~5 by pH paper. The pH was adjusted to ~8 with concentrated NaOH and readjusted to ~8 every hour for 4 hours; the mixture was then allowed to stir overnight. A pale blue solid was obtained by filtration, dried *in vacuo* at 60°C for 48 hours and then ground to a fine powder. Additional details of VO-HA synthesis and characterization are provided in the Supporting Info.

VO²⁺-triphosphate sample [22]

Vanadyl stock solution ([VO²⁺] = 1.808·10⁻² M, 2.00 mL, 0.036 mmol) was added to 48 mL of Ar sparged water. Pentasodium polyphosphate (0.0606 g, 0.127 mmol) was added to the solution. The solution was kept under Ar and stirred for 20 minutes, and the pH adjusted to 5 by slow, drop-wise addition of 0.1 M NaOH. Glycerol (21 mL) was added and the solution stirred for 30 minutes. An aliquot of this solution was then loaded into a 4 mm i.d. quartz cryotube for spectroscopic study. The triphosphate to VO²⁺ ratio was 3.1:1.

Bone samples [21]

Bone samples were taken from diabetic and non-diabetic rats treated chronically (6 weeks) with BEOV at a dose of 0.26–0.29 mmol kg⁻¹ day⁻¹ given in drinking water.

EPR Measurements

The pulsed EPR experiments were carried out using an X-band Bruker ELEXSYS E580 spectrometer equipped with Oxford CF 935 cryostats. Several types of ESE experiments with different pulse sequences were employed with appropriate phase cycling schemes to eliminate unwanted features from the experimental echo envelopes. Among them are two-pulse and two-dimensional four-pulse ESEEM sequences. In the two-pulse experiment ($\pi/2$ - τ - π - τ -echo), the intensity of the echo signal is measured as a function of the time interval τ between two microwave pulses with turning angles $\pi/2$ and π to generate an echo envelope that maps the time course of relaxation of the spin system (in ESEEM) or as a function of magnetic field at fixed τ (in field-sweep ESE). In the 2D, four-pulse experiment ($\pi/2$ - τ - $\pi/2$ - t_1 - π - t_2 - $\pi/2$ - τ -echo, also called HYSORE), the intensity of the echo after the fourth pulse was measured with t_2 and t_1 varied and constant τ . The length of a $\pi/2$ pulse was 16 ns and a π pulse 32 ns. HYSORE data were collected in the form of 2D time-domain patterns containing 256×256 points with steps of 16 ns. Spectral processing of ESEEM patterns, including subtraction of relaxation decay (fitting by 3–6 degree polynomials), apodization (Hamming window), zero filling, and fast Fourier transformation, were performed using Bruker WIN-EPR software.

3. RESULTS

3.1 EPR characterization of the samples

Continuous wave (CW) EPR and field-sweep pulsed EPR measurements of the VO-HA, VO-TPH and VO-bone samples performed at 30 K showed a lineshape with axially symmetric g tensor anisotropy and ^{51}V hyperfine structure (as a typical example, see the spectrum of VO-HA3 in Figure S2). The principal values of the g -tensor and ^{51}V hyperfine tensor are listed in Table 1. These values are typical for square pyramidal oxovanadium(IV) complexes with an O_4 coordination sphere [35,36]. The spectral lineshape in all samples is characteristic of isolated VO^{2+} species with no oligometallic species detected. Based on g - and $A(^{51}\text{V})$ - tensors determined from CW and FS-ESE spectra, the most accurate model of VO-bone is VO-HA, while VO-TPH is significantly different from the *in vivo* sample. Detailed pulsed EPR studies of the two systems, however, give a better indication of the applicability of the VO-TPH and VO-HA systems as models of the *in vivo* coordination of vanadyl ions.

3.2 Relaxation decay of two-pulse ESE and concentration of VO^{2+} ions in the samples

Figure 1 shows two-pulse ESEEM patterns for the VO-bone, VO-TPH and three VO-HA samples. As it is clearly seen from the Figure 1, the relaxation decay of the two pulse ESE, which is determined by the phase-memory relaxation time T_2 , is different in samples studied. The difference is not significant between VO-bone, VO-TPH and VO-HA3 samples but the relaxation decay increases drastically in two other VO-HA samples prepared from vanadyl solutions with larger concentrations.

The relaxation decay of two-pulse ESE in solid matrices as a function of VO^{2+} concentration is described by equation (1).

$$V(\tau) = V(0) \exp(-b\tau) \quad (1)$$

$b = 1/T_2 = b_0 + \alpha C$, C -average concentration of ions in cm^{-3} [37,38].

