Coordination of Vanadyl(IV) Cation in Complexes of *S*-Adenosylmethionine Synthetase: Multifrequency Electron Spin Echo Envelope Modulation Study†

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ABSTRACT: *S*-Adenosylmethionine (AdoMet) synthetase requires two freely dissociable divalent cations for activity, and its activity is greatly stimulated by certain monovalent cations (K^+, Tl^+). Omission of the native Mg^{2+} cations prevents enzyme-catalyzed reactions, although the substrates and products still bind. Vanadyl (oxovanadium, VO^{2+}) serves as a convenient paramagnetic probe of the substrate-independent, divalent cation binding site in the enzyme. In the present study, multifrequency electron spin echo envelope modulation (ESEEM) is employed to explore the cation's coordination sphere in several functionally relevant complexes. In the substrate complex enzyme·VO^{2+}·ATP·methionine·K^+, an equatorially coordinating ^{14}N ligand is found, with A_{iso} = 4.3 MHz. Selective ^{15}N labeling of the ϵ-amino nitrogens of all lysine residues in the protein reveals that lysine is the source of the ligand. A lysine ^{14}N ligand is also observed in the intermediate complex enzyme·VO^{2+}·AdoMet·PPP_i·K^+, with A_{iso} = 4.8 MHz, and in the initial product complex enzyme·VO^{2+}·AdoMet·PP_i·K^+. In the subsequent product, enzyme·VO^{2+}·AdoMet·K^+ (formed upon dissociation of PP_i), the methionyl amino nitrogen of AdoMet coordinates VO^{2+} (A_{iso} = 5.3 MHz), and the lysine ligand is lost. In each complex, the monovalent cation activator can be changed from K^+ to Tl^+ or Na^+ with no effect on the ESEEM spectra. Combination of the ESEEM data from this study with previous CW data [Markham, G. D. (1984) *Biochemistry 23*, 470–478] leads to identification of three of the equatorial ligands to VO^{2+} and places constraints upon the identity of the fourth ligand, in both the substrate and product complexes. A hypothetical outline of changes in the metal coordination scheme during the reaction is presented, based upon these results.

S-Adenosylmethionine (AdoMet)[1] synthetase (ATP:L-methionine *S*-adenosyltransferase) catalyzes a reaction in which the adenosyl group of ATP is transferred from the triphosphate moiety to the sulfur atom of L-methionine (Mudd, 1973). The enzyme subsequently hydrolyzes triphosphate into pyrophosphate and orthophosphate (Mudd, 1963; Markham et al., 1980). The product AdoMet is the alkyl donor in most cellular reactions which require methylation and is the propylamine donor in polyamine biosynthesis (Cantoni, 1975; Tabor & Tabor, 1976). The AdoMet synthetase encoded by the *Escherichia coli metK* gene is tetrameric (molecular weight 180 000; Markham et al., 1980) with one monovalent cation (e.g., K^+) and two divalent cations (e.g., Mg^{2+}, Mn^{2+}) per monomer required for catalytic activity (Markham et al., 1980; Markham, 1981). At one of the divalent sites, the cation binds independently of the presence of substrate, while either substrate or the product $PP(P)_i$ is required for binding of the second divalent metal ion. The spin exchange interaction observed when Mn^{2+} binds to both sites demonstrates that the divalent cations are close together (Markham, 1981); it has been suggested that they may be bridged by a common ligand, such as a polyphosphate group (Markham, 1984). Vanadyl, VO(IV), binds exclusively to the substrate-independent site (Markham, 1984). With Mg(II) in the other divalent site, substantial catalytic activity is obtained. The monovalent cation also binds at the active site, as was shown by observation of an isotropic hyperfine coupling of the nuclear spin of Tl^+ to the unpaired electron of VO^{2+} bound to the enzyme (Markham & Leyh, 1987).

The VO^{2+} cation, with electron spin $S = {}^1/_2$, has proven to be a useful paramagnetic probe in proteins which require divalent cations for their catalytic functions (Chasteen, 1981; Eaton & Eaton, 1990). Continuous-wave (CW) EPR measurements (Markham, 1984) indicate that substrate binding to the AdoMet synthetase·VO^{2+} complex affects the coordination of VO^{2+}. Specifically, when a polyphosphate moiety is present, either in substrate form as ATP or in an intermediate form as PPP_i or PP_i, each of two phosphates donates an oxygen as an equatorial ligand to VO^{2+}. In these complexes, linewidth reductions subsequent to exchange against D_2O suggest the presence of at least one equatorial water ligand. On the other hand, when a polyphosphate moiety is absent (e.g., enzyme·VO^{2+}·K^+ or enzyme·VO^{2+}·AdoMet·K^+), the evidence favors two water oxygens in the equatorial ligand set; when MgATP binds in the absence of methionine there is no indication that any phosphate group coordinates VO^{2+}.

Although the continuous-wave (CW) EPR spectrum of vanadyl is sensitive to its environment in the protein (Chasteen, 1981), inhomogeneous broadening frequently precludes the direct identification of ligands by measurements of splitting

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; ATP, adenosine triphosphate; E, enzyme; [^{15}N]E, *S*-adenosylmethionine synthetase nonspecifically enriched with ^{15}N; PP_i, pyrophosphate; PPP_i, triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DDT, dithiothreitol; CW, continuous wave; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance; DQ, double quantum; shfc, superhyperfine coupling.

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due to superhyperfine couplings. The method of electron spin echo envelope modulation (ESEEM) is well-suited to the measurement of weak superhyperfine couplings (Mims, 1972; Mims & Peisach, 1981), and there have been several recent reports of ESEEM applications to studies of VO^{2+} in proteins (Tipton et al., 1989; Eaton et al., 1988; deBoer et al., 1988; Gerfen et al., 1991). Coordination of VO^{2+} by ^{14}N was discovered in each of these cases, although the assigned source of the nitrogen varied. Tipton et al. (1989), in a study of pyruvate kinase, found that under certain conditions vanadyl bound to the protein-based divalent cation site is coordinated by a nitrogen ligand. This nitrogen was assigned to lysine, on the basis of comparison of nuclear hyperfine and quadrupole coupling parameters obtained from simulations of ESEEM spectra of the enzyme with those from model complexes. Eaton et al. (1988) measured nitrogen couplings to VO^{2+} in the proteins lactoferrin and transferrin. The hyperfine coupling constants obtained by simulation of their data were significantly larger than those found in pyruvate kinase (Tipton et al., 1989). The modulations were assigned to a directly-coordinating imidazole nitrogen from histidine, in the equatorial plane of the vanadyl cation, on the basis of measurements of model complexes. de Boer, et al. (1988) identified an equatorial nitrogen ligand to vanadyl in the enzyme bromoperoxidase, but no assignment was made. Most recently, Gerfen et al. (1991) observed a strong ^{14}N coupling to VO in complex with apoferritin and also assigned it to an imidazole nitrogen of histidine.

