In Vivo Electron Spin−Echo Envelope Modulation (ESEEM) Spectroscopy: First Observation of Vanadyl Coordination to Phosphate in Bone

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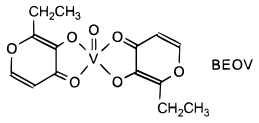
Vanadium compounds have insulin-enhancing properties both in vivo and in vitro.[1] Bis(ethylmaltolato)oxovanadium(IV) (BEOV), an analogue of bis(maltolato)oxovanadium(IV) (BMOV) designed to improve V uptake,[2] has been synthesized and fully characterized. The coordination structure and oxidation state of the VO^{2+} ion in vivo are important in elucidating the biological fate of exogenously added V, which is known to accumulate primarily in bone.[3] These parameters cannot be determined by atomic spectroscopy studies or other previously available methods.[1] Electron spin−echo envelope modulation (ESEEM)[4−7] spectroscopy is used here to determine the final in vivo coordination environment of paramagnetic V(IV) in bone samples from diabetic (DT) and nondiabetic control (CT) rats treated for 6 weeks with BEOV (0.26−0.29 mmol kg^{-1} day^{-1} in the drinking water). Bone samples from non-BEOV (NB) rats were also taken for comparison.



BEOV

Echo-detected EPR spectra (Bruker ELEXSYS spectrometer, $T = 30$ K) of the samples showed signals with an axially symmetrical line shape and ^{51}V hyperfine (hf) structure. DT and CT samples did not differ in the **g** and **A**(^{51}V) hf tensors. Analysis of hf structure gave values of $g_\perp = 1.996 \pm 0.005$, $g_{||} = 1.93 \pm 0.01$ and $A_\perp(^{51}V) = 8$ mT and $A_{||}(^{51}V) = 19$ mT, consistent with those previously reported for square pyramidal oxovanadium(IV) complexes.[8,9] No such signal was observed in the NB samples prepared in the same way. The two-, three-, and four-pulse

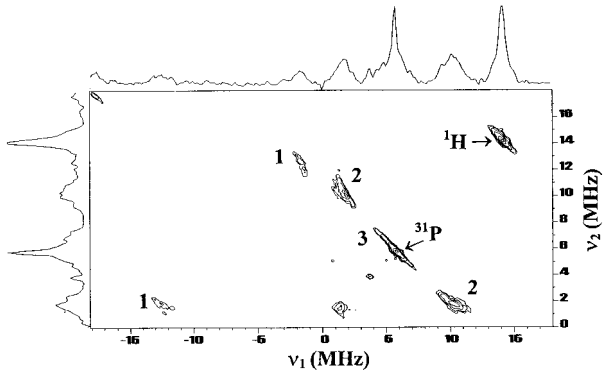


Figure 1. HYSCORE spectrum measured at the extra absorption peak, $m_I^V = -1/2$, of the VO^{2+} EPR spectrum in the DT bone (magnetic field = 331.5 mT; microwave frequency = 9.3252 GHz; time τ between first and second pulses = 120 ns). The spectrum was obtained after Fourier transformation of 2D time-domain patterns containing 256 × 256 points with a step of 16 ns.

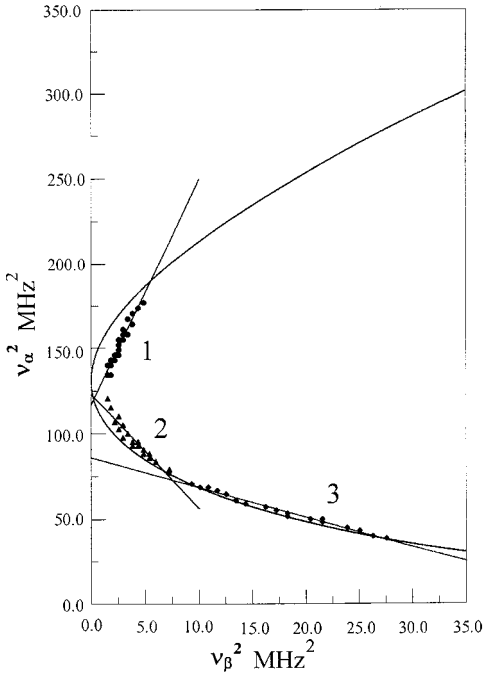


Figure 2. Points of cross-peaks 1−3 from HYSCORE spectra measured at different τ in the ν_α^2 vs ν_β^2 coordinate system. The larger coordinate of each point is arbitrarily chosen as ν_α and the smaller as ν_β. The heavy line is defined by $|\nu_\alpha + \nu_\beta| = 2\nu_I$.

