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Kinetics and Electron Paramagnetic Resonance Spectra of Vanadyl(IV) Carboxypeptidase A†

Robert J. DeKoch, David J. West, James C. Cannon, and N. Dennis Chasteen*

ABSTRACT: This study was undertaken to further investigate the use of the vanadyl ion, VO^{2+}, as a spectroscopic probe of active sites in metalloenzymes. The vanadyl derivative of zinc bovine carboxypeptidase A was prepared and found to hydrolyze the ester hippuryl-L-β-phenyllactic acid and the peptide benzoylglycyl-L-phenylalanine. One vanadyl ion per enzyme molecule was sufficient for full activity. Electron paramagnetic resonance (epr) spectra of polycrystalline samples were examined and three types of binding sites, A, B, and C, were found. The A site corresponds to the active site normally occupied by zinc and does not appear unusual in its geometry about the VO^{2+} group. Two water molecules and two imidazole groups, His-69 and His-196, appear to bind in equatorial positions while the axial position opposite the vanadyl oxygen may be occupied by the carboxylate ion of Glu-72. Epr evidence suggests that the VO^{2+} ion has the same active site coordination geometry in polycrystalline, room temperature, and frozen solution samples. The B sites arise from extraneous binding at protein carboxyl groups. The identity of the C sites is unknown. Binding at B and C sites was observed only with crystalline samples. A binding site (possibly C) in addition to the active site (A) was also observed in solution. EDTA was found to bind to carboxypeptidase A crystals. Addition of *p*-mercuribenzoate or Hg^{2+} to solutions of the vanadyl enzyme results in displacement of the VO^{2+} group from the active site.

We have been investigating the use of the vanadyl ion, VO^{2+}, as a spectroscopic probe of metal binding sites in proteins. In addition to its infrared and visible spectroscopic properties, the vanadyl ion exhibits sharp room temperature and liquid nitrogen electron paramagnetic resonance (epr) spectra which are sensitive to the ligand environment about the VO^{2+} group (Chasteen *et al.*, 1973; Boucher *et al.*, 1969; Kuska and Rogers, 1968; Goodman and Raynor, 1970). Recently, we completed a spectroscopic and chemical study of the isomorphous substitution of VO^{2+} for Zn^{2+} in insulin crystals and were able to show that the binding of the vanadyl ion "paralleled" that of the zinc (Chasteen *et al.*, 1973). Encouraged by these results, we have been extending this technique to other protein systems. Of interest is the applicability of this approach to protein solutions, particularly solutions of metalloenzymes in which the metal is vital to the function of the enzyme. This paper reports results on polycrystalline, room temperature solution, and frozen solution samples of the esterase and peptidase active vanadyl analog of zinc carboxypeptidase A (CPA).[1]

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[1] Abbreviations used are: CPA, carboxypeptidase A; OP, *o*-phenanthroline; HPLA, hippuryl-L-β-phenyllactic acid; BGP, benzoylglycyl-L-phenylalanine; PMB, *p*-mercuribenzoate.

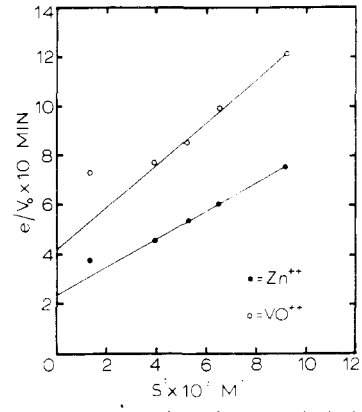

FIGURE 1: Lineweaver-Burk plot of the hydrolysis of HPLA by Zn^{2+}-CPA (●) and VO^{2+}-CPA (○). Reciprocal of moles of substrate hydrolyzed per minute per mole of enzyme *vs.* the reciprocal of the substrate molar concentration. See text for conditions of assay.

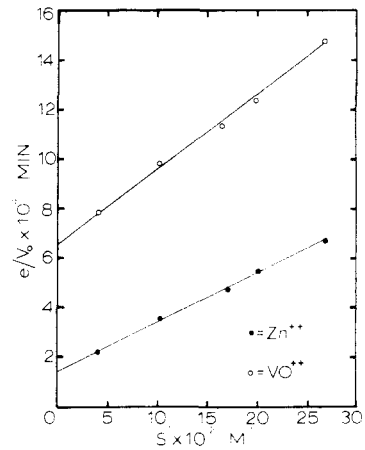

FIGURE 2: Lineweaver-Burk plot of the hydrolysis of BGP by Zn^{2+}-CPA (●) and VO^{2+}-CPA (○). Reciprocal of moles of substrate hydroyzed per minute per mole of enzyme *vs.* the reciprocal of the substrate molar concentration. See text for conditions of assay.