In the present study, three types of enzyme·VO^{2+} complexes are examined: those containing substrates (ATP and/or methionine), those containing the reaction intermediates (AdoMet and PPP_i), and those with an incomplete set of reaction products (i.e., either AdoMet or PP_i or both, but not P_i, which binds weakly). ^{14}N couplings of 4.3–5.3 MHz, indicative of directly-coordinating nitrogen, are found in each case, but the coupling parameters in the complexes containing PP_i, PPP_i, or ATP·methionine are distinctly different from those observed when there are no polyphosphates in the sample. ESEEM measurements utilizing ^{15}N labels in the protein or in AdoMet demonstrate that an exchange of nitrogen ligands occurs when the diphosphate coordination of VO^{2+} is lost.

MATERIALS AND METHODS

Materials. $(^{15}NH_4)_2SO_4$ at 98.8 atom % ^{15}N was obtained from the Monsanto Mound facility. L-[ϵ-^{15}N]lysine, 99 atom % ^{15}N, and L-[^{15}N]methionine, 99 atom % ^{15}N, were purchased from Merck Isotopes. The bacterial strains EMG2 and AT2453 were obtained from the *E. coli* Genetic Stock Center, Yale University. EMG2 is a wild-type *E. coli* K12 strain (Clowes & Hayes, 1968). The genotype of AT2453 is *lysA22 thy-1 spoT1 relA1* Hfr (PO1 of Hayes) (Taylor & Trotter, 1967).

Preparation of [U-^{15}N]AdoMet Synthetase. [U-^{15}N]-AdoMet synthetase was prepared from the protrophic *E. coli* strain JT10pK8. JT10 is a derivative of the *E. coli* strain EMG2 into which a mutation in the *metJ* locus, which encodes the repressor protein for *metK* and for methionine biosynthesis, was introduced; this strain produces ca. 2-fold more AdoMet synthetase than EMG2pK8. JT10 was constructed by bacteriophage P1 cotransduction of the *rpoB* and *metJ* loci into EMG2. The P1 lysate was prepared from strain EWH186 (Hafner, et al., 1977) using standard methods (Miller, 1972). Initially transductants were selected by rifampicin resistance which accompanies the introduction of *rpoB* (Miller, 1972), followed by screening for the cotransduction of *metJ* by testing for the acquisition of resistance to the methionine analog ethionine. Ethionine was present at 20 mM in Vogel–Bonner minimal medium plates (Hafner et al., 1977).

The plasmid pK8 which carries *metK* was introduced into JT10 by standard transformation procedures (Maniatis et al., 1982). The strain was grown to stationary phase at 37 °C in 8 L of minimal A medium (Miller, 1972) containing 0.2% glucose, 0.1% thiamin, and 0.5 g/L $(^{15}NH_4)_2SO_4$, 99 atom % ^{15}N, as sole nitrogen source. Methionine (0.01%) was included with the medium since strains containing pK8 often grow poorly in the absence of methionine. The medium contained 30 μg/mL oxytetracycline to ensure the maintainance of the plasmid. Cells (24 g) were harvested by centrifugation and stored at −70 °C until use. The enzyme was purified as described below.

Preparation of [ϵ-^{15}N-lysine]AdoMet Synthetase. This protocol used the lysine-auxotrophic *lysA* mutant AT2453 (Taylor & Trotter, 1967) as host for pk8. AT2453pK8 was grown to stationary phase at 37 °C in 4 L of Vogel–Bonner medium (Vogel & Bonner, 1956) containing 0.2% (w/v) glucose, 0.1% thiamin, 0.0025% L-[ϵ-^{15}N]lysine, 99 atom % ^{15}N, 0.1% of all other amino acids, and 30 μg/mL oxytetracycline. The other added amino acids served as a buffer against ^{15}N lost from lysine being incorporated into other amino acids, as well as increasing the cell yield. A total of 21 g of cells was obtained by a 4-L growth.

Enzyme Purification. Due to the expense of isotopically enriched enzyme, we sought to expedite the purification of AdoMet synthetase. The enzyme was purified to homogeneity using modifications of published procedures (Markham et al., 1980; Markham, 1981). The procedure follows the 1980 protocol exactly through the phenyl-Sepharose chromatography, with linear adjustments of the column volume according to the amount of cells used. The active fractions from the phenyl-Sepharose column were pooled, diluted with 10% aqueous glycerol containing 0.1% mercaptoethanol to a conductivity of <7 mmho, and chromatographed on aminohexyl-Sepharose 4B (Markham, 1981), with the column volume adjusted for the amount of cells used. The active fractions from the aminohexyl-Sepharose column were loaded directly onto a 2.5- × 7-cm column of Bio-Rad hydroxylapatite equilibrated with 50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 10% glycerol, and 0.1% mercaptoethanol (buffer A). Electrophoretically homogeneous enzyme elutes near the middle of a gradient of 0–50 mM phosphate in 0.3 L of buffer A. This purification protocol yields enzyme of the same specific activity (2.2 μmol min^{-1} mg^{-1}) originally reported while eliminating two chromatographic steps, ion exchange on DEAE-52 cellulose and gel filtration on Sephadex S-300. The yield of enzyme is ca. 10 mg of protein/g of cell paste.

