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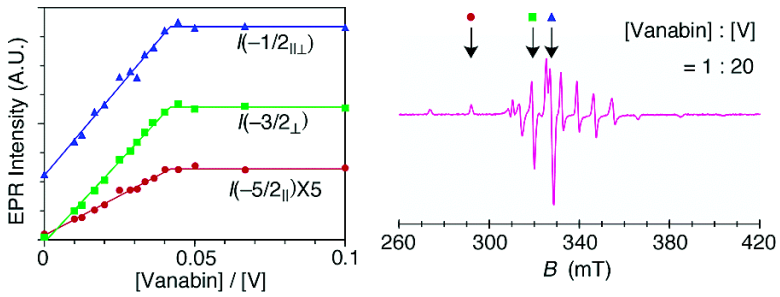
J. Am. Chem. Soc., **2003**, 125 (21), 6352-6353• DOI: 10.1021/ja034507w • Publication Date (Web): 02 May 2003



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Published on Web 05/02/2003

Vanadium-Binding Protein in a Vanadium-Rich Ascidian *Ascidia sydneiensis samea*: CW and Pulsed EPR Studies

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Some of the ascidians (tunicates or sea squirts) belonging to the suborder Phlebobranchia accumulate vanadium ion, although the biological roles of the vanadium are not clear.[1−3] In remarkable cases, the concentration of cellular vanadium reaches 350 mM, corresponding to ca. 10^7 times the concentration of seawater.[4] Clarification of the mechanism of this surprisingly efficient metal-accumulation system is desirable. The fate of vanadium currently known is that vanadium is finally accumulated in vacuoles of the blood cell called vanadocyte (or signet ring cell).[5] Most vanadium ions in the vacuoles are in the V(III) state and seem to exist as a free ion.[6] From vanadocytes of *Ascidia sydneiensis samea*, we have recently isolated some proteins that are likely to be involved in vanadium-accumulation processes.[7,8] Among them, two similar proteins, vanabin1 and vanabin2, which are named for vanadium-binding proteins, turned out to be capable of binding as many as ca. 10 and 20 vanadium ions, respectively, per one protein.[9] We believe that they play an important role in vanadium-transport or -storage processes in the organism. Their amino acid sequences show that they are cysteine- and lysine-rich proteins. Vanabin1 and vanabin2, which are respectively composed of 87 and 91 amino acids, contain 18 cysteines for both and 12 and 14 lysines, respectively.[9] In this study, vanadium-binding properties of vanabin2 have been examined further by EPR spectroscopy. We carried out EPR spectrometric titrations to elucidate the maximum number of vanadium bound to the protein and electron spin−echo envelope modulation (ESEEM) experiments to gain insights into the coordination environments of the vanadium-binding sites. ESEEM, which is a technique based on pulsed EPR, has been proven to be particularly useful for studies of coordination environments of VO^{2+} ions in complexes[10−12] and proteins.[13,14]

Vanabin2 was prepared as reported previously.[9] For preparations of EPR samples, an aqueous solution containing 1:1 equiv of $VOSO_4$ and iminodiacetic acid (IDA) was added to a Tris-HCl buffered NaCl solution (pH = 7.4) of vanabin2. IDA was included to prevent precipitation of vanadium.

X-band CW EPR spectra were recorded for samples containing various ratios of VO^{2+} and vanabin2. Results show that the complex VO−vanabin2 invariably exhibits a usual mononuclear-type VO^{2+} signal (Figure S1). Figure 1 depicts the variation of the EPR signal intensities for the $M_I = -5/2_{||}$, $-3/2_{\perp}$, and $-1/2_{||\perp}$ lines as a function of the ratio [vanabin]/[V]. It is known that the VO^{2+} ion forms EPR-silent polymeric species at neutral pH when no strong ligands coexist.[15] Thus, the EPR signal intensities are proportional to the amount of the VO^{2+} ions bound to the protein. (In fact, the sample for [vanabin]/[V] = 0 exhibited a very weak signal, whose intensity is subtracted in the analysis below.) No other signals were observed

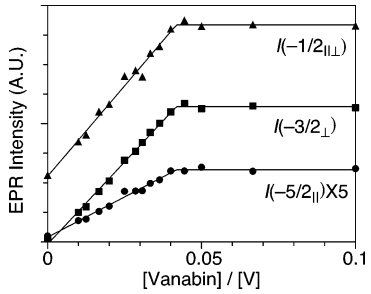

Figure 1. EPR spectrometric titration of VO^{2+} ion bound to vanabin2. EPR intensities of the $M_I = -5/2_{||}$, $-3/2_{\perp}$, and $-1/2_{||\perp}$ lines for various [vanabin]/[V] ratios are plotted. Conditions: T = 77 K; ν = 9.21 GHz; power = 1 mW; modulation (100 kHz) = 1 mT. Final concentrations: [VO] = 100 μM, [vanabin] = 0−10 μM, [Tris] = 10 mM, and [NaCl] = 100 mM in a total volume of 150 μL.