From the e -fold decrease of the two-pulse echo intensity the values of b for different samples were measured and collected in Table 2. The concentration dependence of the relaxation decay is governed through the mechanism of spectral diffusion, resulting from the random modulation of dipolar interaction of VO^{2+} electron spins by the mutual flip-flop transitions. This contribution to the relaxation rate is described by the term αC . The available measurements of the concentration dependence of the relaxation rate for VO^{2+} complexes in glassy matrices [37,39] report, or allow estimate of, the coefficient α as $(1 \pm 0.3) \cdot 10^{-13} \text{ cm}^3 \text{ s}^{-1}$. The value of α indicates that this mechanism produces a measurable contribution to b of the order of $> \sim 0.1 \cdot 10^6 \text{ s}^{-1}$ at concentrations of VO^{2+} larger than 10^{18} cm^{-3} . Term b_0 describes a contribution of other mechanisms to the relaxation decay. Most significant among them is the relaxation from the magnetic nuclei. The value of the term b_0 vary in different samples.

Among the investigated samples, only the concentration of VO^{2+} in VO-TPH was measured analytically with the value of 0.509 mM or $3 \cdot 10^{17} \text{ cm}^{-3}$. For this concentration, the contribution of spectral diffusion to the relaxation rate is still negligible, with a value of $0.03 \cdot 10^6 \text{ s}^{-1}$ that does not exceed the accuracy in its determination from echo decay. This estimate shows that, for the VO-TPH sample, the major part of the relaxation decay is induced by other mechanisms, where the important role belongs probably to the relaxation from the strongly coupled protons of the ligand water molecules [22]. Those contributions are described effectively by the term b_0 .

The sample of VO-bone possesses lower relaxation compared to that of VO-TPH. Therefore, it is reasonable to suggest that $[\text{VO}^{2+}]$ in VO-bone does not exceed 0.5 mM, and that the b_0 value in this sample is lower than in VO-TPH particularly due to lack of ligand water molecules. Thus, using the value $b \approx b_0 = 0.5 \cdot 10^6 \text{ s}^{-1}$ in bone and assuming that this value is close to b_0 in HA samples, one can estimate $[\text{VO}^{2+}]$ in the HA samples, Table 2. This estimate shows that in accordance with the relaxation rates of the HA samples the variation of VO^{2+} concentration for them approaches one order that allows us to study the effect of VO^{2+} accumulation on its coordination state in HA matrix.

3.3 ESEEM experiments

The EPR spectra of the VO^{2+} -complexes (Table 1) did not indicate significant variation of the g -tensor and hyperfine tensor of ^{51}V , likely because only oxygen atoms from phosphate or water ligands form the first coordination sphere of the complexes in all samples. Therefore, the possible structural differences start in the second sphere and do not influence significantly the characteristics of the EPR spectra. The peculiarities of the VO^{2+} coordination in bones and model complexes, however, were clearly resolved in the ESEEM experiments discussed below.

3.3.1 Two-pulse ESEEM—Figure 2 shows two-pulse ESEEM spectra obtained after modulus FT of the echo decays for VO-TPH, VO-bone and VO-HA3 shown in Figure 1. The short relaxation decay of the two-pulse ESE in VO-HA1 and VO-HA2 samples prevents obtaining of resolved FT spectra.

Two-pulse ESEEM patterns were measured at the $m_I^V = -1/2$ component of the hyperfine structure from ^{51}V of the EPR spectrum. The distinctive feature of this component in X-band EPR is the close proximity of singularities corresponding to the perpendicular and parallel orientations of the external magnetic field to the unique axis of the ^{51}V hyperfine tensor. As a result the lineshape of the $m_I^V = -1/2$ component in X-band EPR spectrum resembles more a single peak in contrast to other components possessing extended lineshapes typical for an axially symmetric hyperfine tensor with well-separated parallel and perpendicular singularities [40]. Therefore, the excitation of the $m_I^V = -1/2$ component can be considered as essentially non-selective. This allows one to exploit approaches for analysis of ESEEM spectra used for orientation-disordered (i.e. powder) systems.

The spectra depicted in Figure 2 exhibit discernible features leading to preliminary quantitative conclusions about the vanadyl coordination in the investigated samples. The common features of all spectra (marked on Figure 2) are the peaks at ν_H and $2\nu_H$ and $2\nu_P$ ($\nu_H \sim 14.9$ MHz and $\nu_P \sim 6$ MHz are Zeeman frequencies of ^1H and ^{31}P nuclei, respectively, in the applied magnetic field ~ 350 mT). The spectra of VO-HA3 and VO-bone contain also the line at ν_P with some unresolved features around and broad intensive line with maximum at $\sim 1\text{--}2$ MHz. The line at ν_P is absent in VO-TPH sample. In contrast, the spectrum of VO-TPH contains a doublet of lines at 6.7 MHz and 5.3 MHz centered relative to the ν_P . An additional feature in this spectrum is the peak of a sum combination harmonic at 30.35 MHz shifted to the higher frequencies relative to $2\nu_H = 29.62$ MHz [22]. Similar clearly resolved peaks are not observed in two other spectra. It should be noted, however, that the $2\nu_H$ peak is accompanied by the low amplitude feature at the same frequency in the VO-bone spectrum and by the extended shoulder in the VO-HA3 spectrum.