Preparation of [α-^{15}N]AdoMet. [α-^{15}N]AdoMet was prepared from an overnight incubation containing 6.8 mM ATP, 1.0 mM L-[^{15}N]methionine (99% ^{15}N), 50 mM Hepes-KOH, 50 mM KCl, 10 mM $MgCl_2$, pH 8.0, and 1.5 mg of AdoMet synthetase in a total volume of 10 mL. AdoMet was purified on a 1- × 20-cm column of SP-Sephadex which was initially equilibrated with water. The reaction mixture was loaded directly onto the column, which was then eluted batchwise with successive 40-mL portions of water, 10 mM HCl, 100 mM HCl, and 2 M HCl. AdoMet eluted in the 2 M HCl wash. AdoMet was taken to dryness by evaporation under vacuum at room temperature and was then taken up in 2 mL of H_2O and dried again before dissolution in water and storage at −70 °C The reaction yielded 5 μmol of AdoMet (determined from the absorbance at 259 nm). The [α-^{15}N]-

Table I: Summary of ESEEM Results from VO(IV) Complexes of AdoMet Synthetase

complex	ν_e (GHz)	H_0 (mT)	frequencies[a] (MHz)	A_{iso}[b] (MHz)	assignment
E·VO·ATP·met·K	7.3914	261.9	4.3, 6.8	4.3	lysine ^{14}N
E·VO·ATP·met·K	8.7995	313.5	4.1, 7.1	4.4	lysine ^{14}N
E·VO·ATP·met·K	9.1290	323.2	4.0, 7.2	4.5	lysine ^{14}N
E·VO·AdoMet·PPP$_i$·K	8.1940	290.1	4.0, 7.1	4.8	lysine ^{14}N
E·VO·AdoMet·PPP$_i$·K	9.8650	351.1	3.6, 7.4	4.8	lysine ^{14}N
E·VO·AdoMet·PP$_i$·K	9.1603	325.0	7.2, 3.9	4.6	lysine ^{14}N
E·VO·AdoMet·PP$_i$·K	8.1270	287.8	7.1, 4.2	4.6	lysine ^{14}N
E·VO·MgPP$_i$·K	9.1290	325.3	3.8, 7.2	4.7	lysine ^{14}N
E·VO·AdoMet·K	8.1950	290.0	4.5, 7.7	5.5	AdoMet ^{14}N
E·VO·AdoMet·K	9.8650	352.0	4.2, 8.0	5.4	AdoMet ^{14}N

[a] Estimated error in frequency measurement is ±0.1 MHz. [b] Values calculated from eq 3.

AdoMet was judged pure by chromatography on a mono-S cation-exchange column attached to an FPLC system; the column was eluted isocratically with 0.6 N HCl. The [α-^{15}N]-AdoMet showed a single peak with the same retention time as natural-abundance AdoMet.

Preparation of Samples for EPR/ESEEM. Enzyme was prepared for spectroscopy by gel filtration (Sephadex G-50) into 50 mM Hepes-KOH, 50 mM KCl, and 1 mM DTT, pH 8.0. The protein was concentrated using Amicon Centricon-10 and Centriprep-10 devices. Protein concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 1.3 (mg/mL)$^{-1}$ cm^{-1}. To prepare the VO·enzyme complexes, microliter additions of 10 mM $VOSO_4$ were added to samples containing all other components except Mg(II) (where present); when Mg(II) was required, it was always added as the last component of the sample.

Most samples contained final concentrations of 45 mM Hepes-KOH, 45 mM KCl, and 0.9 mM DTT, pH 8.0. In experiments involving Tl^+, Hepes-$(CH_3)_4N^+$ was used and the KCl and DTT was omitted. Samples of approximately 0.2 mL were contained in 4-mm quartz tubes. Samples were frozen by placing EPR tubes in a stream of N_2 gas at ca. −100 °C.

ESEEM Spectroscopy. Electron spin echo envelope modulation measurements were performed at X-band (8–12 GHz) using a laboratory-built spectrometer. Microwave power for pulses was provided by a 50-W (minimum) pulsed traveling wave tube amplifier (Applied Systems Engineering). The microwave magnetic field was concentrated at the sample by a resonant copper foil strip wrapped around each sample tube (LoBrutto et al., 1986). Small tuning adjustments (as well as resonator matching) were accomplished by rotating the sample tube; larger changes in resonant frequency (for multifrequency measurements) were made by changing the length (the circumferential dimension) of the copper strip. The length of a $\pi/2$ pulse using this system was approximately 20 ns. Sample temperatures during measurement were kept in the 4.2–6.0 K range with an APD Cryogenics LTR continuous-flow liquid helium cryostat. Measurements were made using a three-pulse (stimulated echo) sequence, at a pulse repetition rate of 100 Hz, except where otherwise noted. Spectra were composed of 1024 data points, with each point representing a 10-ns step in $T + \tau$, the time between pulses I and III. Each spectral data point was typically an average of 600–1200 individual spin echoes. Spin echo decay functions were fit to an exponential by the "phase plane" method (Bacon & Demas, 1983); the exponential was then subtracted from the spin echo envelope. The remaining modulation function was multiplied by an apodizing function to compensate for the effects of truncation of the data set and then Fourier transformed. Spectra displayed herein are absolute value cosine Fourier transforms of the modulation functions. Unless otherwise noted, magnetic fields are chosen such that ESEEM spectra originate from the central, overlapping $m_I = -1/2_{\parallel}$ and $m_I = -1/2_{\perp}$ features of the CW spectrum.

For nuclei with $I = 1$, when the isotropic hyperfine coupling dominates nuclear Zeeman and quadrupole terms, as for a ^{14}N ligand to vanadyl; in general only the $\Delta m_I = 2$ "double quantum" (DQ) ^{14}N transitions are sufficiently narrow as to be observed. Their frequencies are given approximately (providing that the hyperfine coupling is nearly isotropic) by (Astashkin & Dikanov, 1985)

$$\nu_{\pm} = 2[((A_{iso}/2) \pm g_N\beta_N H_0)^2 + K^2(3 + \eta^2)]^{1/2} \quad (1)$$

where K and η are the standard quadrupole parameters:

$$K = e^2Qq/4 = P_z/2 \qquad \eta = (P_x - P_y)/P_z \quad (2)$$

P_x, P_y, and P_z are the principle values of the quadrupole tensor. In such cases, one can obtain an immediate estimate of A_{iso} from eq 1:

$$A_{iso} = \frac{\nu_+^2 - \nu_-^2}{8\nu_I} \quad (3)$$

Equation 3 (Reijerse et al., 1989) was used to obtain the values of the isotropic parts of the ^{14}N superhyperfine couplings, which are listed in Table I. These values were also used as starting points for theoretical simulations of the ESEEM spectra. In each case where simulations were performed, it was possible to obtain acceptable fits to the experimental data using values of A_{iso} within ±0.2 MHz of those given by eq 3. However, as the spectra contained only $\Delta m_I = \pm 2$ transitions, the fits were not necessarily unique, even with data obtained at multiple frequencies. Therefore, in order to assure internal consistency, eq 3 was used for comparisons of A_{iso} values between complexes.