in the field range 0−500 mT. The occurrence of plateaus in the region [vanabin]/[V] ≥ ∼1/20 indicates that vanabin2 can bind up to ∼20 vanadium per one protein. This number agrees with that obtained previously by the Hummel−Dreyer method.[9] The EPR parameters of VO−vanabin2 were $g_{||} = 1.9430$ and $A_{||} = 168.7 \times 10^{-4}$ cm^{-1}, falling on the boundary region of the N_2O_2 and $O_2O_2^-$ equatorial donor sets in the $g_{||}-A_{||}$ diagram.[15,16] The signal did not show any appreciable variations in line positions and line widths with the variation of [vanabin]/[V], which indicates that there is only one type of EPR-active VO^{2+} in VO−vanabin2 in the EPR sense. We performed quantitation of the EPR signals to check the amounts of EPR-active VO^{2+}.[17] Double integration of the spectra provided $[V]_{EPR-active}/[V]_{all} = 0.84 \pm 0.07$ for [vanabin]/[V] = $1/20$−$1/2.5$ and 1.04 for [vanabin]/[V] = 1, which confirms that almost all protein-bound vanadium was observed in the EPR spectra. This result also shows that most of the vanadium ions in the protein exist as a mononuclear VO^{2+} form. In Figure 1, the EPR intensities increase almost linearly in the range of [vanabin]/[V] = 0−$1/20$. This suggests that no allosteric effects are present in the binding processes. We therefore analyzed the titration curve by assuming that each binding site behaves independently. From the fittings of the titration data, the number of the vanadium-binding sites per one protein was determined to be $n = 23.9 \pm 0.5$.[18]

Figure 2 shows the two-pulse ESEEM spectrum of VO−vanabin2 ([vanabin]/[V] = 1/20) recorded at the $-1/2_{||\perp}$ line. This type of spectrum is typical of those exhibited by VO^{2+} complexes with equatorial ^{14}N coordination.[10,11] The peaks at 3.9 and 7.1 MHz are attributed to the ^{14}N double-quantum (DQ) lines, and the peaks in the range 0−3 MHz are attributed to single-quantum lines. The three-pulse stimulated-echo (τ = 300 ns) ESEEM spectrum also showed the DQ lines at 3.9 and 7.2 MHz (Figure S2). Measurements were also performed for samples that do not contain IDA. The mixture of vanabin2 and freshly prepared $VOSO_4$ solutions provided the same ESEEM results as in Figure 2, which excludes the

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10.1021/ja034507w CCC: $25.00

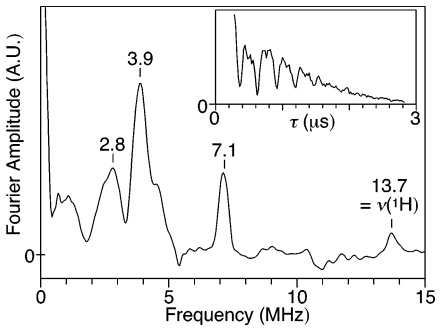

Figure 2. Two-pulse ESEEM spectrum and its time-domain data (inset) of VO−vanabin2 ([vanabin]/[V] = 1/20). Conditions: $T = 77$ K; $\nu = 9.03$ GHz; $B = 320.7$ mT; $t_{\pi/2} = 20$ ns; $t_{\pi} = 40$ ns. Ethylene glycol (75 μL) was added to the VO−vanabin2 solution (150 μL) prepared in the same manner as in the CW EPR experiments except larger [V] and [vanabin] were employed ($[V]_{final} = 626.7$ μM).

possibility that this nitrogen might come from IDA. From the DQ line frequencies, we obtain the ^{14}N hyperfine coupling (HFC) parameter as $A_{iso} = 4.5$ MHz and the nuclear quadrupole coupling (NQC) parameter as $K(3 + \eta^2)^{1/2} = 1.45$ MHz (hence $e^2qQ = 4K = 2.90-3.35$ MHz because $0 \le \eta \le 1$).[19] It has been reported that the ^{14}N HFC parameter distinguishes the types of equatorial nitrogen:[10,11] $A_{iso} \approx 5$ MHz for amine nitrogen, ~6−7 for imine nitrogen (including amidate nitrogen),[20] and 7.2−7.3 for porphyrin nitrogen. According to this criterion, the ^{14}N nucleus in the present data is attributed to amine nitrogen. The e^2qQ value estimated above is also consistent with amine nitrogen coordination.[14] Quantitation of the ESEEM signal was unfortunately not practicable because the ^{14}N ESEEM amplitude is a complex function of HFC, NQC, magnetic field, and their relative orientations. Nevertheless, the deep modulation in the time-domain data (inset of Figure 2) strongly suggests that most EPR-active VO^{2+} ions have amine nitrogen ligands. This also suggests that all or almost all amine nitrogens are used in the vanadium-saturated vanabin, where the EPR-active vanadium number is ~20 (=23.9 × 84%), while vanabin2 has 15 amine nitrogens (14 lysines and one N-terminal amine),

In summary, this study has shown that vanabin2 can bind up to ~23.9 vanadium ions per one molecule with most of the vanadium ions being in a mononuclear state and coordinated by (most likely one) amine nitrogen. Despite its cysteine-rich nature, no evidence was found for thiolate-coordination (the observed $A_{||}$ value differs much from the values for the N_2S_2 and S_2O_2 donor sets, (140−155) × 10^{-4} cm^{-1}).[16] It is interesting that this relatively small protein can bind such a large number of metal ions in a mononuclear fashion. Ferritin and metallothionein are typical examples for the proteins that can bind large numbers of metal ions.[20] However, the metal ions exist as clusters and/or microcrystals in these proteins. This study has shown that this is not the case for vanabin, arousing further interests in the vanadium-binding processes of vanabin.

Acknowledgment. This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#13024255, #14340264, and #14596005).

Supporting Information Available: Representative CW EPR spectra, two-pulse ESEEM spectrum (0−30 MHz range), and a three-pulse ESEEM spectrum (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA034507W