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Structure of the Divalent Metal Ion Activator Binding Site of *S*-Adenosylmethionine Synthetase Studied by Vanadyl(IV) Electron Paramagnetic Resonance†

George D. Markham

ABSTRACT: The structure of the divalent metal ion binding site of *S*-adenosylmethionine synthetase from *Escherichia coli* has been studied by using the vanadyl(IV) ion (VO^{2+}) as probe. VO^{2+} binds at a single site per subunit in the presence or absence of substrates. Single turnover experiments measuring *S*-adenosylmethionine (AdoMet) formation from methionine and the ATP analogue 5'-adenylyl imidodiphosphate show that complexes containing VO^{2+} and either Mg^{2+} or Ca^{2+} as a second metal ion are catalytically active, while a complex containing VO^{2+} alone is inactive. Electron paramagnetic resonance spectra of the enzyme–VO^{2+} complex, as well as complexes also containing AdoMet or methionine, indicate the coordination of two water molecules and at least two protein ligands to the VO^{2+}. In complexes with polyphosphate substrates or products (e.g., enzyme–VO^{2+}–ATP–methionine, enzyme–VO^{2+}–PP_i–Mg^{2+}), EPR spectral changes reveal ligand substitutions on the VO^{2+}, and 8.5-G isotropic superhyperfine coupling to two ^{31}P nuclei can be resolved. ^{17}O superhyperfine coupling from [^{17}O]pyrophosphate indicates coordination of two oxygen atoms of PP_i to the VO^{2+} ion. Thus the polyphosphate compounds are bidentate ligands to the VO^{2+}, demonstrating that the VO^{2+} binds at the active site and suggesting a catalytic role for the protein-bound metal ion.

S-Adenosylmethionine is the primary alkylating agent in biological systems and occupies a central role in cellular metabolism (Cantoni, 1975; Tabor & Tabor, 1976). The biosynthesis of *S*-adenosylmethionine (AdoMet)[1] occurs in a unique enzymatic reaction catalyzed by *S*-adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase) (Mudd, 1973). In the biosynthetic reaction, a substitution at C5' of the ribose of ATP results in formation of the sulfonium compound AdoMet; the tripolyphosphate formed is then hydrolyzed to pyrophosphate and orthophosphate before products are released (Mudd, 1963; Markham et al., 1980). The tetrameric AdoMet synthetase from *Escherichia coli* requires two divalent metal ions (e.g., Mg^{2+}, Mn^{2+}, Ca^{2+}) as well as a single monovalent cation (e.g., K^+) per active site for catalytic activity (Markham et al., 1980; Markham, 1981). One of the divalent metal ion activators binds to the protein in the absence of substrates, while the second divalent metal ion binds in conjunction with the nucleotide substrate or the product PP_i. EPR studies using Mn^{2+}, which binds at both sites, showed that in complexes of enzyme with Mn^{2+}, AdoMet, and either the product PP_i or imidotriphosphate (an analogue of the tripolyphosphate intermediate) the two metal ions were coupled by spin exchange (Markham, 1981). The exchange coupling

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received July 20, 1983.* This work was supported by U.S. Public Health Service Grants GM-31186-01, CA-06927, and RR-05539 and also by an appropriation from the Commonwealth of Pennsylvania.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AMPPNP, 5'-adenylyl imidodiphosphate; PPNP, imidotriphosphate (O_3P–O–PO_2–NH–PO_3); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PP_i, pyrophosphate; PPP_i, tripolyphosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

0006-2960/84/0423-0470$01.50/0

demonstrated that the ions bind in close proximity to one another and suggested that they shared a common ligand. These studies, however, did not specify the groups to which the metal ions bind.

Knowledge of the structure of the divalent metal ion binding sites is required for understanding of the roles of these ions in catalysis. The characterization of these sites would be aided by the use of a spectroscopically active metal ion that bound preferentially at one of the two sites. The divalent oxovanadium(IV), or vanadyl(IV), ion has been used to study the zinc and iron binding sites of a number of proteins [reviewed by Chasteen (1981)]. VO^{2+} has one unpaired 3d electron and exhibits room temperature EPR spectra that are sensitive to the types of ligands coordinated to the metal, as well as to the motional freedom of the ion. In this paper, VO^{2+} is shown to bind exclusively to one of the metal binding sites of AdoMet synthetase, in the absence or presence of substrates. In the presence of a second metal ion (e.g., Mg^{2+}), an enzyme complex containing VO^{2+} is active in catalyzing AdoMet formation. EPR spectroscopic studies are reported that characterize the coordination environment of the metal ion and the changes in ligands that occur when substrates bind to the enzyme.

Materials and Methods

AdoMet synthetase was purified to electrophoretic homogeneity from the *E. coli* K12 strain DM25pKA8 by using the method described previously (Markham et al., 1980). The strain DM25pKA8 produces ca. 5-fold more AdoMet synthetase than the strain used previously (i.e., ca. 400-fold more AdoMet synthetase than a wild-type strain). The construction of the plasmid pKA8, which contains the structural gene for AdoMet synthetase in the plasmid pBR322, is described elsewhere (Tabor et al., 1983); strain DM25 is a *rpoB metJ* derivative of strain CL510 (Tabor et al., 1983). The purified AdoMet synthetase had a specific activity of 2.2 μmol min^{-1} mg^{-1} in the usual assay (Markham et al., 1980). Enzyme concentrations were calculated from the absorbance at 280 nm using the relation that a 1 mg/mL solution has an absorbance of 1.3 in a 1-cm light path and a subunit molecular weight of 43 000.

[*methyl*-^{14}C]Methionine (48.5 mCi/mmol) was obtained from New England Nuclear. AMPPNP, AdoMet, and methionine were purchased from Sigma. AdoMet was purified before use by chromatography on SP-Sephadex (Glaser & Peale, 1978). 99.8% D_2O was obtained from Wilmad. Reagent-grade $VOSO_4$ was purchased from Alfa Inorganics; concentrations of $VOSO_4$ solutions were determined from the absorbance at 750 nm using a molar extinction coefficient of 18 (Fitzgerald & Chasteen, 1974). $Co^{III}(NH_3)_3PPP_i$ was synthesized as described by Cornelius et al. (1977). [^{17}O]PP_i was synthesized from ultrapure PCl_5 (Alfa Inorganics) and 52.4% $H_2{}^{17}O$ (Prochem) as described by Hackney et al. (1980); the actual ^{17}O incorporation of 50 ± 10% was determined by integration of ^{31}P NMR spectra of a sample containing the [^{17}O]PP_i and an internal standard of a known concentration of thiophosphate (Tsai, 1979). NMR measurements were made at 24.3 MHz on a Varian NV14 spectrometer modified for Fourier-transform operation. All other compounds were obtained from commercial sources.

Enzyme was prepared for use by passage through a column of Sephadex G-25 that had been equilibrated with 50 mM Hepes/KOH, pH 8.0, containing 50 mM KCl and 1 mM dithiothreitol. When necessary, the enzyme was concentrated in a collodion bag apparatus. For experiments using $Co^{III}(NH_3)_3PPP_i$, the same buffer was used except that dithiothreitol was not included. For experiments in D_2O, the enzyme solution was dialyzed against three changes of 5 volumes of the same buffer in D_2O. The pH of the D_2O buffer was adjusted by using the relationship that the pH of a D_2O solution is equal to the pH meter reading plus 0.4 (Glasoe & Long, 1960). Kinetic experiments measured the incorporation of [*methyl*-^{14}C]methionine into AdoMet as described previously (Markham et al., 1980).