The doublet of lines in VO-TPH spectrum indicates ^{31}P nucleus(i) with hyperfine coupling of $\sim 1\text{--}1.5$ MHz. Such splitting cannot appear from randomly distributed, remote ^{31}P nuclei; its presence suggests direct coordination of at least one phosphate to the metal ion [22]. This doublet is well-resolved because of the very low concentration (~ 1 mM) of uncoordinated TPH molecules randomly distributed in the sample. In contrast, the spectra of VO^{2+} in HA and bone contain the line at ν_P produced by the weakly coupled ^{31}P nuclei in the VO^{2+} surroundings. ^{31}P coupling ~ 1 MHz was not observed. A peak with a maximum at ~ 1.5 MHz in the low frequency region of the spectra may be a result of other ^{31}P nuclei, strongly coupled with the VO^{2+} unpaired electron. The proton sum combinations with a shift of ~ 0.7 MHz at field strength of ~ 330 mT is typical of equatorial water coordination to vanadyl [41,42].

Thus, the two-pulse spectra indicate the presence of ^1H and ^{31}P nuclei in the surroundings of the VO^{2+} in all studied samples. However, the intense shifted sum combination line in the VOTPH spectrum and difference in the low part frequency region allow the conclusion that the organization of coordination sphere in VO-TPH is different compared to those in VO-

HA and VO-bone. More complete information about the phosphorus and proton environments of the vanadyl ions was obtained from the application of 2D ESEEM.

3.3.2 ^{31}P HYSCORE—The most complete information about ^{31}P and ^1H interactions was obtained from 2D ESEEM (also called HYSCORE) spectra. The ^{31}P part of the representative contour HYSCORE spectra of VO-HA3 and VO-HA1 in comparison with that of VO-TPH are shown in Figure 3. The additional spectra for the VO-bone and VO-TPH previously published by us [21,22] are shown in Supplementary Material (Figure S3). The spectra of all samples considered in this work contain three types of similarly located features, designated P_1 , P_2 , and P_3 . The pair of cross-peaks P_1 appear in the $(+,-)$ quadrant with a maximum at frequencies $\sim[\mp 2; \pm 14]$ MHz, oriented approximately parallel to the diagonal and centered at $A/2 \sim 8$ MHz. The difference between the two coordinates of each peak is close to $2\nu_P$. The two other features P_2 and P_3 are located in the $(+,+)$ quadrant. The P_2 is a pair of ridges extended along antidiagonal and centered symmetrically about the diagonal point of (ν_P, ν_P) with $\nu_P = 6.03$ MHz. The maximum of the peaks corresponds to the splitting $A \sim 9$ MHz. Thus, the HYSCORE spectra of the VO-HA samples, similar to the spectra of VO-TPH and VO-bone, show two strong vanadyl-phosphorus couplings with estimated values of ~ 15 and 9 MHz.

The P_3 feature is located around the diagonal point (ν_P, ν_P) . The shape of P_3 peaks in HYSCORE spectra of HA samples is shown more clearly in the 3D stacked plot presentation in Figure 4, compared with the VO-TPH and VO-bone samples. One can see that, consistent with the two-pulse ESEEM spectra, the P_3 feature is a doublet of lines with the similar splitting ~ 1 MHz in VO-TPH. The P_3 signal has a more complex shape consisting of a sharp diagonal peak at ν_P and extended shoulders with the length up to ~ 1.0 MHz in HA samples and bone. The diagonal peak results at least partly from ^{31}P matrix nuclei weakly coupled with VO^{2+} spins. The clear observation of resolved coupling of ~ 1.0 MHz in VO-TPH was facilitated by the absence of a diagonal peak from weakly coupled matrix nuclei in this sample. Our attempts failed to resolve similar doublets in the HYSCORE spectra of VO-HA and VO-bone recorded at different times τ with the aim to suppress the diagonal peak. This result suggests different coordination models of VO^{2+} in these systems.

3.3.3 ^1H HYSCORE—In addition to the phosphorus lines, the HYSCORE spectra in VO-HA samples possess proton lines grouped around the (ν_H, ν_H) diagonal point (Figure 5). The ^1H HYSCORE spectra of VO-TPH resolve the contribution from three different types of proton, as we previously reported ([22], Figure 5 and Supplementary Material). Two pairs of cross-peaks (H_1 and H_2) can be identified in the ^1H HYSCORE spectrum of the VO-HA3 (Figures 5 and S4). Despite the acquisition of several HYSCORE spectra with various τ times, no third cross-peak could be discerned. One can note, however, that the failure to resolve the third proton signal in VO-HA3 may result from larger individual width of ^1H cross-peaks in the diagonal direction and its closer location then in VO-TPH (see Figure S4). In the spectrum of VO-HA1 only traces of extended proton cross-peaks could be recognized.

In addition to the difference between the P_3 signal in VO-TPH and VO-HA samples, one can note the variation of the relative intensity of P_1 , P_2 and ^1H peaks in these samples. The

relative intensity of different ^{31}P and ^1H peaks is better seen from the 2D stacked presentation of the HYSORE spectra shown in Figures 6 and S5. Despite the similarity in the location of the cross-peaks P_1 and P_2 one can see significant increase of the P_1 peak intensity in VO-HA samples compare to those of VO-TPH. Decrease of the proton peaks intensity, and particularly from the strongly coupled protons of ligand waters relative to the P_2 lines in VO-HA3 sample, leads to a significant increase of P_1 peaks intensity in (+−) quadrant. Complete disappearance of the ^1H lines from the ligand protons in VO-HA1 is accompanied by the further increase of the relative intensity of the P_2 signal compare to P_1 .