ESEEM spectra of DT and CT samples showed similar peaks with minor variations in peak intensity. Since these variations are slight, all data shown in Figures 1 and 2, and their corresponding analyses, relate to the DT rat.

Three-pulse ESEEM spectra of a bone sample from DT (not shown) contain a narrow peak at $\nu_P = 5.7$ MHz and an intense

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Table 1. Parameters Derived from Contour Line Shape Analysis of HYSCORE Spectra

cross-peak	Q_α	G_α, MHz^2	a, MHz	T, MHz
1	13.22 (0.50)	117.5 (5)	−14.25/12.38 (0.25)	1.87 (0.01)
2	−6.67 (0.90)	123.1 (5)	−9.19/7.72 (0.12)	1.47 (0.50)
3	−1.74 (0.12)	86.1 (2)	−3.78/2.40 (0.35)	1.39 (0.02)

line at $\nu_H = 14.1$ MHz equal to the Zeeman frequencies of ^{31}P and ^{1}H, respectively, in an applied magnetic field of 330.1 mT. These lines are produced by weakly coupled matrix phosphorus and proton nuclei around the VO^{2+} species. Extended peaks starting at ~1.5 MHz with shoulders to ~4 MHz (and suggestions of low intensity peaks between 5 and 10 MHz) were also seen.[10]

Complete interpretation of the observed peaks was obtained from the four-pulse, two-dimensional ESEEM technique, HYSCORE (Hyperfine Sublevel Correlation) spectroscopy.[11] The typical contour HYSCORE spectrum (Figure 1) contains peaks from weakly coupled ^{1}H and ^{31}P matrix nuclei located on the diagonal of the (+,+) quadrant at the ν_H and ν_P. In addition to the diagonal peaks, three pairs of cross features can be distinguished, designated as 1, 2, and 3. Cross-peaks 1 appear as extended, weak arcs along the diagonal of the (+,−) quadrant, centered at ~7 MHz. The two coordinates of each point of the arc differ by close to $2\nu_P = 11.4$ MHz. Two other cross features are located in the (+,+) quadrant, centered symmetrically around the diagonal point of $\nu_P = 5.72$ MHz. Cross-peaks 2 have maxima at (10.3, 1.5) MHz. Cross-peaks 3 overlap with the high-intensity diagonal peak at the time τ used in Figure 1; however, suppression of the intensity at the diagonal by adjustment of τ allows their observation as a pair of extended ridges (even partially overlapping with cross-peaks 2) with maxima at (~7.4, ~4.3) MHz. The characteristics of the cross-peaks 1−3 led to their assignment as three phosphorus nuclei with estimated hf couplings of 14, 9, and 3 MHz, respectively.

The contour line shapes of the cross-peaks provide the isotropic a and anisotropic T couplings of ^{31}P nuclei (spin $I = 1/2$)[12,13] when plotted ν_α^2 vs ν_β^2 (Figure 2).[14] The coordinates (ν_α, ν_β) of arbitrary points along the top ridge forming each peak were measured from HYSCORE spectra recorded with different τ values. The slopes and intercepts (Table 1) of ν_α^2 vs ν_β^2 yield[12−14] two sets of a and T with opposite relative signs for each nucleus and with the same $|2a + T|$ value.[15]

These ^{31}P couplings in bones correlate with the phosphorus couplings observed in VO^{2+}−adenosine phosphate complexes.[16,17] Metal−ligand binding for ADP and ATP had a 1:2 stoichiometry (by EPR), with metal chelation occurring via the phosphate groups.[17] EPR spectra of $[VO(ADP)_2]^-$ in frozen solution exhibit additional hf structure assigned to four equivalent phosphorus nuclei with a coupling of 18.54 MHz. Regular hf structure is not observed for the ATP complex due to inequivalent couplings with phosphorus. ENDOR spectra of these complexes, however, have shown features assigned to the ^{31}P coupling of 20.6 MHz only.[17] In contrast, HYSCORE spectra of $[VO(ATP)_2]^{2-}$ contain cross-peaks from ^{31}P with estimated couplings of 14.8 and 9.0 MHz assigned to the β- and γ-phosphorus atoms of ATP.[16]