Carboxypeptidase A was chosen as a model system to further test the vanadyl labeling method since a great deal is known about various metal derivatives (Coleman and Vallee, 1960, 1961; Vallee *et al.*, 1960a; Davies *et al.*, 1968; Latt and Vallee, 1971; Rosenberg *et al.*, 1973) and the crystal structure is known from X-ray data (Lipscomb, 1970). Native carboxypeptidase has a molecular weight of 35,000 and contains one tightly bound zinc ion which is essential for activity. Several metals can be substituted for zinc in the protein structure and also impart activity to the enzyme (Vallee *et al.*, 1960a; Davies *et al.*, 1968).

Experimental Section

All glassware was cleansed of metals by soaking in 1:1 concentrated H_2SO_4–HNO_3 followed by rinsing in doubly distilled deionized water. Polyethylene or polypropylene containers were used where possible. Buffer and substrate solutions were rendered metal free just prior to use by phase extraction with 0.001% dithiophenol carbazone in carbon tetrachloride. All solutions were purged with prepurified nitrogen gas before addition of the vanadyl ion in order to minimize oxidation. Solution transfers were done with syringes and serum-stoppered containers. Stock solutions of $VOSO_4$ (Alfa Inorganics) were standardized spectrophotometrically by using the decadic molar extinction coefficient of 17.6 M^{-1} cm^{-1} at 750 nm for solutions with concentrations less than 0.65 M (Chasteen *et al.*, 1973). 1.0 M $VOSO_4$ stock solutions contained nominally 0.2 ppm zinc as determined by atomic absorption spectroscopy.

Bovine carboxypeptidase A (EC 3.4.2.1), prepared chromatographically by the method of Cox *et al.* (1964), was purchased from Sigma (Cat. CO261) and used without further purification. The apoenzyme was prepared by dialysis against *o*-phenanthroline (OP) (Vallee *et al.*, 1960a). The dialysis tubing was prepared by steam heating in distilled-deionized water for 2 hr with two changes of water followed by overnight soaking in 0.1 M Tris-HCl–1.0 M NaCl–1 × 10^{-3} M OP, at pH 7.0. The enzyme was dialyzed at 4° against the above solution for 2–5 days with changes of dialysate at 24-hr intervals. A 100:1 dialysate to protein solution containing ~10^{-3} M protein was employed and the zinc content of the dialysate was monitored at each change. When no zinc could be detected in the OP dialysate, the OP was removed from the bag by dialysis against 1.0 M NaCl–0.1 M Tris-HCl (pH 7.0) for 36 hr with three changes of buffer at 12-hr intervals.

The apoenzyme had a residual activity 3% of that of the native enzyme. The native enzyme was dialyzed against 0.5 M Tris-HCl (pH 7.0), 1.0 M NaCl, and 1 × 10^{-4} M $ZnCl_2$ for 24 hr to remove excess toluene preservative.

The enzyme was reconstituted by dialysis at 4° under nitrogen against 1 × 10^{-4} M VO^{2+} or Zn^{2+}, 0.5 M Tris-HCl (pH 7.5), and 1.0 M NaCl, and with or without 1 × 10^{-4} M $Na_2S_2O_4$. Alternately, 1 equiv of metal ion was added to the apoenzyme just prior to the assay. The stock vanadyl enzyme was kept on ice during the 35–45 min required for the assays. Enzyme reconstituted by either method gave identical kinetic results. The vanadyl enzyme contained 0.027 g-atom of zinc/mol of enzyme.

Some samples for epr measurements were prepared by soaking ~20 mg of Zn-CPA crystals in 1 ml of citrate (10^{-2} M, pH 6.0), *o*-phenanthroline (10^{-2} M, pH 7.0) or Na-EDTA (5 × 10^{-3} M pH 7.0) for 48 hr with one change of chelate solution at 24 hr. The apoenzyme crystals, containing less than 5% of the original zinc, were washed six times with 1-ml portions of distilled-deionized water with 0.5 hr soaking intervals between washings to remove the chelating agent. The citrate and *o*-phenanthroline but not all the EDTA were removed by this procedure (see Results and Discussion). The apoenzyme was dissolved and reconstituted with metal as described above.

The ester substrate hippuryl-L-β-phenyllactic acid, HPLA (Cyclo Chemical), was employed in the concentration range (1.0–7.5) × 10^{-4} M with 1 × 10^{-9} M enzyme in each 3-ml assay volume of 1.0 M NaCl–0.05 M Tris-HCl (pH 7.5). The hydrolysis of the substrate in 1-cm pathlength quartz cells (serum stoppered, nitrogen purged) was followed spectrophotometrically at 254 nm with a Cary 14 spectrophotometer equipped with a cell compartment thermostated at 25°. The substrate solution was frequently checked for contaminating metal ions by assaying the apoenzyme. Initial slopes of absorbance *vs.* time were converted to enzyme turnover numbers by using the change in extinction, $\Delta\epsilon$ = 592 M^{-1} cm^{-1}, due to substrate hydrolyses (McClure *et al.*, 1964) and the value of the enzyme concentration.