3.3.4 Analysis of HYSORE Spectra—Contour lineshape analysis described in detail previously [21,22] was applied to extract the isotropic a and anisotropic hyperfine (T) couplings from both the ^{31}P and ^1H cross-peaks in VO-HA. The square of each pair of ^1H and ^{31}P cross-peak frequencies (ν_α , ν_β) can be plotted as $(\nu_\alpha)^2$ versus $(\nu_\beta)^2$ (Figure S6) transforming the contour line shape into a straight line segment whose slope Q_α and intercept G_α can then be used to obtain simultaneously two possible sets of a and T couplings in approximation of axial hyperfine tensors. The resulting two sets of ^{31}P and ^1H hyperfine couplings are listed in Table S1 for VO-TPH and VO-HA3. Contour lineshape analysis of several HYSORE spectra of VO-HA3 formed a plot very similar to that for VO-TPH, with slight differences in Q_α and G_α values leading to minor differences in determined a and T constants. The spectra of VO-HA1 and VO-HA2 give values similar to those for VO-HA3.

Generally, each VO-HA P_1 - P_3 cross-peak can be roughly correlated to a cross-peak set observed in VO-TPH. The anisotropic constants of VO-HA showed very little variance, suggesting a relatively uniform distance of ^{31}P atoms around the vanadyl ion. The structural interpretation of the relative magnitudes of the a and T values for the ^{31}P and ^1H nuclei reported in Table S1 is detailed in the Discussion.

4. DISCUSSION

4.1 Hydroxyapatite and Vanadyl Interactions

The structural similarity between hydroxyapatite and the mineral fraction of bone has been known since powder X-ray and neutron diffraction patterns of HA were compared against those of ground bone powder a half century ago [43,44]. While the patterns shared some similarities, a number of key differences existed between the laboratory and natural products. Firstly, biological HA is generally less crystalline than is synthetic HA [45]. This observation is predominately due to ion substitutions in the lattice; several monovalent and divalent metal ions can substitute for Ca^{2+} (e.g. Na^+ , Sr^{2+}), while carbonate and halogen anions can replace phosphates. The second major difference is in overall composition. Biological samples studied by Parfitt had a 1.3:1 $\text{Ca}^{2+}:\text{PO}_4^{3-}$ ratio while for synthesized HA, the ratio was 1.67 which agrees with the unit cell formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ [29]. It is likely that this ion deficiency allows for the high degree of lattice substitution. Bone and HA samples possess two monolayers of water molecules, held in place by hydrogen bonding to exposed hydroxides [30]. The water layers impart a degree of selectivity to ion substitution by limiting access to the crystal lattice to ions of a certain size. Na^+ , for

instance, can incorporate into the lattice, while K^+ ions do not penetrate the hydration shell [29].

The interactions of vanadium with HA are dictated by the oxidation state of the metal ion. Vanadium(V), not surprisingly because of its structural analogy to phosphate, incorporates into the lattice, although not without causing charge-compensating vacancies and modifications in unit cell parameters [46]. The interaction of VO^{2+} is not clearly defined. Through a precipitation method, it appears that VO^{2+} ions can enter the crystal lattice for Ca^{2+} [47], although this result is in conflict with similar VO^{2+} solution-solid interactions with alumina and anatase (both hydroxyl-bearing solid phases) which found surface-binding only [48,49]. Other study reported only pH-dependent surface binding of vanadyl ions to HA through specific adsorption to surface hydroxides [26].

4.2 Comparison between the VO-HA and VO-TPH

The VO-TPH and VO-HA model systems yield similar 1H and ^{31}P coupling constants by HYSCORE spectroscopies, including number, type and relative magnitude. Only difference is the failure to detect in VO-HA3 spectra cross-peaks equivalent to H_3 in VO-TPH with $T \sim 1$ MHz, suggesting that no water molecules sit above the $V=O$ bond [22]. This observation is consistent with the state of each model; VO-TPH was a frozen solution sample and VO-HA3 was a solid state sample that had been dried to remove adsorbed water.