The simulations were performed using a local modification of software provided by the Biotechnology Resource in Pulsed EPR Spectroscopy at Albert Einstein Medical College, Bronx, NY, and described by Magliozzo et al. (1987). The simulation program is based upon the density matrix formalism of Mims (1972) and employs the angle-selection methods developed originally for ENDOR by Hurst, Henderson, and Kreilick (1975). The software was adapted to run in FORTRAN on a RISC coprocessor plug-in board hosted by a DOS-based personal computer.

RESULTS

Enzyme·ATP·Methionine Complexes. Three-pulse, frequency-domain ESEEM spectra of the complex enzyme·VO(IV)·ATP·[*methyl*-^{3}H]methionine·K^+, obtained at 7.4, 8.8, and 9.1 GHz, are displayed in Figure 1 (experimental data for this study are summarized in Table I). The spectra were

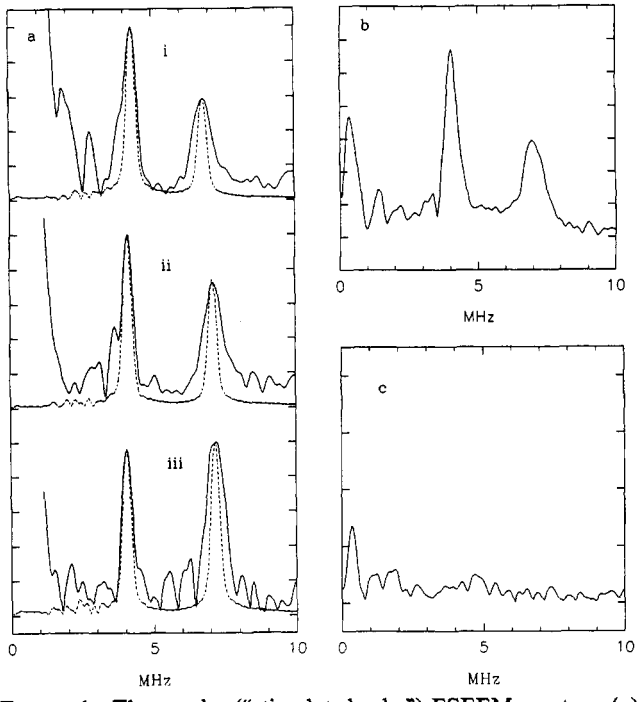

FIGURE 1: Three-pulse ("stimulated echo") ESEEM spectra. (a) E·VO^{2+}·ATP·[*methyl*-^{3}H]methionine·K^+: (i) ν_e = 7.3914 GHz, H_0 = 261.9 mT, τ = 190 ns; (ii) ν_e = 8.7995 GHz, H_0 = 313.5 mT, τ = 190 ns; (iii) ν_e = 9.1290 GHz, H_0 = 325.6 mT, τ = 190 ns. Dashed lines are simulated spectra, obtained using the parameters e^2Qq = 3.7 MHz, η = 0.3, $A_{xx} = A_{yy} = A_{zz}$ = 4.3 MHz. (b) E·VO^{2+}·ATP·[*methyl*-^{13}C]methionine·K^+: ν_e = 9.1109 GHz, H_0 = 323.2 mT, τ = 130 ns. (c) [ϵ-^{15}N-lysine]E·VO^{2+}·ATP·methionine·K^+: ν_e = 8.7567 GHz, H_0 = 310.9 mT, τ = 190 ns.

obtained from the large, central feature of the CW spectrum of the complex, which derives from both the $m_I = -1/2_{\parallel}$ and $m_I = -1/2_{\perp}$ transitions of the ^{51}V hyperfine pattern. Other portions of the CW spectrum yielded much weaker spin echo amplitudes and were not further studied. The most prominent features of the ESEEM spectra are transitions near 4 and 7 MHz. The ESEEM frequencies fall symmetrically about the free ^{31}P resonant frequency in the applied DC magnetic field (326.5 mT; cf. Table I) of the third spectrum, taken at 9.1290 GHz. However, multifrequency measurements reveal that the transitions do not track upward with increasing field in the manner expected of ^{31}P; instead, the difference between the transition frequencies increases very slightly with increasing H_0. This is characteristic of DQ nuclear transitions of ^{14}N, as described by eq 1. The spectra show no evidence of deuterium superhyperfine couplings, which would indicate proximity of the methionine methyl group to the vanadyl cation; no reproducible features are visible in the spectral region below 4 MHz. A sample containing fully protonated methionine with a ^{13}C-enriched methionine methyl carbon (Figure 1b) yields a similar spectrum. Again, the isotopic label is intended to detect unpaired electron spin density on or near the methyl group, but no ^{13}C modulations are evident. Thus, all of the frequency components shown in Figure 1 are attributed to ^{14}N. Simulations of two of the spectra of Figure 1a (dashed lines) support this assignment. The isotropic hyperfine coupling parameter most consistent with these spectra is A_0 = 4.4 MHz. When all lysine residues in the protein are labeled with ^{15}N at N_ϵ (Figure 1c), the ^{14}N transitions vanish entirely, proving that the modulations in Figure 1a,b arise from one or more lysine ligands to VO^{2+}.