EPR spectra were recorded on a Varian E109 spectrometer interfaced to a Varian E935 data acquisition system. Temperature was regulated at 4 °C (or −70 °C) with a Varian temperature controller. Spectra were recorded at a modulation amplitude of 5 G and were generally the average of four scans (4096 data points each); in a few cases, a spectrum consisting of four scans of buffer was subtracted in order to correct a slightly sloping base line. g values were calculated relative to diphenylpicrylhydrazyl (g = 2.0036).

VO^{2+} Binding. In experiments with VO^{2+}, solutions were generally handled under N_2 atmosphere; transfers and additions were made with gas-tight syringes. However, parallel experiments showed no detectable difference when manipulations were conducted in air, possibly as a result of the presence of 1 mM dithiothreitol in the solution. VO^{2+}–enzyme complexes were formed by addition of microliter volumes of freshly prepared 10 mM $VOSO_4$ to enzyme solutions. For binding experiments, the reported intensities were measured as the peak to peak height of the most intense feature of the spectrum [usually the $-^1/_2$ perpendicular line, following the notation of Chasteen (1981)], although the intensity of all spectral features increased proportionally. No EPR signal was observed when $VOSO_4$ in the concentrations used herein (0.1–5 mM final concentration) was present in buffer that did not contain a chelating agent (e.g., AdoMet synthetase, ATP, or PP_i), consistent with the report that in the absence of chelating agents addition of $VOSO_4$ to Hepes buffer solutions results in formation of an EPR silent aggregate (Chasteen, 1981). Thus the height of the EPR signal reflects the amount of VO^{2+} bound to a chelating agent.

VO^{2+} EPR. The details of the EPR spectra of VO^{2+}–protein complexes have been reviewed recently (Chasteen, 1981), and only the general features are described here. VO^{2+} EPR spectra typically display axially symmetric g tensors and coincident, axially symmetric hyperfine coupling to the ^{51}V nucleus (100% natural abundance, $I = {}^7/_2$). The symmetry axis is along the V=O bond. Hyperfine coupling is largest for molecules oriented so that the symmetry axis is parallel to the applied magnetic field; this orientation has the smaller g value. The magnitudes of the g values ($g_\parallel$, $g_\perp$) and the ^{51}V hyperfine coupling constants ($A_\parallel$, $A_\perp$) are sensitive to the type of ligands coordinated in the plane perpedicular to the V=O bond ("equatorial ligands") but are relatively insensitive to the nature of the ligand trans to the V=O bond. The variation in g values and hyperfine coupling constants with ligands of known structure has been tabulated by Chasteen (1981). It is particularly advantageous that the data demonstrate an additive relationship, so that a complex containing, for example, two carboxylate groups and two water molecules as ligands will have g values and hyperfine coupling constants intermediate between the complexes containing either solely water or carboxylate ligands (Chasteen, 1981). Thus, in favorable cases it is possible to deduce the equatorial first coordination sphere ligands from the EPR spectral parameters.

Vanadyl(IV) EPR spectra are sensitive to motion on a time scale from 0.01 to 70 ns; for slower motion, the spectra are essentially rigid limit powder spectra (Bruno et al., 1977). The spectra of solutions of VO^{2+} bound to large proteins, in which

the VO^{2+} rotates on the time scale of the entire protein, are similar to the spectra of frozen samples, particularly at reduced temperatures and high protein concentrations.

Ligand Superhyperfine Coupling. The study of VO^{2+} complexes with AdoMet synthetase relies heavily upon the analysis of the interaction of the VO^{2+} electron spin with the nuclear spins of groups that are ligands to the VO^{2+}, i.e., superhyperfine coupling. This interaction can provide definitive evidence for direct coordination of ligands to the metal ion. Superhyperfine coupling results from two distinct types of magnetic interactions. There is a scalar, through bond, interaction due to delocalization of the electron spin into ligand orbitals with s character. This scalar coupling is isotropic. The second type of interaction is a through space dipolar interaction between the electron and nuclear spins. The dipolar interaction is anisotropic and, in a point-dipole approximation, falls off as $1/r^3$, where r is the distance between the metal and ligand atoms. The topic of ligand superhyperfine coupling has been reviewed by Goodman & Raynor (1970).

The EPR spectra of VO^{2+}–AdoMet synthetase complexes show superhyperfine coupling in three different fashions. The most straightforward is the resolved splitting of the ^{51}V hyperfine lines into triplets in the enzyme–VO^{2+}–PP_i–Mg^{2+} and related complexes. In this case, the superhyperfine coupling results from interaction with two spin $^1/_2$ nuclei, since the intensities of the triplet are in the ratio 1:2:1 (Goodman & Raynor, 1970). The magnitude of the coupling constant is directly measurable from the spacings of the triplet features. The second case is the interaction with protons of water molecules that results in unresolved coupling that is manifest as inhomogeneous line broadening. For unresolved superhyperfine splitting the observed line width (ΔH) is approximated by

$$\Delta H^2 \sim \Delta H_i^2 + \Delta H_{hf}^2$$

where ΔH_i is the intrinsic line width and ΔH_{hf} is the width of the superhyperfine coupling pattern (Norris et al., 1971). As a result of the smaller magnetic moment of the deuteron, replacement of H_2O by D_2O results in a decrease in the observed line width through a reduction in ΔH_{hf}. The magnitude of the reduction per equatorially coordinated water molecule has been calibrated by Albanese & Chasteen (1978). Calculations with perpendicular lines are preferred because the widths of perpendicular lines have less contribution from possible superhyperfine coupling to the ligand trans to the V=O bond. The third way in which superhyperfine coupling is manifest is in interaction with the oxygens of ^{17}O-enriched PP_i. Coupling to the ^{17}O (spin $^5/_2$) is unresolved and results in inhomogeneous broadening. However ^{17}O is not 100% abundant, and the spectra of complexes containing ^{17}O consist of a superposition of spectra of complexes containing ^{17}O and those with the residual ^{16}O. For a fractional ^{17}O enrichment x, the fraction of each spectrum is x and $1 - x$ for ^{17}O and ^{16}O, respectively, when a single oxygen interacts. When two equivalent oxygens are coupled, there are three species $^{17}O_2$, $^{17}O^{16}O$, and $^{16}O_2$, with relative proportions x^2, $2x(1 - x)$, and $(1 - x)^2$. The analysis of spectra to determine the number of interacting oxygens involves subtraction of the appropriate fraction of a spectrum of a sample containing only ^{16}O from the spectrum of the ^{17}O-enriched sample of the identical spin concentration [cf. Reed & Leyh (1980) and Bray & Gutteridge (1982)]. When the number of atoms interacting is underestimated, too large a fraction of the ^{16}O spectrum is subtracted, resulting in negative features in the difference spectrum. A correctly deconvoluted spectrum will show broadening of the lines; however, the spectral peaks and troughs remain in the same places in the ^{17}O and ^{16}O spectra.