4.2.1 ^{31}P Couplings—HYSCORE spectra of VO-TPH and VO-HA each show the presence of three ^{31}P couplings with significantly distinct isotropic hyperfine constants. They can be compared against those obtained in analogous systems [50,51]. Three similar ^{31}P constants were also reported for VO^{2+} used as a spin probe for the Mg^{2+} binding site of wild type F1 ATPase (TF1) [51]. CW EPR and ENDOR studies of VO^{2+} complexes with adenosine diphosphate (ADP) and ATP reported ^{31}P hyperfine couplings ~ 18.5 – 20.6 MHz [50]. These data could be used to suggest the preferable relative signs of a and T from the two possibilities indicated in Table S1, at least for the strongest ^{31}P couplings. The features with large splittings of 18–20 MHz observed in the powder EPR and ENDOR spectra of VO-ADP and VO-ATP most likely corresponds to the perpendicular component of the hyperfine tensors. The two sets of hyperfine constants derived from the analysis of the P_1 cross-peaks in VO-TPH give $A_{\perp} = |a - T| = 17.8$ and 12.1 MHz, respectively, while for VO-HA3 the values are 18.6 and 12.6 MHz. Only the larger of the two possible a values listed in Table S1, -15.9 MHz and -16.6 MHz, approach the reported EPR and ENDOR splittings (corresponding to opposite signs of a and T).

The range in T values for the ^{31}P cross-peaks is greater for the VO-TPH sample. This perhaps reflects less restriction on the orientations of the phosphates relative to the orbitals of the vanadium center. In VO-HA, phosphates are not tethered together and therefore can be oriented to energetically favorable positions with shorter V-O-P distances. Magnitudes of isotropic couplings also show some degree of variation between VO-TPH and VO-HA, but considering the size of these couplings they likely do not represent major differences in compound structure, but rather the simple discrepancy between a frozen solution and a true powder sample.

4.2.2 ^1H Couplings—Many studies have been conducted to analyze the hyperfine couplings of protons adjacent to the vanadyl ion [41,42,52–59]. Proton couplings for coordinated water and alcohol molecules in VO^{2+} complexes in single crystal [52], powder [42], zeolite ZSM-5 [59], frozen aqueous [41,42,53,54] and alcohol [55–57] solutions, as well as in frozen protein solution [58] have been investigated in detail. In general, the magnitude of the isotropic coupling constant is dependent on the orientation of the proton relative to the equatorial plane of the vanadyl ion. The anisotropic coupling constant is also sensitive to the orientation of the proton to the molecular axis system; protons oriented in an axial position have smaller T values (Table 4). Thus, the magnitude of the isotropic and anisotropic coupling constants can be used to gain insight into the overall geometric arrangement of the coupled protons.

Based on the most relevant studies [41,42,52,58,59], the value of $|T| = 4.0\text{--}4.3$ MHz found for cross-peaks H_1 in VO-TPH and VO-HA3 belongs to protons from equatorially coordinated water(s) [22].

According to several studies, protons from axially coordinated water molecules typically possess $T = 3.1\text{--}3.4$ MHz and isotropic constants close to zero. Cross-peaks H_2 , H_3 in VO-TPH and H_2 in VO-HA3 define anisotropic couplings that are smaller than those determined for protons of water ligands. The protons producing them, therefore, must arise from different sources. Previous ENDOR spectra of VO^{2+} in frozen aqueous and alcohol solutions show the presence of couplings assigned to the proton involved in a hydrogen bond to the vanadyl oxo ($\text{V}=\text{O}\cdots\text{H}$) [55]. Splittings of $A_{\parallel} = 4.34$ MHz and $A_{\perp} = 1.40$ MHz were reported by Mustafi and Makinen [55]; calculated values of $A_{\parallel} = |a + 2T| = 4.8$ MHz and $A_{\perp} = |a - T| = 1.2$ MHz for proton H_3 (based on $a = 2.39$ MHz and $T = 1.22$ MHz) compare favorably to these values. Therefore H_3 coupling likely arises from a hydrogen-bound proton attached to the vanadyl oxygen [22].

The hyperfine parameters obtained for cross-peaks H_2 do not correlate with any values previously reported for the protons of ligands in the first coordination sphere. The anisotropic components $|T|$ for cross-peaks H_2 are smaller than typical values obtained for both equatorial and axial ligands and so it is likely that these peaks arise from one or more proton(s) bound as hydroxyls on the triphosphate ligand. The most significant observation of the HYSCORE studies of the ^{31}P and ^1H coupling constants is that no axially-bound protons are detected. Coupled with the detection of three distinct ^{31}P couplings, it is consistent with experimental data to invoke a solution structure with some other ligand (i.e. phosphate) coordinating in the axial position.

4.2.3 Structure of the Complexes— ^{31}P and ^1H couplings indicate a mixed coordination state of the VO^{2+} ions in both TPH and HA3 samples. The magnitude of these couplings allows for some structural conclusions to be made about the complexes in solution and the solid state. The HYSCORE and ESEEM results for VO-TPH are entirely consistent with equatorial water coordination to VO^{2+} as previously reported (Table 4) [22]. From the data presented, a solution structure was proposed, which includes a facial, tridentate triphosphate coordinated to the vanadyl ion, along with two water molecules located near to the equatorial plane [22]. While previous studies of these systems have been reported, the

axial phosphate interaction has not been previously detected. All other previous work with vanadyl has reported only equatorial phosphate coordination, corresponding to the β - and γ -phosphates of ATP. The proposed structure is consistent, however, with several X-ray crystal structures of other metal complexes with triphosphate (two containing Co(III) [60,61] and one Cd(II) [62]) or ATP (including Mn(II) [63,64], Mg(II), Ca(II) [65], Ni(II) [66], Cu(II) and Zn(II) [67]) as tridentate ligands. An example of one such complex is shown in Figure S8. Evidence also exists for the formation of a tridentate triphosphate Rh(III) complex from NMR spectroscopy and HPLC [68]. The triphosphate moiety is coordinated in a facial fashion, analogous to the VO-TPH model structure.