Hippuryl-L-phenylalanine (Sigma), otherwise known as benzoylglycyl-L-phenylalanine, or BGP, was used as the peptide substrate. The assay was performed as for HPLA except that a substrate concentration of (3.75–25.0) × 10^{-4} M and an enzyme concentration of 3.7 × 10^{-7} M were used;

TABLE I: Kinetic Parameters of Vanadyl and Zinc Carboxypeptidases.[a]

Enzyme	Esterase (HPLA) K_M(M) $\times 10^4$	V_{max} (min^{-1}) $\times 10^{-4}$
Native Zn^{2+}-CPA	2.5	4.3
Reconstituted Zn^{2+}-CPA	2.3	4.1
Reconstituted VO^{2+}-CPA	1.9	2.3
Enzyme	**Peptidase (BGP) K_M(M) $\times 10^3$**	**V_{max} (min^{-1}) $\times 10^{-3}$**
Native Zn^{2+}-CPA	1.2	6.4
Reconstituted Zn^{2+}-CPA	1.2	6.4
Reconstituted VO^{2+}-CPA	0.45 ± 0.5	1.5

[a] Assay conditions: 25°, 1 M NaCl–0.05 M Tris-HCl (pH 7.5), HPLA concentration in the range (1.0–7.5) $\times 10^{-4}$ M and BGP in the range (3.75–25.0) $\times 10^{-4}$ M. Errors are nominally ±0.1 except where noted differently.

$\Delta\epsilon = 280\ M^{-1}\ cm^{-1}$ at 254 nm (Davies *et al.*, 1968). Protein concentrations were determined spectrophotometrically at 278 nm where $\epsilon = 6.42 \times 10^4\ M^{-1}\ cm^{-1}$ (Simpson *et al.*, 1963).

Polycrystalline vanadyl carboxypeptidase samples for epr analysis were prepared by soaking native carboxypeptidase (12–20 mg) in 1.0 ml of 0.15 M $VOSO_4$ (ph 3.0 adjusted with NaOH) or 1 ml of 0.025 M $VOSO_4$–0.1 M sodium citrate (pH 3.0–7.0) for 48 hr at 4° under nitrogen. The soaking solution was carefully layered over the crystals to avoid dissolving the protein. The product was washed vigorously with four 1-ml portions of distilled-deionized oxygen-free water. A small amount of protein dissolved in the washing procedure. Samples were dried under nitrogen. Less than 0.05 g-atom of zinc/mol of enzyme remained as determined by atomic absorption spectroscopy. Some polycrystalline samples were prepared by soaking native CPA for 24 hr in 0.01 M citrate (pH 6.0), and 0.004, 0.002, or 0.001 M $VOSO_4$. These samples contained nominally 0.1 g-atom of zinc and up to 0.45 g-atom of VO^{2+}.

The vanadyl content of the protein samples was determined by atomic absorption spectroscopy (Galbraith Laboratories) or alternately by precipitating the protein with concentrated HCl and measuring the epr signal height of the released VO^{2+} (Fitzgerald and Chasteen, 1974a).

Epr spectra were measured on Varian E-4 and E-9 spectrometers. Electron g values were determined relative to 1,1-diphenyl-2-picrylhydrazyl radical, $g = 2.0036$, or Varian strong pitch, $g = 2.0028$. The accuracy of this method was checked by running a number of compounds of known g value and hyperfine splitting.

Results and Discussion

Kinetics. Lineweaver–Burk plots for esterase and peptidase activities are shown in Figures 1 and 2. Each point is an average of determination for three different enzyme preparations. The apparent kinetic parameters, V_{max} and K_M, for native and reconstituted zinc and vanadyl enzymes are summarized in Table I.

In Figure 1 the points which deviate from the straight line at low reciprocal substrate concentrations, S^{-1}, are due to enzyme inhibition by the ester substrate, HPLA. This phenomenon has been observed for all enzymatically active metal derivatives of CPA (Davies *et al.*, 1968). The substrate concentration range employed here corresponds to the intermediate range of Davies *et al.* (1968).

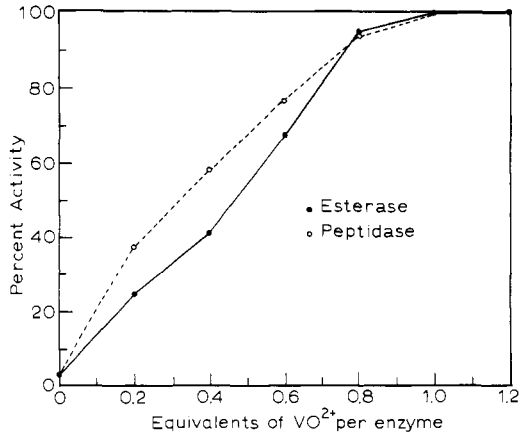

FIGURE 3: Per cent activity of the vanadyl enzyme restored as a function of VO^{2+} added to the apoenzyme. For esterase assays the enzyme and HPLA concentrations were 1.21×10^{-8} and 7.20×10^{-4} M. For peptidase assays the enzyme and BGP concentrations were 1.16×10^{-7} and 3.62×10^{-3} M. See text for other conditions.