Results

VO^{2+} Binding to AdoMet Synthetase. Addition of $VOSO_4$ to solutions of AdoMet synthetase resulted in the appearance of the EPR spectrum shown in Figure 1B. The spectrum is distinctly different from spectra of small-molecule complexes, which are typified by the spectrum of the vanadyl–pyrophosphate complex (Figure 1A). The spectrum of the enzyme–VO^{2+} complex was recorded at various concentrations of VO^{2+}, and the height of the most intense line in the spectrum is plotted vs. the VO^{2+} to enzyme subunits concentration ratio in the inset of Figure 1. The data fit a model for formation of a 1:1 VO^{2+}–enzyme complex with a dissociation constant of 0.1 mM.

To confirm that the VO^{2+} binds to one of the two previously described divalent metal ion activator binding sites, $VOSO_4$ (1.2 mM final concentration) was added to a solution that contained 1.1 mM enzyme subunits, 1.1 mM 5′-adenylyl imidodiphosphate, 3 mM methionine, and 2.2 mM $MgCl_2$. Under these conditions, before VO^{2+} addition both metal activator binding sites were occupied by Mg^{2+}, which dissociates very slowly ($t_{1/2} \sim 15$ min) from the enzyme–Mg^{2+}_2–AdoMet–PPNP complex that is formed (Markham et al., 1980). No VO^{2+} EPR signal was detected (<10% of the signal observed when $VOSO_4$ was added before $MgCl_2$), consistent with VO^{2+} and Mg^{2+} binding to the same sites.

To determine whether VO^{2+} could bind to both divalent metal ion binding sites, EPR spectra were recorded when various amounts of $VOSO_4$ were added to solutions containing enzyme plus either AdoMet and PP_i or AMPPNP and methionine, since AdoMet synthetase binds two divalent metal ions in the presence of these combinations of substrates (Markham, 1981). EPR titrations are shown in Figure 2 (the spectra of the corresponding complexes are shown in Figure 4 and clearly show that a complex with a macromolecule is formed). In each case, the titration data show that only one VO^{2+} binds per subunit. When Mg^{2+} was then added to the enzyme–VO^{2+}–AMPPNP–methionine complex, the amplitude of the spectrum increased, enabling titrations to determine the stoichiometry of Mg^{2+} interaction (Figure 2). Extrapolation of the linear portions of the titration curve indicates that binding of 1 equiv of Mg^{2+} promotes complete spectral change. Addition of Mg^{2+} to the enzyme–VO^{2+}–AdoMet–PP_i solution had no effect on the spectrum.

When PP_i was added to solutions of the enzyme–VO^{2+} complex, the spectrum of the resultant mixture (Figure 3A) was virtually identical with that of a VO^{2+}–PP_i complex (see Figure 1A), and only minor contributions from a macromolecular complex are evident. EPR spectra showed that addition of $MgCl_2$ to the solution resulted in VO^{2+} binding to the protein (Figure 3B), and titrations showed that 1 equiv of $MgCl_2$ is sufficient to promote binding of all the VO^{2+} to the enzyme (data not shown). The spectrum of the solution containing enzyme, VO^{2+}, PP_i, and Mg^{2+} (Figure 3B) is different from that of the binary enzyme–VO^{2+} complex, consistent with presence of enzyme-bound PP_i (see below). These results suggest that VO^{2+} has higher affinity for PP_i than for the enzyme and that a vanadyl–pyrophosphate complex does not bind to the protein. When the experiment was repeated with PPP_i instead of PP_i, a similar result was obtained, except that the spectrum of the enzyme, VO^{2+}, and PPP_i mixture showed no detectable signal from a macromolecular complex, consistent with the anticipated higher affinity of PPP_i than PP_i for VO^{2+}. Subsequent addition of 1 equiv of $MgCl_2$ to the PPP_i mixture resulted in all of the VO^{2+}

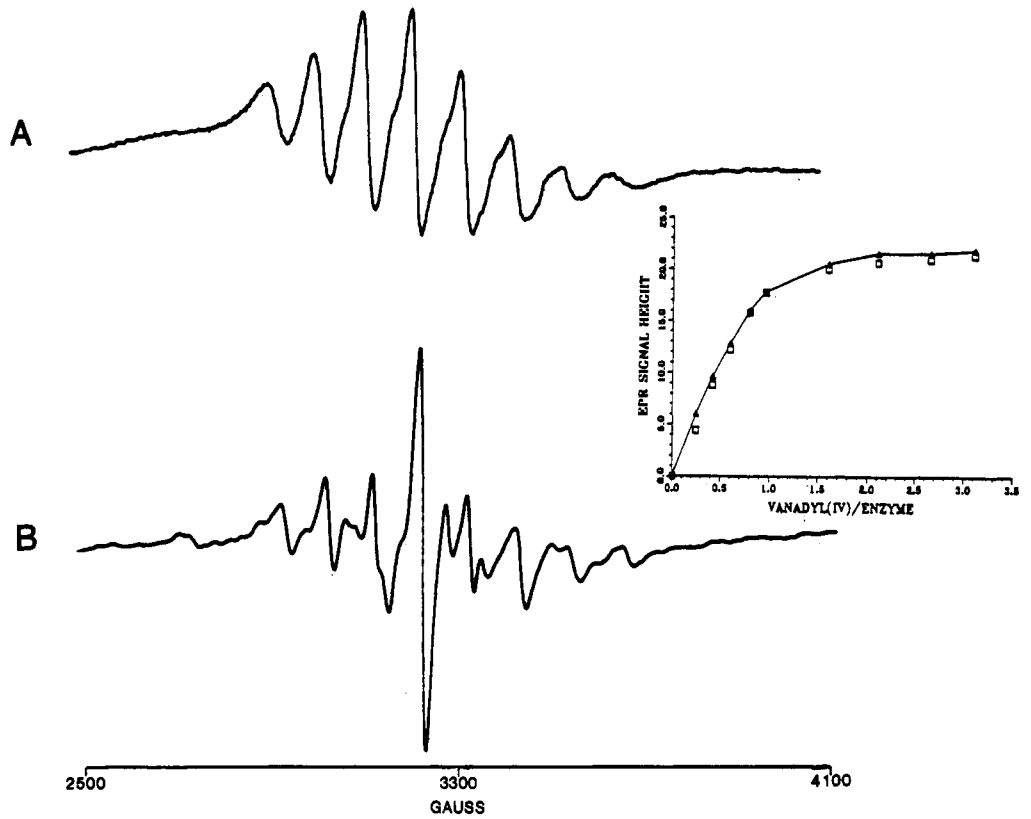

FIGURE 1: Interaction of VO^{2+} with AdoMet synthetase. Part A shows the spectrum of a solution containing 1.6 mM $VOSO_4$ and 1.6 mM PP_i. Part B shows the EPR spectrum of a solution containing 2 mM AdoMet synthetase subunits and 1.6 mM $VOSO_4$. The inset shows the amplitude of the most intense feature of the spectrum of part B plotted as a function of a $VOSO_4$ to enzyme subunits concentration ratio. (△) Experimental points; (□) points calculated from a model for 1:1 complex formation and a dissociation constant of 0.1 mM. Solutions contained 1.4 mM enzyme and various amounts of $VOSO_4$. Solutions also contained 50 mM Hepes/KOH, pH 8.0, 50 mM KCl, and 1 mM dithiothreitol; experiments were performed at 4 °C.