The mineral phase of bone is composed of a roughly crystalline calcium phosphate polymer hydroxyapatite (HA), with a unit cell of $Ca_{10}(PO_4)_6(OH)_2$.[18,19] Biological HA is characterized by numerous ion vacancies as well as by two monolayers of water molecules on the bone surface. Incorporation of vanadyl ions into the crystal lattice, as opposed to interaction with phosphates and hydroxides on the mineral surface, is not differentiable here. Values of maximum phosphorus hf couplings in bones similar to those in $[VO(ADP)_2]^-$ and $[VO(ATP)_2]^{2-}$ suggest at least partial coordination of VO^{2+} via phosphate oxygens, leading to unpaired spin density on phosphorus atoms.[20] Thus, vanadyl interacts with the HA fraction of bone and is located at least between the hydration shell and bone surface. An intense peak at ν_H (by ESEEM) is evidence of weakly coupled protons around the ion. Also, the proton hyperfine coupling of ~3 MHz (from HYSCORE, not shown) derives from a hydrogen-containing ligand, such as H_2O or hydroxide.[6,21]

The ESEEM observations of VO^{2+}−phosphorus coordination in bones can be interpreted in two ways. One possibility is that all of the complexes could have the same structure, that is, all three phosphorus nuclei belong to ligands from the same complex. The three couplings could be via a facial VO^{2+} tris(phosphate) complex, with two other ligands (i.e. H_2O and hydroxide) present, resulting in an overall octahedral structure.[20] Or, vanadyl ions could form several complexes with different structures, but with minor variations in **g** and ^{51}V hf tensors, distinguished by the phosphorus couplings, resulting in a superimposability of ESEEM spectra. Differentiation between these could come from high-field EPR or ESEEM experiments, with samples prepared at varying times after BEOV administration (assuming that these complexes do not all form at the same rate). Multinuclear species such as dimers and trimers, as well as mixed valent species, are not consistent with the observed spectra and the relaxation time of the echo decay, both of which are typical for isolated VO^{2+} complexes.

In summary, we report herein on the first observation of VO^{2+} interacting with the inorganic fraction of bone, by ESEEM spectroscopy. Complete resolution of the ^{31}P lines in 2D spectra led to separate measurement of the isotropic and anisotropic hf couplings of the coordinated phosphates to VO^{2+}. Clearly, the paramagnetic fraction of vanadium present in bones after BEOV administration has changed since its initial coordination. The vanadyl interacts with the HA fraction of bones, and is partially coordinated by phosphate oxygens; the three distinct ^{31}P couplings indicate at least three different V−O−P binding moieties, from one or several complexes. The use of model complexes (experiments in progress) will help elucidate the structure(s) of the VO^{2+}−phosphate complex(es), furthering our understanding of the biological storage and mobilization of vanadium in vivo.

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(10) Spectra measured on the $g_\parallel$ and $g_\perp$ components of the hf structure show matrix peaks in addition to a low-frequency peak; however, the form and intensity of this latter peak vary due to orientation selection.

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(14) The contour line shape in the 2D spectrum from $I = 1/2$ nuclei is described by the following:[13] $\nu_\alpha = \{Q_\alpha\nu_\beta^2 + G_\alpha\}^{1/2}$ where $Q_\alpha = [T + 2a - 4\nu_I)/(T + 2a + 4\nu_I]$ and $G_\alpha = 2\nu_I\,[4\nu_I^2 - a^2 + 2T^2 - aT]/[T + 2a + 4\nu_I]$.

(15) The relative sign of the a and T values are indeterminate here. Additional multifrequency experiments would lead to such a choice.

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(20) Variation of the hf couplings for three P atoms can be attributed to different O═V−O(−P) angles. The isotropic constant is proportional to the unpaired 3s spin density and the coefficient 13306 MHz computed for a unit 3s electron.[22] The anisotropic coupling results from dipole−dipole interaction (mainly from the V−P distance), and indirect spin transfer on a 3p orbital of P. A V−P distance of 3.44 Å[17] for a V−O−P fragment in ADP complexes corresponds to $T = O.79$ MHz for point dipoles; computed T for a unit 3p electron is 367 MHz. Thus, the isotropic coupling should be far more sensitive to structural variations than the anisotropic coupling. Similar behavior has already been found for protons in a VO−aqua complex.[21]

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