Three distinct ^{31}P isotropic constants in VO-TPH, VO-HA and VO-bone can be explained by differences in V-O-P bond angles relative to the V=O bond. ^{31}P isotropic constants, like those for ^1H , would be expected to vary with how equatorial are the atoms, that is, the degree of orbital overlap between the metal center and the orbitals of the phosphorus atoms [52]. Less orbital overlap leads to a decreased contact interaction and spin density on the ^{31}P atoms. Unit spin density for the 3s orbital of phosphorus leads to a calculated isotropic coupling constant of 13306 MHz [69]. Therefore, the isotropic couplings 9–15 MHz for P_1 and P_2 correspond to spin density of the order $(0.67 - 1.1) \cdot 10^{-3}$ on the ^{31}P nuclei. Unit spin density on the 1s orbital of hydrogen atom produces ^1H isotropic constant 1420 MHz that would lead to significantly higher spin density $(5.6 - 7.0) \cdot 10^{-3}$ for the protons with the isotropic coupling $\sim 8 - 10$ MHz of equatorial water ligands.

In contrast, ^{31}P anisotropic coupling of phosphate ligands is determined by two factors - dipole-dipole coupling and indirect spin transfer. If the V-P distance of 3.44 Å, determined by Mustafi *et al.* [50], from molecular modeling is used as an estimate of the V-P distance in the VO-TPH sample, a dipolar coupling of 0.79 MHz is obtained through the point dipole approximation. Unpaired spin density in the 3p orbital determines the second contribution. The computed value for a 3p electron is 367 MHz, approximately 36 times less than that determined for the 3s orbital [69]. Thus, it would be expected that the isotropic coupling would be much more sensitive to changes in coordination state (i.e. axial vs. equatorial) than the anisotropic coupling constant. This prediction is indeed what is observed in the coupling values determined for cross-peaks P_1 - P_3 , which exhibit a large variation in isotropic coupling from ~ 1 to 15 MHz, while the T component is relatively stable between $\sim 1 - 2$ MHz. The anisotropic coupling constants for VO-HA are even less variant. Similar observations were made by Atherton and Shackleton with proton anisotropic coupling constants for the $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$ complex, in which the anisotropic variation did not exceed 1.5 times [52]. These values are also consistent with tridentate TPH coordination. Axial phosphate coordination to vanadyl has never been spectroscopically characterized, and has some implications for the related coordination of divalent metal ions in ATP-utilizing enzymes. The low isotropic coupling and relatively invariant anisotropic coupling (compared to the other two cross-peaks) of the P_3 cross-peaks therefore supports the presence of an axial phosphate coordinated to the vanadyl ion.

Density functional (DFT) calculations confirmed the weak influence of an axial ligand on the characteristics of the EPR spectrum resulting from low spin density transfer to this ligand [70]. Calculations for $[\text{VO}(\text{H}_2\text{O})_5]^{2+}$, $[\text{VO}(\text{H}_2\text{O})_4]^{2+}$ and $[\text{VO}(\text{NH}_3)_4(\text{H}_2\text{O})]^{2+}$

yielded anisotropic hyperfine coupling values of $|T| \sim 4$ MHz for equatorial protons and $|T| \sim 3$ MHz for axial protons with isotropic constants of ~ 9 – 10 MHz and ~ 0 MHz, respectively [70,71]. These results correlate very well with experimental results reported in Tables 3 and 4 and provide further support for the assignment of the weakest ^{31}P coupling as an axially-ligated phosphate residue. One can suggest that the observed ^{31}P isotropic constant of $|a| \sim 1$ – 2 MHz for axial ligand (Table 3 and S1) in TPH arises from a more complex mechanism of spin density distribution which does not take place in isolated aqua and phosphate axial ligands. Likely, a major contribution to the observed constant is the transfer of spin density to the ^{31}P of the axially coordinated phosphate from the metal center through the coordinated oxygen, as well as through the chain of equatorial ^{31}P -O donors. This suggestion finds support in the different shape of the P_3 signal in VO-HA and VO-bone which does not show the isotropic splitting (Figure 4). The observed extended shoulders of the P_3 could result from a purely anisotropic interaction with the phosphorus of axially located ligand that is confirmed by one possible solution with $a \sim 0$ MHz in VO-HA (Table S1). In this situation the lineshape of P_3 is complicated by the partial overlap of two cross-ridges from phosphorus of the axial ligand and the matrix peak possessing larger intensity in VO-bone than in VO-HA samples. An estimate of the anisotropic component T for the axial ligand can be obtained from the length of shoulders around the narrow diagonal peak, assuming that it is close to $A_{\parallel} = 2T$. An analysis of VO-HA and VO-bone spectra recorded at different times τ gives values of $T \sim 1.3$ – 1.6 MHz in these two systems. Further computational work would be required to analyze spin density transfer on isolated and chemically-bound phosphate ligands.