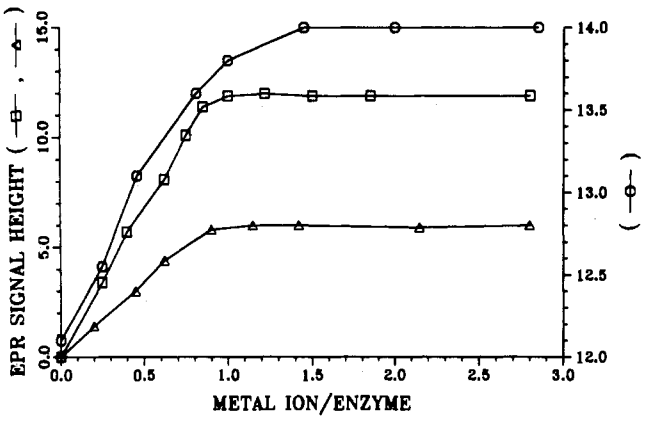

FIGURE 2: Metal binding to AdoMet synthetase in the presence of substrates and products. The data are plotted as the height of the most intense feature of the spectrum vs. the concentration ratio of metal ion [VO^{2+} for (△) and (□); Mg^{2+} for (○)] to enzyme subunits. (△) Height of the most intense feature of the spectrum obtained when various amounts of $VOSO_4$ were added to a solution that contained 1.3 mM enzyme, 1.4 mM AdoMet, and 1.3 mM PP_i plotted vs. the VO^{2+} to enzyme subunit concentration ratio. (□) Plot of the most intense feature of the EPR spectrum formed when various amounts of $VOSO_4$ were added to a solution containing 2 mM AdoMet synthetase subunits, 2 mM AMPPNP, and 4 mM methionine. (○) Increase in amplitude of the most intense line of the EPR spectrum of the enzyme–VO^{2+}–AMPPNP–methionine complex when various amounts of $MgCl_2$ were added to a solution containing 1.2 mM enzyme, 1.2 mM AMPPNP, 2.5 mM methionine, and 6 mM $VOSO_4$. Other conditions are the same as in Figure 1.

binding to the protein, giving a spectrum identical with that of the complex formed in the presence of PP_i and $MgCl_2$. The VO^{2+} also bound to the enzyme when 1 equiv of AdoMet instead of Mg^{2+} was added to the mixture of equimolar enzyme, VO^{2+}, and PP_i (or PPP_i), indicating increased affinity of the enzyme for VO^{2+} and polyphosphate in the presence of AdoMet. Identical spectra were obtained when either Mg^{2+}, AdoMet, or both were added to the solution of enzyme, VO^{2+}, and PP_i.

When the stable coordination complex $Co(NH_3)_3PPP_i$, an inhibitor of the enzyme[2] with a K_i of 3 μM, was added to the enzyme–VO^{2+} complex, EPR spectra showed that VO^{2+} remained bound to the enzyme. The EPR spectrum of the resultant mixture (Figure 3C) was the same as the spectrum of the mixture of enzyme, VO^{2+}, PP_i, and $MgCl_2$. This result is consistent with the interpretation that a VO^{2+}–polyphosphate complex does not bind to AdoMet synthetase but that a metal–polyphosphate complex does bind to the enzyme–VO^{2+} complex.

Activation of AdoMet Synthetase by VO^{2+}. When VO^{2+} was used as sole divalent metal ion in an assay for AdoMet formation from either ATP or AMPPNP, no AdoMet was detected (<5% of a single turnover in 5 min). This was not unexpected in light of the previous report that both divalent metal ions are required for catalytic activity (Markham, 1981). The question of whether a complex containing VO^2 and Mg^{2+} was catalytically active was approached by measuring a single enzyme turnover with AMPPNP as substrate. When AMPPNP and methionine react to form enzyme-bound AdoMet and PPNP, both products and divalent metal ions are bound in an enzymic complex that dissociates very slowly. Thus, an individual metal ion can partake in only one catalytic event, and the stoichiometry of metal required for activation can be determined.

[2] G. D. Markham, unpublished results.

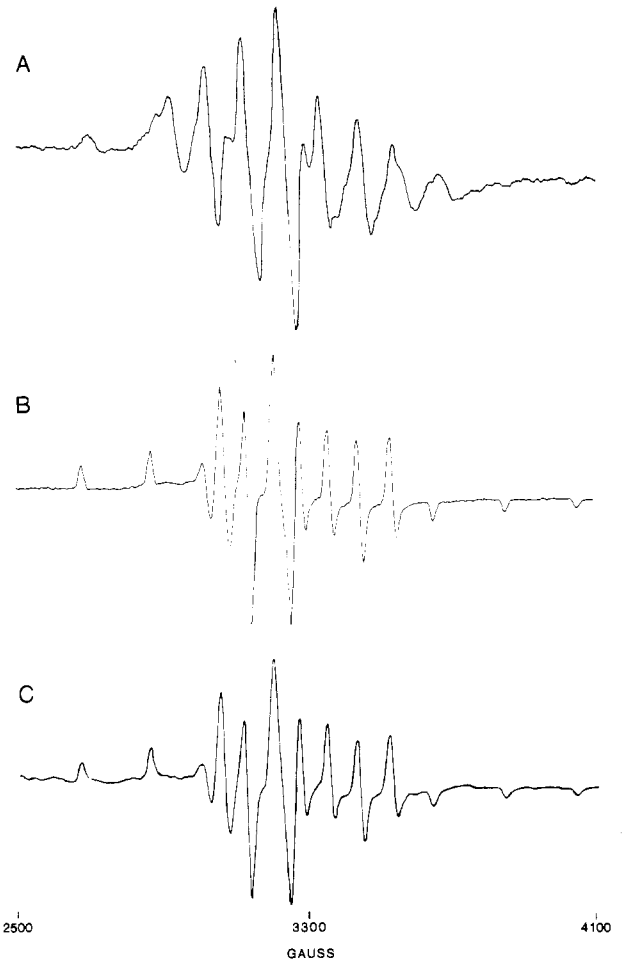

FIGURE 3: EPR spectra of VO^{2+}-AdoMet synthetase complexes. Solutions contained 1.3 mM enzyme and 1.2 mM $VOSO_4$. Spectrum A was obtained when pyrophosphate (1.3 mM final concentration) was added to a solution of the enzyme–VO^{2+} complex. Spectrum B was obtained after $MgCl_2$ (1.3 mM final concentration) was added to the above solution. Spectrum C was observed when the solution of enzyme and $VOSO_4$ also contained 1.3 mM $Co(NH_3)_3PPP_i$. Other conditions were as in the legend to Figure 1.

When $MgCl_2$ was titrated into a mixture containing 1.0 mM enzyme subunits, 1.0 mM AMPPNP, and 1.1 mM methionine, maximal AdoMet formation of 1 equiv/subunit occurred with addition of 2 equiv of Mg^{2+}/subunit, confirming the previous results obtained with Mn^{2+} (Markham, 1981). When Mg^{2+} was added to a mixture containing the same components plus 1.0 mM $VOSO_4$, AdoMet was also formed;[3] titration with $MgCl_2$ indicated that maximal AdoMet formation is obtained with 1 Mg^{2+} ion per subunit. Since EPR experiments under these conditions demonstrated that VO^{2+} remained enzyme bound in the presence of Mg^{2+}, the data show that a complex containing both VO^{2+} and Mg^{2+} is catalytically active. In the presence of VO^{2+} plus Mg^{2+}, the amount of AdoMet formed in the burst reaction was 60% of the amount formed in the presence of Mg^{2+} alone; the amount of AdoMet formed per enzyme subunit did not alter when the concentration of methionine was doubled or when the concentrations of all components were decreased 2-fold.