Complexes with S-Adenosylmethionine and PPP_i. In the ESEEM spectrum of the complex enzyme·VO^{2+}·AdoMet·PPP_i·Tl^+ (Figure 2a), a pair of peaks occurs at 4.0 and

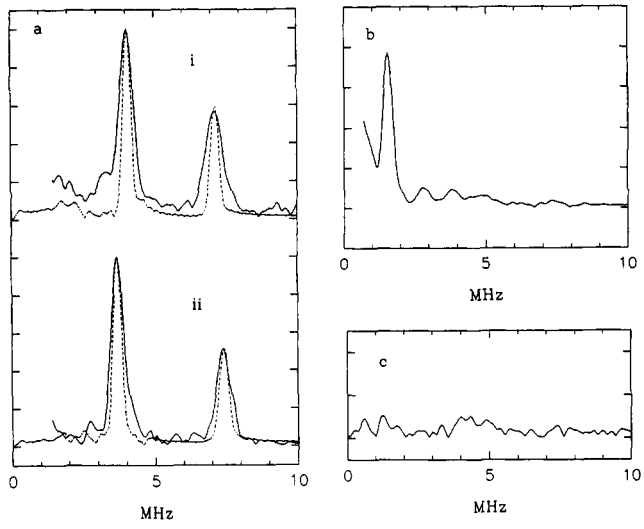

FIGURE 2: Three-pulse ESEEM spectra. (a) E·VO^{2+}·AdoMet·PPP_i·Tl^+: (i) ν_e = 8.1940 GHz, H_0 = 290.1 mT; (ii) ν_e = 9.8650 GHz, H_0 = 351.1 mT. Dashed lines are simulated spectra, obtained using the parameters e^2Qq = 2.9 MHz, η = 0.4, $A_{xx} = A_{yy}$ = 4.9 MHz, A_{zz} = 4.5 MHz. (b) [^{15}N]E·VO^{2+}·AdoMet·PPP_i·Tl^+: ν_e = 9.9861 GHz, H_0 = 355.4 mT. (c) [ϵ-^{15}N-lysine]E·VO^{2+}·AdoMet·PPP_i·K^+: ν_e = 8.4217 GHz, H_0 = 299.0 mT. τ = 190 ns in each case.

7.2 MHz when ν_e = 8.1940 GHz and H_0 = 290.1 mT. The assignment of these peaks to ^{14}N is confirmed by repeating the measurement at ν_e = 9.8650 and H_0 = 351.1 mT. Simulation of these features (dashed lines) yields A_0 = 4.8 MHz, in agreement with the direct calculation. If the same complex with Tl^+ in place of K^+ is examined at the same H_0 and microwave frequency, the same modulation frequencies and relative amplitudes occur (data not shown). No evidence of modulations from any monovalent cation's nucleus was seen under any conditions, nor does the choice of monovalent cation (K^+, Tl^+) have any influence on modulation frequencies from other nuclei, in this complex or in any of the complexes studied. In Figure 2b, uniform labeling of the enzyme with ^{15}N is seen to eliminate the ^{14}N transitions altogether, although an intense peak appears at the ^{15}N Larmor frequency. The presence of this peak provides an indication of the overall enrichment of the protein in ^{15}N and also demonstrates that the spin echo is of adequate amplitude to detect ESEEM where it is present. The spectrum of the complex (with K^+) selectively labeled with ^{15}N at all lysine residues is virtually featureless (Figure 2c), under conditions which previously produced intense ^{14}N peaks. Thus in this complex, too, there is a lysine ligand to VO^{2+}.

Enzyme·VO^{2+}·AdoMet·PP_i·K^+ Complex. Results obtained from this complex (data not shown), which contains both of the main products of the enzymatic reaction, are nearly identical to those (just described) results obtained from the complex containing the product, AdoMet, and the reaction intermediate, PPP_i. We obtain A_0 = 4.6 MHz for the complex with PP_i. Thus the hydrolysis of PPP_i does not appear to alter the coordination of the substrate-dependent divalent cation.

Enzyme·VO^{2+}·$MgPP_i$·K^+ Complex. In the complex enzyme·$VO^{2+}$$MgPP_i$·$K^+$, a pair of ^{14}N DQ transitions is observed once again; their frequencies correspond to A_0 = 4.7 MHz (Figure 3a). The initial assignment to ^{14}N was again made by use of multifrequency ESEEM (not shown). These peaks disappear in the fully ^{15}N-labeled enzyme complex (Figure 3b); again, the ^{15}N Larmor peak is present when the entire enzyme is labeled. No nuclear transitions are observed when the enzyme is labeled with ^{15}N at lysine residues only (Figure

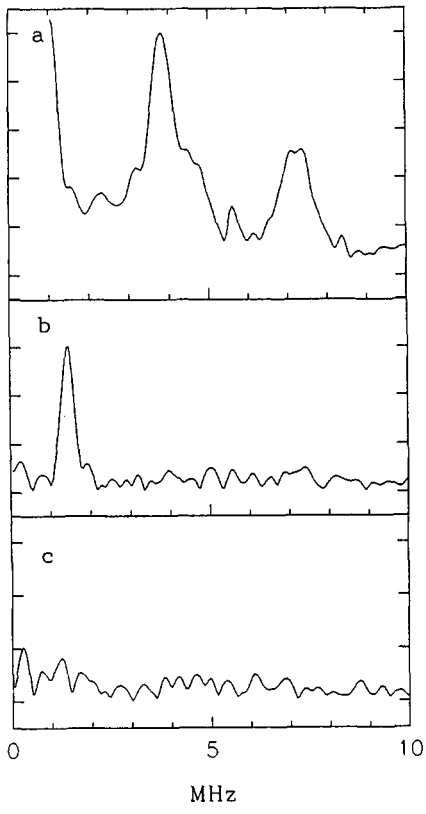

FIGURE 3: Three-pulse ESEEM spectra. (a) E·VO^{2+}·$MgPP_i$·K^+: ν_e = 9.1290 GHz, H_0 = 325.3 mT. (b) [^{15}N]E·VO^{2+}·$MgPP_i$·K^+: ν_e = 8.9450 GHz, H_0 = 317.6 mT. (c) [ϵ-^{15}N-lysine]E·VO^{2+}·$MgPP_i$·K^+: ν_e = 8.5469 GHz, H_0 = 303.5 mT. τ = 190 ns in each case.