4.2.4 Comparison to In Vivo Coordination in Bone—Tables 3 and 4 present the ^1H and ^{31}P hyperfine coupling constants for the *in vivo* bone sample and for comparison with two models, VO-TPH and VO-HA. The two largest ^{31}P couplings are conserved in VO-TPH, VO-HA, bone, VO-ATP and VO-TF1-ATP [51], with only slight variation in the P_3 lineshape, indicating a common tridentate binding motif in all samples for triphosphate moieties to the vanadyl ion. It is clear that both the isotropic and anisotropic coupling constants for all systems correlate very well, indicating a high degree of structural similarity between the vanadyl complexes and suggesting three structural motifs of P-O coordination with VO^{2+} , two equatorial and one axial. The VO-TPH sample demonstrated that it is feasible for a single triphosphate moiety to generate three distinct vanadyl-phosphate coupling constants in a 1:1 solution complex between triphosphate and VO^{2+} . Hydroxyapatite has also shown to be a suitable model for the bone mineral and its coordination to vanadyl ions. The similarities between the systems present the possibility that *in vivo* vanadyl coordination in bone is relatively uniform.

On the other hand, the experiments with the HA samples containing different amount of adsorbed VO^{2+} , demonstrate additional peculiarities of ion-adsorbent interaction which may be also expected *in vivo*. HYSORE spectra of HA samples show varying relative intensities of P_1 and P_2 lines as well as intensities of ^1H lines, especially lines from protons of coordinated water molecules. This result suggests that the number of equatorial phosphate ligands in HA could be different depending on the water content of the sample and $[\text{VO}^{2+}]$; the complexes of different structure may contribute to the spectra of VO-HA and VO-bone.

This suggestion follows from the variation of ^1H and ^{31}P peak intensity in different VO-HA samples. Clear indication of water coordination is observed in HA3 possessing extended cross-ridges in HYSORE spectra. Similar cross-ridges are almost not seen in the spectra of HA1 and HA2 samples with larger $[\text{VO}^{2+}]$.

A previous study of the interaction of VO^{2+} with crystalline calcium hydroxyapatite in aqueous solution concluded that ions are adsorbed on HA [26]. The model proposed for the adsorbed complex includes four equatorial water ligands and an axial oxygen ligand from the surface (Figure S9). This model, however, is inconsistent with our observations of strong ^{31}P couplings indicating equatorial phosphate coordination. Observation of these couplings together with the ^1H lines possessing characteristics of equatorial water ligands in HA3 sample would suggest partial equatorial phosphate coordination even for the surface adsorbed ions as for the model in Figure S9. However, an opposite trend of ^1H (decrease) and ^{31}P (increase) peak intensities in the HYSORE spectra (Figure 6) for larger concentrations of adsorbed VO^{2+} probably corresponds to a diffusion of vanadyl ions inside the HA and its preferable equatorial coordination with phosphate oxygen atoms. The EPR spectrum of VO^{2+} [26] consists of broad asymmetric lines without any resolved hyperfine structure. It suggests that the concentration of adsorbed VO^{2+} in [26] significantly exceeds the concentration of even our most concentrated sample HA2.

The major peculiarity of the HYSORE spectra is the absence of abundant proton couplings in the bone sample while the VO-TPH HYSORE spectra contained 3 sets of cross-peaks $\text{H}_1\text{-H}_3$. The bone sample, however, did possess a relatively strong ^1H matrix line and observable cross-peaks with the coupling ~ 3 MHz. On the other hand, two pulse spectra do not show any well-resolved shifted sum-combination lines from the protons with significant anisotropic coupling $T \sim 3$ MHz or higher excluding the equatorial and axial water coordination. It is most likely that this coupling may belong to the H_3 protons found in the VO-TPH though H_2 protons seen in VO-TPH and VO-HA3 cannot be completely excluded. One can also suggest that such proton coupling is not homogeneous throughout the sample and hence not all complexes produce the cross-peak signals for these protons. The HYSORE spectrum of VO-bone also shows the increased intensity of the ^{31}P diagonal peak probably reflecting the more dense phosphate packing in bone (Figure S5). This peak overlaps with the broader signal with the total width of wings of about ~ 3 MHz from the ^{31}P of axial ligand. Thus it suggests axial phosphate interaction with the VO^{2+} in bone, while the two stronger couplings correlate well with the possible four equatorial phosphate ligands with coupling constants P_1 and P_2 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Two-pulse ESEEM patterns of VO-HA, VO-TPH and VO-bone samples.

Figure 2.
Modulus FT two-pulse ESEEM spectra of the pattern shown in Figure 1.

Figure 3.