Vanadyl CPA has esterase and peptidase V_{max} values which are 53 and 23%, respectively, of the value for the native zinc enzyme. This compares, for example, with the Co^{2+}-CPA which is 132 and 133% as active as the native enzyme (Davies *et al.*, 1968).

There is considerable variation in the literature values of V_{max} and K_M for the hydrolysis of BGP and HPLA by Zn^{2+}-CPA (see Table II of Davies *et al.*, 1968). Our values for BGP fall within the range of those reported; however, the value of $K_M = 2.5 \times 10^{-4}$ M for HPLA is considerably higher than the highest literature value of 8.8×10^{-5} M (Bender *et al.*, 1965). Our V_{max} value is also larger: 43,000 min^{-1} *vs.* 34,700 min^{-1}. The reason for this is not clear; we note that Bender *et al.* performed their assays at a NaCl concentration of 0.5 M compared to 1.0 M in our work.

The relative activity as a function of equivalents of VO^{2+} added to the apoenzyme is shown in Figure 3. Full peptidase and esterase activity is obtained at 1 equiv of VO^{2+}/mol of enzyme. Addition of a 50-fold excess of VO^{2+} gives no further increase in activity. Evidently the VO^{2+} ion binds tightly to the enzyme. This is in contrast to several other metal derivatives, most notably Mn^{2+}, which require an excess of metal to ensure complete metal binding and maximum activity (Davies *et al.*, 1968).

To be sure that the vanadyl ion did not oxidize at the concentration employed in the assays ($\sim 10^{-7}$–10^{-9} M enzyme), assays were run with and without 1×10^{-4} M of the reducing agent dithionite.[2] No differences in results were obtained for either the Zn^{2+} or the VO^{2+} derivatives in the presence or absence of dithionite. Thus it appears that the activity is imparted to the enzyme by the species VO^{2+}.

The activity (V_{max}) toward HPLA for various active divalent metal derivatives follows the order $Cd^{2+} > Mn^{2+} > Co^{2+} > Zn^{2+} > Ni^{2+} > VO^{2+} > Hg^{2+}$. Likewise, the relative activity toward BGP is $Co^{2+} > Zn^{2+} > VO^{2+} > Mn^{2+}$.

[2] We have shown that dithionite, $Na_2S_2O_4$, reduces $NaVO_3$ to VO^{2+} under the conditions of the assay.

TABLE II: Epr Parameters of Vanadyl-Labeled Proteins.

Protein[a]	Probable Ligands	$g_{\|}$ (±0.001)	$g_{\perp}$ (±0.002)	g_0[b] (±0.002)	$A_{\|}$[c] (±0.7)	$A_{\perp}$[c] (±1.1)	A_0[c,d] (±1.1)
Carboxypeptidase[e] (A site) polycryst.	2 histidines[f]– 1 glutamate	1.944	1.977	1.966	166.6	62.3	97.1
Carboxypeptidase A[e] Frozen soln (77°K)	2 histidines– 1 glutamate	1.944	1.978	1.967	165.9	61.1	96.0
Carboxypeptidase A[e,g] room temp soln	2 histidines– 1 glutamate	"1.945"	"1.980"	"1.968"	"162.6"	"63.8"	"96.7"
Carbonic anhydrase[h] (A site) polycryst.	3 histidines[i]	1.943	1.977	1.965	167.7	62.1	97.3
Insulin[j] (A sites) polycryst.	3 histidines[k]	1.943	1.977	1.965	167.1	61.7	96.8
Carboxypeptidase A[e] (B sites) polycryst.	1 carboxyl[e] group	1.936	1.978	1.965	175.8	67.3	103.5
Insulin[j] (B sites) polycryst.	1 glutamate[i]	1.938	1.978	1.965	177.0	67.5	104.0

[a] All bovine proteins. [b] Calculated from $g_0 = (g_{\|} + 2g_{\perp})/3$. [c] Units of 10^{-4} cm^{-1}. [d] Calculated from $A_0 = (A_{\|} + 2A_{\perp})/3$. [e] This work. [f] Lipscomb, 1970. [g] The epr parameters for the room temperature solution, 0.2 M Tris-Cl (pH 7.5), are placed in quotation marks since they represent *apparent* parameters obtained from a partially rotationally averaged spectrum. [h] Fitzgerald and Chasteen, 1974b. [i] X-Ray results on carbonic anhydrase have been summarized by Lindskog *et al.*, 1971. [j] Chasteen *et al.*, 1973. [k] Blundell *et al.*, 1971.