A more qualitivative demonstration that a complex containing both VO^{2+} and another metal ion is catalytically active was obtained with Ca^{2+} as the second metal ion. Ca^{2+} is a poor activator of AdoMet formation from AMPPNP, with the half-time for a single enzyme turnover being in excess of 2 h. When VO^{2+} and Ca^{2+} were added in amounts equimolar with enzyme subunits to a solution of enzyme, AMPPNP, and methionine (conditions as for the $MgCl_2$ experiments), there was a substantial increase in AdoMet synthesis compared to either VO^{2+} or Ca^{2+} alone. In contrast to either Mg^{2+} alone or a Mg^{2+}/VO^{2+} mixture, with a Ca^{2+}/VO^{2+} mixture AdoMet formation was linear with time. A single turnover was observed in 4 min with the VO^{2+}/Ca^{2+} mixture, whereas in this time no detectable AdoMet was formed (<5% of a single turnover) with either VO^{2+} or Ca^{2+} alone.

EPR Spectra of VO^{2+}–Enzyme Complexes. Figures 4–6 show EPR spectra of complexes of AdoMet synthetase, VO^{2+}, and substrates. Figure 4A presents the spectrum of a frozen sample of the enzyme–VO^{2+} complex. The spectrum is distinctly different from the solution spectrum of Figure 1B, and the changes upon freezing (a decrease in the apparent perpendicular hyperfine coupling constant and an increase in the apparent parallel hyperfine coupling constant) indicate that the solution spectrum at 4 °C reflects mobility of the bound VO^{2+}. Since it is probable that the motion is not isotropic, a rotational correlation time is not readily estimated; however, comparison of the solution spectrum with published spectra [cf. Chasteen (1981)] suggests a correlation time on the order of 20 ns, which is substantially less than the value of ~110 ns predicted by a Stokes law calculation for the entire protein.

When AdoMet was added to a solution of the enzyme–VO^{2+} complex, the spectrum shown in Figure 4B was observed. The spectrum has a clearly solid-state line shape, and line positions did not alter when the sample was frozen. The observation of the same spectrum for solution and frozen samples is consistent with VO^{2+} rotating with a correlation time comparable to that of the entire 160000 molecular weight protein. The peak positions in the enzyme–VO^{2+}–AdoMet complex are the same as those of the frozen spectrum of the enzyme–VO^{2+} complex, indicating that binding of AdoMet reduces the motional freedom of the VO^{2+} but has little effect on the ligands coordinated to the bound ion. The lines are substantially narrower in the presence of AdoMet, suggesting a more uniform environment of the VO^{2+}. g values and hyperfine coupling constants for complexes of VO^{2+} with AdoMet synthetase are collected in Table I. Addition of PP_i to the enzyme–VO^{2+}–AdoMet complex resulted in substantial increases in the ^{51}V hyperfine coupling constants and changes in g values (Table I); the spectrum is essentially identical with that of the enzyme–VO^{2+}–PP_i–Mg^{2+} complex and is not altered in either line position or intensity by the addition of $MgCl_2$.

When the nucleotide substrate ATP and Mg^{2+} were added to the VO^{2+} complex, the solution spectrum shown in Figure 4C was obtained; Figure 4D shows the spectrum of the frozen sample. The alterations in the spectra upon freezing indicate that addition of nucleotide plus Mg^{2+} to the enzyme–VO^{2+} complex does not reduce the motional freedom of the bound VO^{2+} to the extent that $MgPP_i$ binding does. The line positions in the frozen enzyme–VO^{2+}–ATP–Mg^{2+} complex are similar to those of the enzyme–VO^{2+} complex, showing that nucleotide addition had little effect on the environment of the bound VO^{2+}. Similar spectra were obtained when AMPPNP was used in place of ATP or when nucleotide was added without Mg^{2+} (not shown). Addition of ATP and methionine (4 mM), in the presence or absence of Mg^{2+}, resulted in spectra that were identical with the spectra of the enzyme–VO^{2+}–AdoMet–PP_i complex. Methionine (4 mM) alone had no affect

[3] The time course of AdoMet formation showed burst kinetics with the mixture of VO^{2+} and Mg^{2+}, as well as with Mg^{2+} alone. In both cases, the burst, which reflects the formation of enzyme-bound products (Markham et al., 1980), is complete within 100 s.