Contour ^{31}P HYSCORE spectra of VO-HA3, VO-HA1 and VO-TPH samples showing the phosphorus cross-peaks P_1 - P_3 . The spectra were measured at the maximum intensity of the $m_I^V = -1/2$ peak of the EPR spectrum. $T = 30$ K. Time τ is equal 192 ns for all spectra, VO-HA1: magnetic field 347.8 mT, microwave frequency 9.710 GHz; VO-HA3: 346.8 mT, 9.685 GHz; VO-TPH: 347.9 mT, 9.713 GHz.

Figure 4.

Stacked plot presentation of the (+,+) quadrant of ^{31}P HYSCORE spectra of VO-TPH, $\tau=120$ ns (a) and 240 ns (b), VO-HA3, $\tau=128$ ns (c) and 256 ns (d), VO-HA2, $\tau=120$ ns (e); VO-bone, $\tau=120$ ns (f). Magnetic field 347.9 mT (VO-TPH), 346.8 mT (VO-HA3), 351.4 mT (VO-HA2), 331.5 mT (VO-bone).

Figure 5.

Contour ^1H HYSCORE spectra of VO-HA3, VO-HA1 and VO-TPH samples showing the proton cross-peaks. The spectra were measured at the maximum intensity of the $m_I^V = -1/2$ peak of the EPR spectrum. Temperature was 30 K. Experimental parameters are the same as in for spectra in Figure 3 caption.

Figure 6. 2D stacked presentation of HYSORE spectra showing relative intensities of the peaks from ^1H and ^{31}P nuclei. Experimental parameters are the same as for spectra in Figure 3.

Table 1EPR characteristics of studied VO^{2+} complexes.

Sample	$g_{\perp} (\pm 0.005)$	$g_{\parallel} (\pm 0.01)$	$A_{\perp} (\pm 0.5)^a$	$A_{\parallel} (\pm 0.5)^a$
VO-TPH ^b	1.983	1.92	202	83
VO-HA3 ^c	1.996	1.92	198	80
VO-bone ^d	1.996	1.93	190	80

^a in units of mT^b from [22]^c powder sample with minimal concentration of VO^{2+} (see next section), HA1 and HA2 samples possess spectra with practically similar g- and A-tensor characteristics but more broad individual lineshapes^d from [21]

Table 2

The rates of relaxation decay and estimated concentrations in the samples.

Sample	$b \cdot 10^6 \text{ s}^{-1}$	Concentration, mM (cm^{-3})
VO-bone	0.53 ± 0.02	$<0.5 (3 \cdot 10^{17})$
VO-TPH	0.67 ± 0.05	$0.5 (3 \cdot 10^{17})$
VO-HA3	1.02 ± 0.02	$8.3 \pm 2.5 (0.5 \cdot 10^{19})$
VO-HA2	5.4 ± 0.02	$81 \pm 24 (4.9 \cdot 10^{19})$
VO-HA1	2.85 ± 0.02	$38 \pm 12 (2.3 \cdot 10^{19})$

Table 3

Comparison of hyperfine coupling constants obtained by HYSCORE study of VO-TPH, VO-HA and VO-treated bone.^a

Cross-Peak	<i>a</i> , MHz	<i>T</i> , MHz
TPH P ₁	−15.9 / 14.0	1.94
TPH P ₂	−8.82 / 7.33	1.48
TPH P ₃	−2.01 / 0.88	1.14
HA P ₁	−16.6 / 14.6	2.00
HA P ₂	−8.10 / 6.33	1.77
HA P ₃	~0	~1.5 ^b
Bone P ₁	−14.25 / 12.38	1.87
Bone P ₂	−9.19 / 7.72	1.47
Bone P ₃	~0	~1.5 ^b

^aVO-TPH data are from [22], VO-bone data are from [21]

^b_T value is estimated from the length of showlders accompanying diagonal peak (ν_P, ν_P) in HYSCORE spectra.

Table 4

Isotropic ($|a|$) and anisotropic ($|T|$) ^1H coupling constants of various VO^{2+} complexes compared to the proton couplings of VO-TPH and VO-HA.

$ a $, MHz	$ T $, MHz	Assignment	Reference
~ 0	3.3	H_2O (axial)	52
0 – 9	4.2 – 5.0	H_2O (equatorial)	
0	3.4	H_2O (axial)	41,42
0.65 – 8.6	4.23 – 4.69	H_2O (equatorial)	
--	4.34	H <i>cis</i> to V=O	58
(–1) – 12	3.8 – 4.8	H_2O (equatorial)	59
8.17 or 3.87	4.29	H_1 TPH	
		H (equatorial)	
6.9 or 4.15	2.79	H_2 TPH	
		Remote ^1H , Perhaps from $-\text{O}-\text{P}-\text{OH}^a$	22
3.66 or 2.39	1.22	H_3 TPH	and this work
		$\text{V}=\text{O}\cdots^1\text{H}-\text{O}^a$	
4.11 or 2.00	2.13	H_2 HA3	
		Remote ^1H , perhaps from $-\text{O}-\text{P}-\text{OH}^a$	
6.5 or 2.50	4.00	H_1 HA3	
		H (equatorial)	

^a see text for assignment