Presently there is no simple explanation of the above orders (Davies *et al.*, 1968).

There must be considerable flexibility in the ligand environment at the active site to even accommodate such a variety of metal ions, including the oxycation vanadyl. Recently Rosenberg *et al.* (1973) have presented evidence from optical spectral measurements and magnetic susceptibility data that Ni^{2+} has an octahedral geometry in CPA whereas Co^{2+} is either five-coordinate or in a distorted tetrahedron

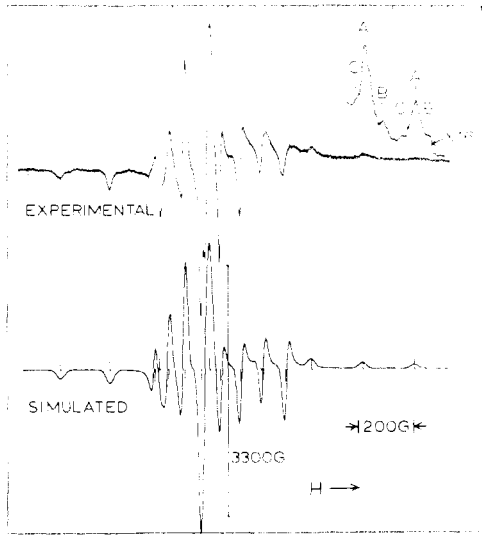

FIGURE 4: Experimental and simulated first-derivative X-band epr spectra of polycrystalline VO^{2+}-CPA at room temperature. The vertical lines in the simulated spectrum denote parallel and perpendicular resonance fields of the "A" site. Simulation of the A site spectrum is based on a Gaussian line-shape function with $\sigma_{\|} = 14$ G, $\sigma_{\perp} = 9$ G, and the epr parameters given in Table II. The A, B, and C designate "parallel" resonances arising from three different magnetic environments of the VO^{2+} ion. Sample prepared by soaking Zn^{2+}-CPA in 0.025 M $VOSO_4$–0.1 M citrate buffer (pH 5.0). The resonances of the A site (active site) dominate the spectrum.

(Latt and Vallee, 1971; Rosenberg *et al.*, 1973). As will be discussed later, vanadyl CPA probably has a square bipyramidal geometry which is characteristic of nearly all vanadyl complexes (Selbin, 1965, 1966). However, a few instances of deviations from this geometry are known (Chasteen *et al.*, 1969).

The epr and visible spectroscopic properties of most metalloenzymes are unusual when compared to model compounds and nonenzymatic metalloproteins. Williams and Vallee have suggested that metal sites in metalloenzymes are in an "Entatic state" poised for catalytic action (Williams and Vallee, 1968; Williams, 1971). In this model, the metal ion coordination sphere presumably is strained.

In contrast to most of the data available on other metalloenzymes (Williams, 1971), the spectral properties of esterase and peptidase active vanadyl CPA are not unusual when compared with model vanadyl complexes.[3] We have shown (*vide infra*) that the enzyme bound VO^{2+} exhibits epr parameters (believed to be largely dependent on the equatorial ligands) which are consistent with data obtained on model compounds. Furthermore the epr parameters (see Table II) of vanadyl carboxypeptidase (A site) are very similar to those of nonenzymatic vanadyl insulin (A sites) in which the vanadyl ion only has a structural role in maintaining the insulin hexamer. Our data, to be discussed in more detail later in this report, therefore do not suggest an unusual coordination of the equatorial ligands in vanadyl CPA. As mentioned above, the active nickel carboxypeptidase apparently has a normal octahedral coordination geometry (Rosenberg *et al.*, 1973).

The above discussion applies to the resting enzyme. It is

[3] Compilations of epr parameters of vanadyl complexes can be found in Kuska and Rogers (1968) and Goodman and Raynor (1970). The reader is referred to the paper on vanadyl insulin (Chasteen *et al.*, 1973) for discussion of the spectroscopic properties of vanadyl chelates and for leading references.

possible that the coordination geometry substantially changes during the catalytic event.

Epr Spectra of Frozen Solutions and Polycrystalline Samples. The epr spectrum of a polycrystalline sample of VO^{2+}-CPA prepared at pH 5.0 is shown in Figure 4. Epr spectra nearly identical with that in Figure 4 were obtained with frozen solutions at 77°K prepared in the pH range 6.2–8.5. The spectrum in Figure 4 is characteristic of randomly oriented magnetically dilute VO^{2+} groups and reflects the near axial symmetry typical of vanadyl complexes. The parameters summarized in Table II were determined as previously described (Chasteen *et al.*, 1973).

The spectrum in Figure 4 is composed of signals which arise from at least three magnetically distinct binding sites labeled A, B, and C. We believe that the A signals, to be discussed later, originate in the binding of VO^{2+} at the active site.