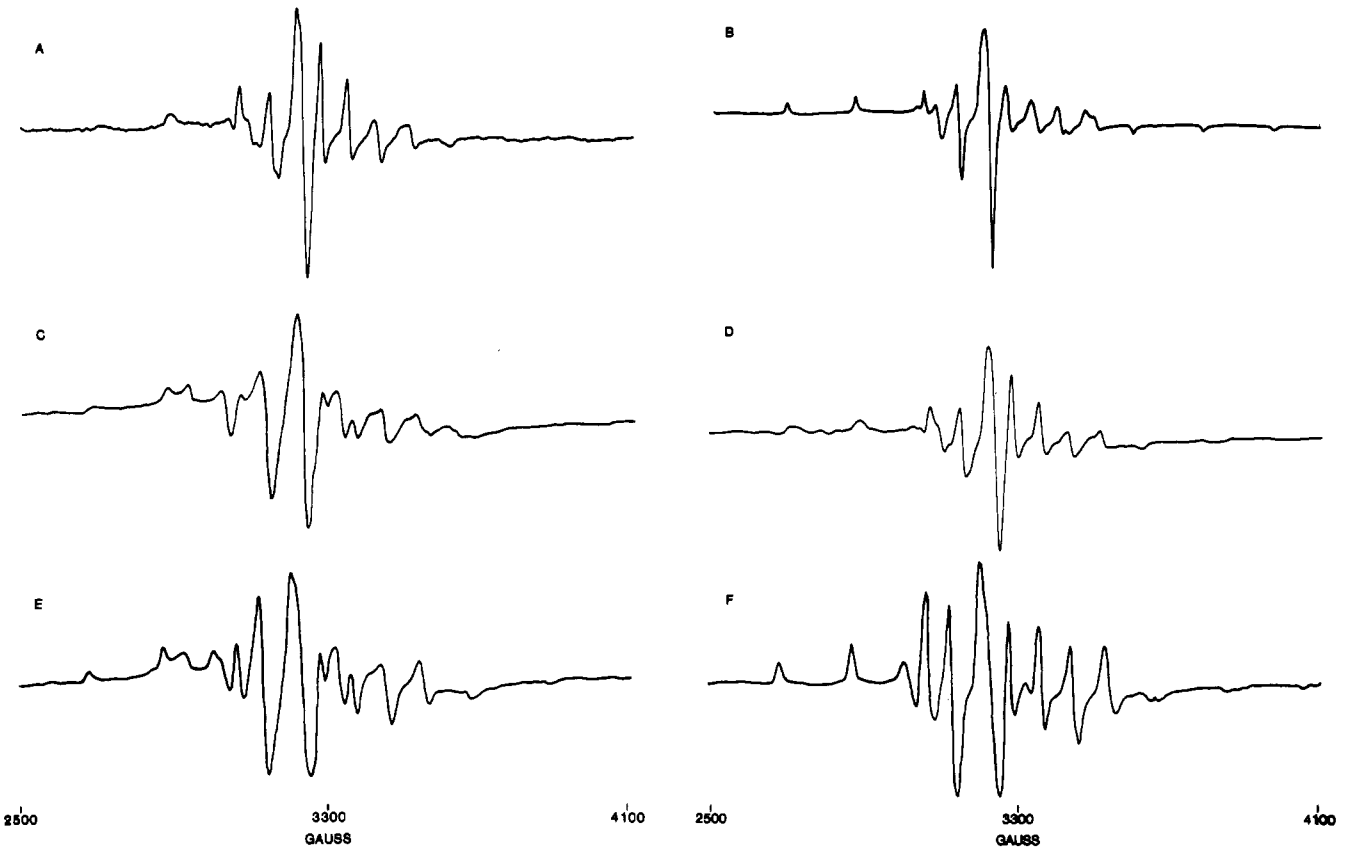

FIGURE 4: EPR spectra of VO^{2+}–AdoMet synthetase complex in the presence of substrates and products. Solutions contained 1.1 mM enzyme and 1.0 mM $VOSO_4$. When present, other components were 2.4 mM AdoMet, 1.1 mM ATP, 1.1 mM AMPPNP, 4 mM methionine, and 1.3 mM $MgCl_2$. Part A shows the spectrum of a frozen solution of the enzyme–VO^{2+} complex. Part B shows the spectrum obtained in solution when 2.4 mM AdoMet was also present. Parts C and D show spectra of solution and frozen samples, respectively, containing enzyme, VO^{2+}, ATP, and $MgCl_2$. Part E shows the spectrum of a solution containing methionine, enzyme, $VOSO_4$, and AMPPNP. Part F shows the spectrum obtained when $MgCl_2$ was added to the solution of part E.

Table I: Spin Hamiltonian Parameters for VO^{2+}–AdoMet Synthetase Complexes[a]

complex	$A_{\parallel}$ ($\times 10^3$ cm^{-1})	$g_{\parallel}$	$A_{\perp}$ ($\times 10^3$ cm^{-1})	$g_{\perp}$
enzyme-VO^{2+}	16.1	1.95	6.1	2.00
enzyme-VO^{2+} [b]	16.4	1.95	5.8	1.99
enzyme-VO^{2+}-AdoMet	16.4	1.95	5.80	2.00
enzyme-VO^{2+}-AdoMet-$PP_i \pm Mg^{2+}$	17.5	1.94	6.76	1.97
enzyme-VO^{2+}-PP_i-Mg^{2+}	17.5	1.94	6.75	1.97
enzyme-VO^{2+}-ATP-Mg^{2+} [b]	16.4	1.95	5.8	1.99
enzyme-VO^{2+}-ATP-methionine	17.5	1.94	6.76	1.97
enzyme-VO^{2+}-AMPPNP-methionine	17.5	1.94	6.95	1.97
enzyme-VO^{2+}-AdoMet-PPNP-Mg^{2+} [c]	17.6	1.94	6.85	1.97
enzyme-VO^{2+}-AdoMet-PPNP-Mg^{2+} [d]	17.9	1.92	6.95	1.98

[a] Values are for solution spectra unless otherwise noted. Spin Hamiltonian parameters were calculated from the experimental spectra using the equations of Bleaney (1951). Values for solution spectra are partially averaged due to molecular rotation and thus are lower limits on the principal values of $A_{\parallel}$ and $g_{\parallel}$ and upper limits on $A_{\perp}$ and $g_{\perp}$. [b] Parameters from frozen solutions. [c] More abundant form. [d] Less abundant form.

on the spectrum of the enzyme–VO^{2+} complex.

The spectrum of the enzyme–VO^{2+}–AMPPNP–methionine complex is shown in Figure 4E. The spectrum changed upon addition of Mg^{2+}, which results in formation of the enzyme–VO^{2+}–AdoMet–PPNP–Mg^{2+} complex (Figure 4F). The line positions of the enzyme–VO^{2+}–AMPPNP–methionine complex alter upon freezing, and in the frozen spectrum the line positions are the same as in the spectrum of the complex containing Mg^{2+}, for which freezing does not effect the line positions. The parallel features in the high-field region of the spectrum of the enzyme–VO^{2+}–AMPPNP–methionine complex, with or without the addition of Mg^{2+}, show the presence of two sets of lines with approximately a 2:1 intensity ratio, indicating the presence of two slightly different environments for the VO^{2+}. The ratio of the intensities of the two sets of lines was the same when the methionine concentration was increased 4-fold or when Mg^{2+} was added, and the ratio was not altered by freezing the sample. It is possible that the two environments seen by EPR are related to the finding that only 60% of the maximal amount of product is formed under these conditions. The hyperfine coupling constants and g values for these complexes are listed in Table I.

Spectra in D_2O. In VO^{2+} complexes containing coordinated water molecules, a substantial part of the line widths frequently originate in unresolved superhyperfine coupling to water protons. Reduction in line widths in D_2O solutions can be used to estimate the number of coordinated water molecules (Albanese & Chasteen, 1978). When the spectrum of the enzyme–VO^{2+}–AdoMet complex was obtained in D_2O, line widths decreased substantially while line positions remained

Table II: Line Widths of the Enzyme-VO^{2+}-AdoMet Complex in H_2O and D_2O[a]

hyperfine line	line width (G)		predicted line-width reduction per coordinated water (G)[b]	calcd no. of water ligands
	H_2O	D_2O		
$-^7/_2\parallel$	4.9	3.7		
$-^5/_2\parallel$	4.9	3.8		
$+^1/_2\perp$	17.8	15.0	1.6	1.8
$-^3/_2\perp$	14.4	11.3	1.6	1.9

[a] Data for solutions containing 1.1 mM enzyme, 1.1 mM $VOSO_4$, and 2.4 mM AdoMet in either H_2O or 99.8% D_2O. Line widths are reported as peak to peak line width for perpendicular lines and half-width at half-height for parallel lines. The estimated uncertainty in line widths is ±0.5 G. [b] From data of Albanese & Chasteen (1978). The values were calculated from frozen solution spectra of the $VO(H_2O)_5^{2+}$ complex. The widths of the parallel lines contain significant contributions for the axially coordinated water ligand, while widths of the perpendicular lines are affected primarily by the equatorial water ligands. Thus, only the model data for the perpendicular lines can be used to evaluate the number of equatorially coordinated water molecules.