3c); the spin echo amplitude is comparable to that obtained from the sample with a natural abundance of ^{14}N.

Complexes with S-Adenosylmethionine but without Polyphosphate Ligands: Enzyme·VO·AdoMet·M$^+$. In Figure 4a, ESEEM spectra of the E·VO^{2+}·AdoMet·Tl^+ complex at 8.19 and 9.86 GHz are shown. As before, the field dependence of the resonant frequencies demonstrates that they arise from ^{14}N. Simulations (dashed lines) yield an isotropic hyperfine coupling of 5.4 MHz, also in good agreement with calculated values (Table I). Figure 4b shows the corresponding spectrum of a sample with enzyme containing [ϵ-^{15}N]lysine, at 8.95 GHz. The intense peaks at 4.2 and 7.8 MHz correspond to a 5.6-MHz coupling. These peaks still do not disappear upon substitution of uniformly ^{15}N-labeled enzyme (Figure 4c). The peak at the ^{15}N Larmor frequency demonstrates the presence of a large number of weakly-coupled ^{15}N nuclei and indicates the success of the labeling process. The ^{14}N transitions observed here must arise from the product, AdoMet, as there is no other source of nitrogen in the complex. The line widths in the frequency domain ESEEM spectra of this complex are sufficiently narrow, and the frequencies observed are sufficiently different from those seen in the presence of PPP_i, as to assure that the lysine transitions are absent, not simply underlying the AdoMet ^{14}N transitions. When the complex is formed with AdoMet enriched in ^{15}N at the α-amino group, the ^{14}N modulations disappear, as shown in Figure 4d. This result confirms that the α-amino nitrogen gives rise to the transitions in Figure 4a–c.

In contrast to observations made in complexes containing polyphosphate groups, Tl^+ superhyperfine coupling has not been observed in CW EPR spectra in the absence of polyphosphate. However, as in the complexes with polyphosphates, the choice of monovalent cation (K^+ or Tl^+) does not affect the ESEEM spectra of the E·VO^{2+}·AdoMet·M$^+$

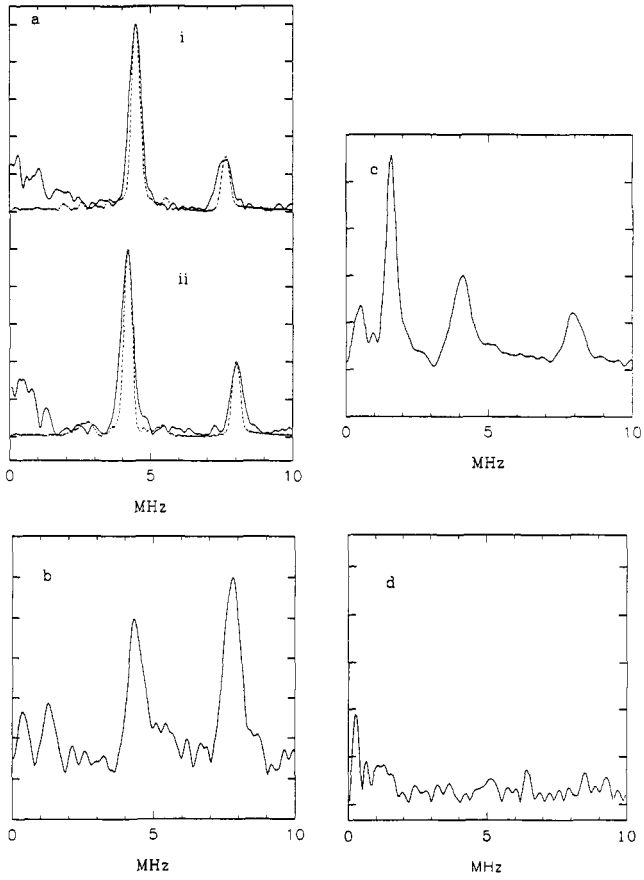

FIGURE 4: Three-pulse ESEEM spectra. (a) E·VO^{2+}·AdoMet·Tl^+: (i) ν_e = 8.1950 GHz, H_0 = 290.0 mT; (ii) ν_e = 9.8650 GHz, H_0 = 352.0 mT. Dashed lines are simulated spectra obtained using the parameters e^2Qq = 3.0 MHz, η = 0.3, $A_{xx} = A_{yy}$ = 5.2 MHz, A_{zz} = 5.5 MHz. (b) [ϵ-^{15}N-lysine]E·VO^{2+}·AdoMet·K^+: ν_e = 9.9920 GHz, H_0 = 354.7 mT. (c) [^{15}N]E·VO^{2+}·AdoMet·K^+: ν_e = 8.9453 GHz, H_0 = 313.0 mT. (d) E·VO^{2+}·[^{15}N-methionine]AdoMet·K^+: ν_e = 9.5668 GHz, H_0 = 339.5 mT. τ = 190 ns in each case.

complexes (Figure 5). Thus our data do not indicate the proximity of the monovalent cation to the VO^{2+} in this complex.

Enzyme·VO^{2+} and Other Complexes. Only very weak spin echoes are obtained from the E·VO^{2+} complex, and no reproducible modulation frequencies are observed, within the available signal-to-noise. This result is consistent with the observation of a poorly-defined, broad CW spectrum from this complex. Liquid solution spectra of E·VO^{2+} are indicative of much shorter rotational correlation times than that obtained from a Stokes law calculation for the entire protein (Markham, 1984). Complexes of the form E·VO^{2+}·methionine or E·VO^{2+}·ATP also exhibit both poorly-resolved CW EPR spectra and weak spin echo signals, while the E·VO^{2+}·ATP·methionine complexes described above generally have narrow CW absorption lines which yield well-defined spectral parameters, as well as intense electron spin echoes.