The B site signals become appreciable only in polycrystalline samples prepared at pH 3.0–4.0 (*cf.* Figures 4 and 5). This suggests that the B sites result from extraneous binding at protein carboxyl groups which generally have low pK_a's in the range 3.5–5.0. This is further substantiated by the fact that the epr parameters of the B sites in carboxypeptidase are nearly identical with those of the B sites in insulin (Table II) where binding at glutamyl carboxyl groups was confirmed (Chasteen *et al.*, 1973). Loss of binding at the B sites above pH 5.0 is probably due to weak interaction with these sites and competition of OH^- and buffer for the available VO^{2+} in the soaking solution. Interpretation of spectra of vanadyl-labeled polycrystalline protein has been discussed previously in some detail (Chasteen *et al.*, 1973).

The spectrum due to the C site(s) was not sufficiently intense in any sample to determine accurate epr parameters. This site represents a very small amount of VO^{2+} bound to the protein; its origin remains obscure.

Structure of the A (Active) Site. Of particular interest is the identity of the A site. Identical signals (epr parameters) due to this site have been observed with every polycrystalline sample prepared in the pH range of 3.0–7.5 and dominate the spectrum above pH 5.0. In all samples which contained one or less VO^{2+} per protein, the spectrum was nearly completely due to the A site.[4] Furthermore, the epr parameters of the A site from polycrystalline samples are very similar to those obtained with frozen solutions of 1:1 VO^{2+}/apoenzyme at pH 7.5, the assay conditions (Table II). The characteristic A site spectrum is not observed in frozen solutions containing the VO^{2+} ion and native Zn^{2+}-CPA. Thus, it is reasonable to assign the A site as the active site normally occupied by zinc.

The protein ligands of the active site have been shown by X-rays (Lipscomb, 1970) to be imidazole nitrogens of His-69 and His-196 and the carboxyl oxygen of Glu-72. Presumably, a fourth ligand, a water molecule, completes a distorted tetrahedral geometry about the Zn^{2+} ion, although five-coordination cannot be ruled out.

The isotropic vanadium nuclear hyperfine coupling constant, A_0, and the "rule of average environment" can provide evidence for the coordinating ligands about the VO^{2+} group in the active site. This approach has worked well with insulin (Chasteen, *et al.*, 1973). We choose as model compounds $VO(H_2O)_5^{2+}$ ($A_0 = 106.4 \times 10^{-4}$ cm^{-1}) with four equatorial water molecules (Kuska and Rogers, 1968), VO(porphyrin) ($A_0 = 88.2 \times 10^4$ cm^{-1}) with four equatorial nitrogen atoms (Kivelson and Lee, 1964), and $VO(malonate)_2^{2-}$ ($A_0 = 93.7 \times 10^{-4}$ cm^{-1}) with four equatorial carboxyl groups (Wüthrich, 1965a). The differences in the A_0 values of these compounds largely reflect the differences in the equatorial ligands.

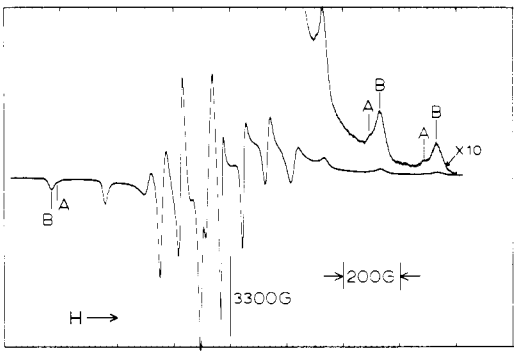

FIGURE 5: Experimental first-derivative X-band spectrum of polycrystalline VO^{2+}-CPA. Sample prepared by soaking polycrystalline Zn^{2+}-CPA in 0.1 M $VOSO_4$ (pH 3.0). The resonances of the B sites (carboxyl groups) dominate the spectrum; however, contributions from the A site are clearly seen.

For equatorial coordination of two water molecules and two imidazole nitrogens in the active site of vanadyl CPA we calculate an A_0 value of $A_0 = [2(106.4 + 2(88.2)]/4 \times 10^{-4} = 97.3 \times 10^{-4}$ cm^{-1} which is in good agreement with the experimental value of $A_0 = (97.1 \pm 1.1) \times 10^{-4}$ cm^{-1}. Other possible coordination geometries which place the Glu-72 carboxyl group in equatorial position give poorer agreement between calculated and experimental A_0. These results are compatible with the coordination of two imidazole groups with Zn^{2+} in the native enzyme (Lipscomb, 1970).

The epr parameters of vanadyl-labeled insulin, carbonic anhydrase (high pH form, inactive), and carboxypeptidase are summarized in Table II. The coordination geometries about zinc in all three native proteins are known to be significantly different (see Table II for references to the X-ray work) and yet the epr parameters for the A sites of all the vanadyl derivatives are essentially the same. We believe that this is likely due to the equatorial coordination of two imidazole groups to the VO^{2+} ion in all three proteins. In the case of vanadyl insulin, the equatorial coordination of two water molecules and two imidazole groups has been assigned with reasonable certainty (Chasteen *et al.*, 1973).