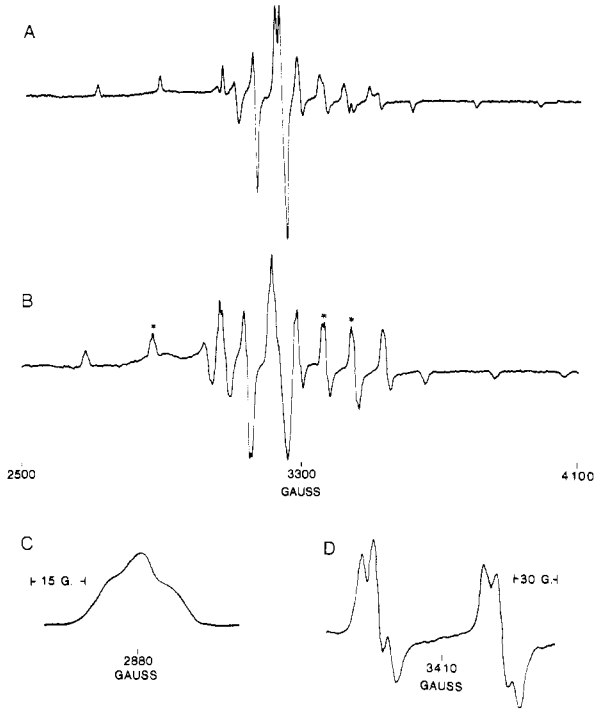

FIGURE 5: Spectra of AdoMet synthetase complexes in D_2O. Part A shows the spectrum of the enzyme-VO^{2+}-AdoMet complex, and part B shows the spectrum of the complex to which PP_i had been added. Solutions contained 1.1 mM enzyme, 1.2 mM $VOSO_4$, 2.4 mM AdoMet, and, when present, 1.1 mM PP_i. Parts C and D show expansions of the $-^5/_2$ parallel line and the $+^3/_2$ and $+^5/_2$ perpendicular lines of the spectrum of part B. The lines that are shown as expansions in parts C and D are marked with an (*) in part B. Other conditions are described in the legend to Figure 1.

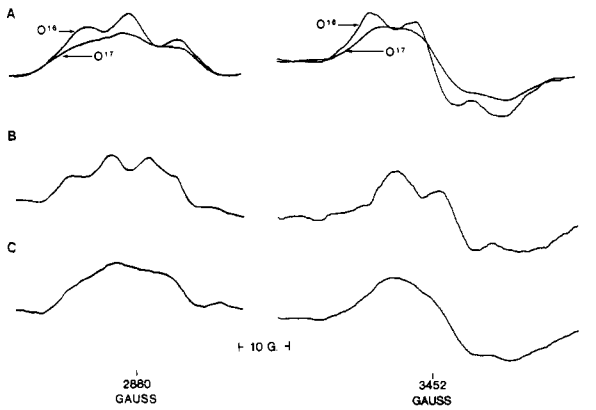

FIGURE 6: Effect of 50% ^{17}O-enriched PP_i on the spectrum of the enzyme-VO^{2+}-AdoMet-PP_i complex in D_2O. Solutions contained 0.7 mM enzyme, 0.7 mM $VOSO_4$, 0.8 mM AdoMet, and 0.7 mM PP_i in 99.8% D_2O. Part A shows experimental spectra for the $-^5/_2$ parallel line (left) and $+^5/_2$ perpendicular line (right). Parts B and C show spectra deconvoluted for models of one coordinated oxygen [^{17}O spectrum − 0.5(^{16}O spectrum)] and two oxygens coordinated [^{17}O spectrum − 0.25(^{16}O spectrum)], respectively.

unaltered (Figure 5A), indicating that the structure of the VO^{2+} binding site was not perturbed by substitution of D_2O for H_2O. The widths of several spectral lines in H_2O and D_2O are listed in Table II. By use of the literature values for the reduction per water ligand in width of the perpendicular lines (which are affected primarily by protons on water ligands in the equatorial plane of the VO^{2+} ion), a value of 1.8 (±0.6) water molecules in the first coordination sphere of VO^{2+} in the enzyme-VO^{2+}-AdoMet complex is calculated.

When the spectrum of the enzyme-VO^{2+}-AdoMet-PP_i complex was recorded in D_2O, the line width also decreased, and each ^{51}V hyperfine line was observed to be split into a triplet (Figure 5B–D). For both the parallel and perpendicular ^{51}V hyperfine lines, the triplet features are spaced at 8.5-G intervals, and intensities of the triplet features are ca. 1:2:1. Thus, the splitting results from superhyperfine coupling to two spin $^1/_2$ nuclei with equal coupling constants of 8.5 G. The same splittings were observed in D_2O solutions of enzyme-VO^{2+}-PP_i-Mg^{2+}, enzyme-VO^{2+}-AdoMet-PP_i-Mg^{2+}, and enzyme-VO^{2+}-ATP-methionine complexes. Since the coupling is isotropic, it arises primarily from unpaired electron density on the nucleus to which the electron is coupled and results from a through bond interaction.

Since the ^{31}P nuclei of PP_i were obvious possibilities for two spin $^1/_2$ nuclei that might interact with the VO^{2+} ion, spectra were obtained for samples containing PP_i enriched to ~50% at each oxygen with the spin $^5/_2$ ^{17}O isotope. Figure 6A shows spectra of the $-^5/_2$ parallel and $+^5/_2$ perpendicular lines for samples of identical VO^{2+} concentration containing [^{16}O]- or [^{17}O]PP_i. The EPR spectra of the sample containing [^{17}O]PP_i showed significant broadening of each hyperfine line and a reduction in amplitude compared to the concentration-matched sample containing [^{16}O]PP_i. In order to determine the number of interacting ^{17}O's, the spectra were deconvoluted as described under Materials and Methods for one or two coordinated oxygens (parts B and C of Figure 7, respectively). A comparison of Figure 6B,C with the experimental spectra in Figure 6A indicates that the electron spin interacts with 2 oxygen atoms.

Analysis of Hamiltonian Parameters in Terms of Ligands to VO^{2+}. One of the primary values of VO^{2+} as a probe is that the g values and ^{51}V hyperfine coupling constants reflect the types of ligands coordinated in the equatorial plane of the ion. The contributions for ligands of various types have been compiled by Chasteen (1981), who notes that the contribution of a given type of ligand is essentially independent of the other ligands present.

For the AdoMet synthetase complexes, two distinct sets of Hamiltonian parameters are seen (Table I). One class of

complexes, illustrated by the enzyme–VO^{2+}-PP_i–Mg^{2+} complex, has ^{51}V hyperfine coupling constants and g values that are comparable to the calculated values for ligands of two water molecules and two phosphate groups[4] ($A_{\parallel} = 17.7 \times 10^{-3}$ cm^{-1}, $g_{\parallel} = 1.92$). These complexes contain either bound PP_i (plus either AdoMet or Mg^{2+}) or ATP and methionine, and the observed ^{31}P superhyperfine coupling confirms coordination of two phosphate groups; the line-width reduction in D_2O is consistent with water coordination. The enzyme–VO^{2+}–AMPPNP–methionine complex and the corresponding complex resulting from Mg^{2+} addition also have ^{51}V hyperfine coupling constants and g values consistent with coordination by two water molecules and two phosphate groups.

The second group of parameters comes from the enzyme–VO^{2+}, enzyme–VO^{2+}–AdoMet, and enzyme–VO^{2+}–Mg^{2+}–ATP complexes. The ^{51}V hyperfine coupling constants are much smaller than for the complexes discussed above, indicating substantial difference in the types of ligands to the VO^{2+}. For the enzyme–VO^{2+}–AdoMet complex, line-width reductions in D_2O show that two of the ligands are water molecules. Possible combinations of protein ligands may be deduced from the observed hyperfine coupling constants and g values and the predicted contribution of two water molecules. Calculated values for the complexes in which the two protein ligands are phenolate groups ($A_{\parallel} = 16.9 \times 10^{-3}$ cm^{-1}, $g_{\parallel} = 1.94$), a carboxyl and a thiolate group ($A_{\parallel} = 16.6 \times 10^{-3}$ cm^{-1}, $g_{\parallel} = 1.95$), or an imidazole and an hydroxyl group ($A_{\parallel} = 16.7 \times 10^{-3}$ cm^{-1}, $g_{\parallel} = 1.95$) are in the range of the experimental values. No data that distinguish among these possibilities could be obtained by EPR.