DISCUSSION

In each complex where a polyphosphate moiety is present (as ATP, PPP_i, or PP_i), the ^{14}N double-quantum transitions observed in samples with natural isotopic abundances vanish when the protein is prepared with [^{15}N]lysine, but the corresponding ^{15}N frequencies are not observed. It is well understood that ^{14}N, with its higher nuclear spin (I = 1) and nonzero nuclear quadrupole moment, produces a more intense ESEEM effect than the ^{15}N nucleus ($I = {}^1/_2$). Consequently,

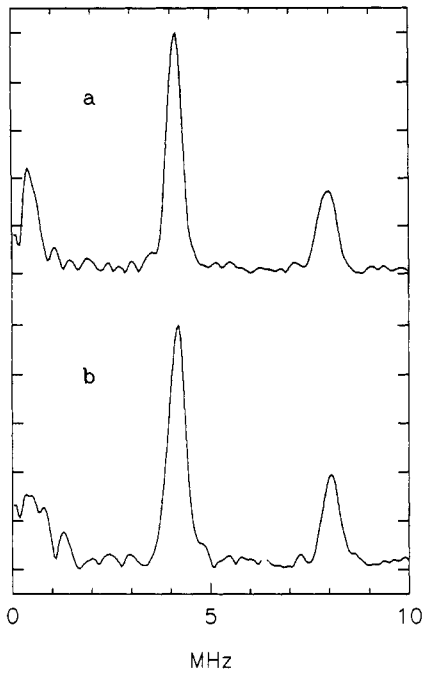

FIGURE 5: Three-pulse ESEEM spectra. (a) E·VO^{2+}·AdoMet·K^+: ν_e = 9.865 GHz, H_0 = 352.0 mT. (b) E·VO^{2+}·AdoMet·K^+: ν_e = 9.856 GHz, H_0 = 352.0 mT. τ = 190 ns in each case.

when the magnitude of the applied magnetic field is less than optimal for nitrogen modulation depth, as in this case, it is not surprising that ^{14}N modulations may be detectable when ^{15}N modulations are not. There are precedents for this effect, both in imidazole nitrogen of a nitric oxide·heme·imidazole complex (Peisach et al., 1979) and in nitrogen of methionine sulfoximine coordinated to Mn(II) in glutamine synthetase (Eads, et al., 1988). It is essential to note that the amplitudes of the electron spin echoes obtained from the [^{15}N]lysine samples were in no case significantly weaker than those from the natural-abundance samples, and so the absence of ^{14}N modulations cannot be explained by poor signal-to-noise ratios in the ^{15}N samples. Therefore, the data presented prove that the 4–5-MHz ^{14}N superhyperfine couplings arise from lysine.

The magnitudes of the observed ^{14}N couplings indicate that N_ϵ of lysine coordinates the VO^{2+} ion directly (Eaton & Eaton, 1990, and references therein). Since the single unpaired electron of the VO^{2+} ion resides largely in a nonbonding d_{xy} orbital, A_{iso} for an axially coordinated nitrogen would likely be much different from the 4–7-MHz couplings that have been observed from equatorial ^{14}N ligands thus far (Reijerse et al., 1989; Tipton et al., 1989), based upon symmetry considerations.[2] Therefore, lysine N_ϵ probably lies in or near the equatorial plane of VO^{2+}.

Only two peaks are seen in the lysine three-pulse ESEEM spectrum, suggesting the presence of either one lysine ligand or two or more equivalent ones. The ^{51}V hyperfine couplings observed (Markham, 1984) rule out the latter possibility. This conclusion is reached by use of the additivity relation described by Chasteen (1981). It is assumed that the size of the largest principal value of the ^{51}V hyperfine coupling tensor is governed only by the equatorial ligands to VO^{2+} and that the contribution of each ligand is independent of those of the other three. That is

$$A_{\parallel calc} = \sum_i n_i A_{\parallel,i}/4 \quad (4)$$

where i counts the *types* of equatorial ligands, n_i is the number of equatorial ligands of type i, and $A_{\parallel,i}$ is the unique principal value of the (axial) ^{51}V hyperfine coupling tensor that is obtained when all four equatorial ligands are of type i. These values are available in the literature for most commonly-occurring cases (Holyk, 1979; Chasteen, 1981). Coordination by two phosphate oxygens was demonstrated in the enzyme·VO complexes with ATP plus methionine and with AdoMet plus PPP_i (Markham, 1984); thus only two equatorial ligands are unaccounted for. If there are two phosphate oxygen ligands, for which $A_{\parallel,i} = 171 \times 10^{-4}$ cm^{-1} (Parker et al., 1970), and two lysine ϵ-amino nitrogens, for which $A_{\parallel,i} = 160.3 \times 10^{-4}$ cm^{-1}, then one obtains $A_{\parallel calc} = 166 \times 10^{-4}$ cm^{-1}. If the fourth equatorial ligand were oxygen from water ($A_{\parallel,i} = 182.6 \times 10^{-4}$ cm^{-1}) rather than a second lysine, then $A_{\parallel calc} = 171 \times 10^{-4}$ cm^{-1}. The experimental value of $A_\parallel = 175 \times 10^{-4}$ cm^{-1} (Markham, 1984) clearly favors the latter case. A carboxylate oxygen ($A_{\parallel,i} = 170.9 \times 10^{-4}$ cm^{-1}) as the fourth equatorial ligand yields $A_{\parallel calc} = 168 \times 10^{-4}$ cm^{-1} and is also preferred to a second lysine, but the discrepancy with the measured value would then be considerable. Thus water is the most probable fourth ligand. If it is assumed that lysine N_ϵ is an axial ligand, then it is unlikely that there is a second lysine, since it would be equatorial by default and thus inequivalent to the first lysine. Interestingly, one can match the observed $A_\parallel$ perfectly by assuming that the single lysine ligand is axial and that the third and fourth equatorial ligands are oxygens, one water and one carboxylate. In this case, $A_\parallel = A_{\parallel calc} = 175 \times 10^{-4}$ cm^{-1}; such an interpretation cannot be ruled out entirely, as in principle there could be coincidental similarity between equatorial and axial ^{14}N couplings. Nevertheless, for the reasons given above, the interpretation of equatorial coordination by lysine is favored, and we estimate that the most probable set of equatorial ligands is two phosphate oxygens, one oxygen from water, and one lysine nitrogen.

Line narrowing has been observed in E·VO^{2+}·ATP·methionine·K^+ complexes prepared in D_2O, perhaps due to an equatorial water ligand (Markham, 1984). But in view of the ESEEM results presented in this work, it is also probable that at least some the narrowing arises from exchange of the $-RNH_2$ protons of lysine. van Willigen (1980) has characterized water proton couplings to $VO(H_2O)_5^{2+}$ in frozen solution. ENDOR studies of AdoMet synthetase whose results can be compared with those from $VO(H_2O)_5^{2+}$ are underway.