The third protein ligand necessary to completely define the metal site geometry probably coordinates axially to vanadyl ion, if at all. However, in vanadyl complexes the axial ligand opposite the vanadyl oxygen has a much less pronounced effect on the epr parameters than do the equatorial ligands. The axial ligand field is largely dominated by the rather short vanadium–vanadyl oxygen bond length of ~1.6 Å (Chasteen *et al.*, 1969). The vanadium is typically displaced ~0.5 Å out of the plane of the equatorial ligands toward the vanadyl oxygen; this results in weak coordination of the sixth ligand (see footnote 22 of Chasteen *et al.*, 1969; Selbin, 1965, 1966). As a consequence, it is not possible from our data to draw conclusions about the axial ligand coordination in vanadyl-labeled proteins. The sixth position may be occupied by a protein ligand or a water molecule. If the coordination of a protein ligand in the sixth po-

[4] The vanadium content of various polycrystalline samples ranged from as little as 0.02 VO^{2+}/protein and up to 9.0 VO^{2+}/protein.

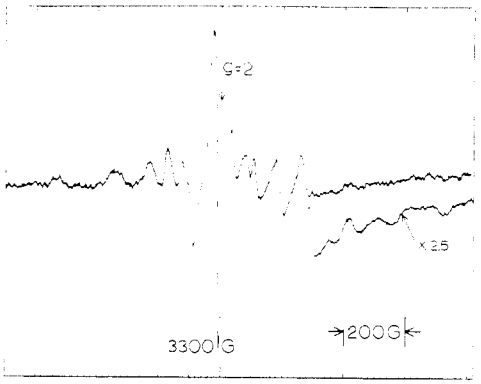

FIGURE 6: Experimental first-derivative X-band epr spectrum of a room temperature solution of VO^{2+}-CPA. 5.3×10^{-4} M VO^{2+}-CPA in 1 M NaCl–0.02 M Tris, pH adjusted down to 6.2 with HCl.

sition deviates from the apical position of a bipyramidal structure, we would not expect it to show up in the epr spectrum.

Epr Spectra of Room Temperature Solutions. One distinct advantage of VO^{2+} spectroscopy is that the spectrum can be readily observed with room temperature solutions. In contrast, most other transition metal probes (*e.g.*, Co^{2+}, Mn^{2+}) often must be studied at low temperature to obtain reasonably well resolved epr spectra.

The epr spectrum of a room temperature solution of VO^{2+}-CPA is shown in Figure 6. This spectrum is quite anisotropic but is distinctly different from the anisotropic spectrum characteristic of frozen solution or polycrystalline samples (*cf.* Figures 4 and 6). This difference can be explained in terms of partial motional averaging in solution of the g and hyperfine tensors. For example, the intense central peak in the room temperature solution spectrum of Figure 6 arises from the near coincidence of the parallel and perpendicular principal resonance fields of the $M_I = -\frac{1}{2}$ line[5] (separation ~33 G); this line has the smallest anisotropy of any of the hyperfine lines and consequently is more completely rotationally averaged.

Motional averaging is further demonstrated by the apparent "$A_\parallel$" and "$A_\perp$" from the solution spectrum. With a decrease in rotational correlation time "$A_\parallel$" should decrease and "$A_\perp$" increase as their values approach the isotropic hyperfine constant A_0. The value of "$A_\parallel$" obtained from the room temperature solution spectrum is indeed smaller than the value obtained from frozen solutions, 179.1 G compared to 182.7 G. Correspondingly, "$A_\perp$" from the room temperature solution spectrum is larger, 66.9 G *vs.* 66.2 G, than from the frozen solution. These results suggest a means of estimating the rotational correlation time of the binding site in the protein. Experiments along these lines are in progress in this laboratory.

In order to compare spectra from solids and solutions in terms of structural similarities or differences in the metal site, we again refer to the isotropic hyperfine coupling constant, A_0.[6] From the room temperature solution spectrum we obtain $A_0 = 96.7 \times 10^{-4}$ cm^{-1} compared to 96.0×10^{-4} and 97.1×10^{-4} cm^{-1} from frozen solution and polycrystalline samples, respectively. Thus the epr data strongly suggest that no gross differences in the metal coordination likely occur in the three types of samples.[7] This is reassuring in that the results for frozen solutions and crystals are often used to explain the properties of proteins in solution.