Discussion

The binding of VO^{2+} at only one of the two divalent metal activator sites of *S*-adenosylmethionine synthetase has allowed EPR studies that help to define the structure of the metal ion binding site. Since a complex containing VO^{2+} and another divalent metal ion, such as Mg^{2+} or Ca^{2+}, is active in catalyzing AdoMet formation, VO^{2+} appears to be a valid probe of the role of the protein-bound divalent metal activator.

The ^{51}V hyperfine coupling constants and g values of the EPR spectra of the enzyme–VO^{2+}–AdoMet complex indicate that groups from the protein bind in the equatorial plane of the VO^{2+} ion. Since the ^{51}V hyperfine coupling constants are similar for the binary enzyme–VO^{2+} complex and the enzyme–VO^{2+}–AdoMet complex, it appears that the coordination scheme is the same for both complexes. Analysis of the spectra of the enzyme–VO^{2+}–AdoMet complex was facilitated by narrower lines, and this complex was studied more extensively. The magnitude of the line-width reduction when D_2O replaces H_2O suggests that two equatorial ligands are water molecules, implying the presence of two equatorial protein ligands. There is a possibility of a third protein ligand located trans to the V=O bond; however, EPR spectra are essentially insensitive to the nature of the ligand in this position. The protein ligands to the VO^{2+} are not definitively identified from the EPR data; however, comparison with model-compound data shows that relatively few combinations of ligands are compatible with both the observed ^{51}V hyperfine coupling constants and g values and the finding that two ligands are water; possible ligands are two phenolate groups of tyrosine, a cysteine sulfur and a carboxyl group, or a histidine side chain and a hydroxyl group from a serine or threonine. Other types of experiments will be required to distinguish among these possibilities and other possibilities for which model-compound data are not available. It should be remembered that the VO^{2+} is bound at a site that normally binds Mg^{2+} and that sulfur ligands to Mg^{2+} are uncommon. Therefore, the other two ligand combinations appear to be more probable.

The spectra of the enzyme–VO^{2+} complex are not altered when either methionine or nucleotide plus Mg^{2+} bind to the enzyme, suggesting that the ligands to the VO^{2+} do not change. However, when methionine and nucleotide (ATP or AMPPNP) are present together, there are substantial increases in the ^{51}V hyperfine coupling constants. In fact, the change in the ^{51}V coupling constants is so large that it must be due to at least two ligand substitutions. On the other hand, the small differences in the g values and ^{51}V hyperfine coupling constants between the complexes with AMPPNP and the complexes with the normal substrates and products (ATP, PP_i) are of the magnitude reported for variations among complexes with different carboxylate ligands to VO^{2+} (Chasteen, 1981) and may be due to alterations in coordination geometry rather than differences in the types of groups bound to the VO^{2+}. The equal, isotropic superhyperfine coupling to two ^{31}P nuclei in the enzyme–VO^{2+}–ATP–methionine, enzyme–VO^{2+}–PP_i–Mg^{2+}, enzyme–VO^{2+}–PP_i–AdoMet, and enzyme–VO^{2+}–PP_i–AdoMet–Mg^{2+} complexes establishes that two phosphate groups of ATP and PP_i are directly coordinated to the VO^{2+} ion. This finding, combined with the g values and ^{51}V hyperfine coupling constants, demonstrates that the ligands in the equatorial plane of the VO^{2+} are two phosphates of PP_i and two water molecules. The presence of two water ligands is supported by the extent of the line narrowing when D_2O replaces H_2O as solvent. The assignment of the resolved superhyperfine coupling to the ^{31}P of PP_i is confirmed by the line broadening obtained with [^{17}O]PP_i. Analysis of the magnitude of the line broadening due to interaction with the spin $^5/_2$ ^{17}O nucleus demonstrates that two ^{17}O atoms interact with the electron spin.

Superhyperfine coupling to ligand nuclei is rarely observed in vanadyl(IV) complexes. However, it is noteworthy that in vanadyl(IV) complexes with bidentate phosphorothioate ligands ^{31}P superhyperfine coupling constants as large as 50 G have been reported (Goodman & Raynor, 1970; Wasson, 1971; Miller & McLung, 1973); these complexes have V–S–P bonds, and it seems that coupling through V–O–P linkages has not previously been resolved. Since the observed ^{31}P superhyperfine coupling is isotropic, the magnitude of the interaction reflects the extent to which the unpaired electron is delocalized onto the phosphorus ligand. The 8.5-G coupling is only 0.2% of the value for an electron in a pure phosphorus 3s orbital (Wasson, 1971). Nevertheless, the magnitude of the ^{31}P superhyperfine coupling on the enzyme is substantially larger than the value for vanadyl–PP_i or vanadyl–ATP complexes in solution, for which phosphorus coupling has not been resolved (Parker et al., 1970; Sakurai et al., 1982), indicating that the bonding on the enzyme is different from that in solution. The ^{17}O superhyperfine coupling to [^{17}O]PP_i appears to be on the order of 1–2 G. This ^{17}O coupling constant is similar to the value of 1.1 G calculated for water in the $VO(H_2O)_5^{2+}$ complex from ^{17}O NMR measurements (Reuben & Fiat, 1969).

Since the superhyperfine coupling is the same for both phosphorous atoms of PP_i, the general environment of the two phosphoryl groups may be similar. The binding data show that PP_i alone does not bind strongly to the enzyme–VO^{2+} complex, while PP_i binding is observed in the presence of Mg^{2+},

[4] Hyperfine coupling constants and g values for phosphoryl ligands were calculated from spectra of frozen solutions of the $VO(PP_i)_2$ complex at pH 9.0 [so that only monomeric $VO(PP_i)_2$ is present (Parker et al., 1970)]. Values calculated were $A_{\parallel} = 17.1 \times 10^{-3}$ cm^{-1} and $g_{\parallel} = 1.91$.

and the substitution-inert coordination complex $Co(NH_3)_3PPP_i$ binds in the absence of Mg^{2+}. These data suggest that the PP_i serves as a doubly bidentate bridge between the VO^{2+} and the other metal ion. This bidentate coordination could provide a pathway for the spin exchange between the two Mn^{2+} that is observed when Mn^{2+} occupies both binding sites. The use of bidentate coordination of the α-phosphoryl group of the nucleotide to facilitate C–O bond cleavage during AdoMet formation has been previously suggested (Markham, 1981). This coordination scheme is consistent with the data reported here, particularly the finding that the coordination of the VO^{2+} is essentially the same in the inactive enzyme–VO^{2+}–nucleotide–methionine complex and the product complex formed after Mg^{2+} addition, which suggests that VO^{2+} binds to the phosphoryl groups before reaction to form AdoMet. Thus, the present results support the notion that the metal ion that binds to the protein in the absence of substrates plays a role in catalysis.

Registry No. AMPPNP, 25612-73-1; PP_i, 14000-31-8; ATP, 56-65-5; VO^{2+}, 20644-97-7; Mg, 7439-95-4; *S*-adenosylmethionine synthetase, 9012-52-6.

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