The identity of the remaining (axial) ligand to VO in each of the various AdoMet synthetase/VO complexes under study is a more difficult problem because axial ligands are not known to exert a similar, systematic effect on the ^{51}V hyperfine tensor. The absence of additional ^{14}N components in the ESEEM spectra may indicate an oxygen ligand, but axial ligand couplings may simply be too weak to measure by ESEEM. It is also unclear whether the axial ligand is donated by solvent, by the protein, or by the substrate (or product).

Significant differences are observed in the magnitudes of the lysine ^{14}N superhyperfine couplings in substrate complexes (A_0 = 4.4 MHz, Figure 1) vs product complexes (A_0 = 4.8 MHz, Figure 2; also cf. Table I). The larger coupling is close to that attributed to lysine coordinating vanadyl in pyruvate kinase (Tipton et al., 1989). As the identity of the lysine ligand in both complexes in the present study is clear, these results demonstrate that subtle differences in environment

[2] A possible example of an axial (histidine) ^{14}N ligand coupling to VO^{2+} was observed by ENDOR in D-xylose isomerase; the reported coupling, obtained from a parallel feature, was 13 MHz (Bogumil et al., 1991). It is not clear what portion of this coupling is isotropic, as ^{14}N ENDOR transitions from perpendicular features could not be assigned.

can have substantial effects on couplings to the vanadyl cation from a given ligand. Note that at least three of the five ligands to VO^{2+}, two phosphate oxygens and one lysine nitrogen, are the same in the E·VO^{2+}·ATP·methionine·K^+ and E·VO^{2+}·AdoMet·PPP_i·K^+ complexes. In view of the fact that the **g** and ^{51}V hyperfine tensors are identical in these two complexes (Markham, 1984), it is possible that all five ligands are the same.

The 5.4-MHz coupling obtained from the enzyme·VO^{2+}·AdoMet·K^+ (or Tl^+) complex arises from the product, AdoMet, and specifically from the α-amino nitrogen of the methionine moiety. The coupling is significantly above the range of the lysine couplings observed in the presence of PPP_i or ATP plus methionine but is still below the range of those couplings assigned to histidine and imidazole nitrogens (6–7 MHz). Amino ^{14}N ligand couplings to VO^{2+} can therefore vary over at least a range of 1 MHz, like the histidine/imidazole ^{14}N couplings.

The identification of ligands by specific isotopic substitutions in the present study generally lends support to recent interpretations of ESEEM from similar systems in which isotopically labeled proteins were not available. The ^{14}N couplings observed in AdoMet synthetase all arise from amino nitrogen, and none has A_{iso} larger than 5.6 MHz. While the total number of VO–N couplings reported is not large, it is notable that to date there remains a separation of about 0.5 MHz between the largest primary amine and smallest imidazole/histidine ^{14}N A_{iso} values reported. Thus, for example, an observation of DQ frequencies which yield A_{iso} = 4.9 MHz can be taken as strongly suggesting an amino ^{14}N ligand, most probably lysine (in protein complexes), while a 6.2-MHz isotropic coupling would almost surely arise from a histidine ^{14}N ligand.

A model of the catalytic cycle of AdoMet synthetase must be compatible with the following observations: (1) In the presence of diphosphate coordination of VO^{2+}, lysine is also a ligand to the cation; thus lysine coordination occurs in the substrate complex, in the intermediate complex, and in the product complex, enzyme·VO^{2+}·AdoMet·PP_i·K^+. (2) In the (partial) product complex, enzyme·VO^{2+}·AdoMet·K^+, the lysine ligand is lost and the α-amino nitrogen of AdoMet coordinates the divalent cation. (3) In the complexes with polyphosphates, the fourth equatorial ligand to VO^{2+} is probably an oxygen, most likely either water or carboxylate. Previous kinetic and stereochemical studies have indicated that the basic chemistry of the reaction involves methionine sulfur's nucleophilic attack upon $C_{5'}$ of ATP (Parry & Minta, 1982; Markham et al., 1987). Thus, one reasonable hypothesis is that in the enzyme·VO^{2+}·ATP·methionine·K^+ complex, one of the two undetermined ligands to VO^{2+} is methionine carboxylate oxygen. It is known that the product PP_i arises from the α- and β-phosphates of ATP, while the γ-phosphate is the source of P_i. Therefore, we favor a model in which the α- and β-phosphates donate the phosphate oxygen ligands to VO^{2+}. In that case, $C_{5'}$ of ATP would be four bond lengths from VO^{2+}, and methionine sulfur would be six bond lengths away, rendering $C_{5'}$ easily accessible to sulfur. After the transfer of the adenosyl moiety to methionine, the immediate ligands to VO would be the same, as suggested by the observed spin Hamiltonian parameters. The stereochemistry of the site would likely be different, though, accounting for the change in the lysine ^{14}N superhyperfine coupling. After PPP_i is hydrolyzed and PP_i dissociates, yielding the E·VO^{2+}·AdoMet·K^+ complex, the lysine ligand would be lost and AdoMet methionine nitrogen would coordinate the cation. AdoMet could be either a monodentate or a bidentate ligand at this point, with oxygen coordinating equatorially, axially, or not at all.

The idea of coordination of VO^{2+} by methionine carboxylate oxygen in the E·VO^{2+}·ATP·methionine and E·VO^{2+}·AdoMet·PPP_i complexes has several virtues. First, as described above, it allows for positioning of methionine prior to the reaction in a manner that facilitates adenosyl transfer. Second, it helps to account for coordination of VO^{2+} by methionine nitrogen subsequent to dissociation of PP_i, as the methionine end of the (rather large) AdoMet product would already be in place; third, it is consistent with the absence of ^{13}C and ^{2}H modulations in samples with an isotopically substituted methionine methyl group. Other models may of course have the same desirable properties, and a test of this hypothesis awaits preparation of samples with ^{17}O and ^{13}C labels in the carboxylate of methionine or AdoMet, which is in progress.

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