However, Johansen and Vallee (1971, 1973) and Johansen *et al.* (1972) have found a major change in coordination of the metal site in an arsanilazotyrosine-248 derivative of Zn^{2+}-CPA upon dissolving the protein crystals. This change in coordination is accompanied by marked changes in the absorption and circular dichroism spectra of the Zn–arsanilazoenzyme; yellow crystals dissolve to produce a red solution. The modified Tyr-248 coordinates to the zinc ion in solutions but not in crystals (Johansen and Vallee, 1971, 1973). We prepared the Zn–arsanilazoenzyme by the procedures of Johansen and Vallee and observed the same color changes. Moreover, we observed that the red Zn^{2+}-CPA solutions in quartz epr sample tubes retained their red color upon sudden freezing in liquid nitrogen. In contrast, when we prepared the apoarsanilazoenzyme and added 1 mol of VO^{2+} at pH 8.2, the solution retained the yellow color characteristic of the apoarsanilazoenzyme and the crystalline Zn–arsanilazoenzyme; when these yellow solutions were frozen they showed no color change and exhibited an epr spectrum identical with that of VO^{2+}-CPA with unmodified tyrosine-248. Apparently Tyr-248 does not coordinate to the vanadyl ion. Thus it appears that each case must be examined individually; that is, the extent of differences in protein conformation in crystals and solution quite likely vary from one system to the next. Since both the vanadyl and zinc arsanilazo derivatives of CPA are modified enzymes, it is impossible from the present data to state what actually takes place in the native zinc enzyme.

We have examined the VO^{2+} hyperfine splittings of room temperature and frozen (77°K) solution samples in the pH range 6.2–8.5 with and without Tris buffer. The spectra all have the same epr parameters which suggests that only one major species is present in solution, and that buffer does not appreciably bind at the metal site. However, samples studied below pH 7.0 show a definite broadening which may be indicative of the presence of a low pH form of the active site. The species in low pH solutions have not been investigated in detail.

Binding of EDTA to CPA Crystals. Zinc can be completely removed from native CPA crystals by soaking them in 5×10^{-3} M NaEDTA (pH 7.0) for 48 hr with two changes of chelate solution. We have found that repeated washing and soaking of the apoenzyme crystals in distilled-deionized water over a period of 3 days is insufficient to remove all the EDTA which apparently tenaciously binds to the crystals. The presence of EDTA is evidenced by the appearance of additional epr lines due to a VO^{2+}-EDTA complex when the apoenzyme crystals are reconstituted with VO^{2+}. Prolonged washing reduces the amount of EDTA present but does not completely remove it. The kinetic pa-

[5] The hyperfine lines are labeled $M_I = -\frac{7}{2}, -\frac{5}{2}, \ldots +\frac{7}{2}$ from low to high field. This is consistent with the fact that the nuclear hyperfine coupling is believed to be negative (Abragram *et al.*, 1955).

[6] To obtain A_0 we must assume that the equation $A_0 = \frac{1}{3}A_\parallel + \frac{2}{3}A_\perp$ (expressed in cm^{-1}) applies to the partially averaged solution spectrum encountered here. This is probably a good approximation since the room temperature solution spectrum apparent "$A_\parallel$" and "$A_\perp$" values are not greatly different from those of the frozen solutions or polycrystalline samples.

[7] The $A_0 = 96.0 \times 10^{-4}$ cm^{-1} from the frozen solution (77°K) is slightly, but significantly, smaller than the values from the room temperature solution and polycrystalline samples. This reduction probably is a temperature effect; a reduction of the same magnitude has been observed upon cooling vanadyl acetylacetone in toluene (Wilson and Kivelson, 1966) and for vanadyl bovine carbonic anhydrase at 77°K (Fitzgerald and Chasteen, 1974b).

rameters of the vanadyl and zinc enzymes reconstituted from the apoenzyme prepared in this way are different from those obtained with reconstituted apoenzyme from OP dialysis (DeKoch *et al.*, 1973).

Ulmer and Vallee (1971) have likewise observed EDTA binding to alkaline phosphatase of *E. coli*. Thus, there is increasing evidence that this chelating agent should be avoided, when possible, in preparing apoenzymes from metalloenzymes.

Binding of Ag^+, p-Mercuribenzoate, and Hg^{2+}. In the early work on the determination of the zinc ligands in CPA it was observed that the apoenzyme could be titrated with 1 equiv of Ag^+ or *p*-mercuribenzoate (PMB), but the native zinc enzyme would bind neither of these reagents (Coombs *et al.*, 1964; Vallee *et al.*, 1960b). It was concluded that one sulfhydryl group was probably coordinated to the zinc. However, the subsequent X-ray work (Lipscomb, 1970) and the amino acid sequence (Neurath *et al.*, 1969) indicated that this was not the case. In fact, amino acid sequence revealed that there are no cysteinyl-SH groups in carboxypeptidase A. The results of a recent Cl^- nmr study of Hg^{2+} binding to Zn^{2+}-CPA and apo-CPA are also consistent with the lack of a sulfhydryl group in the enzyme (Bryant, *et al.*, 1969).

To further clarify the binding of these "sulfhydryl specific" reagents to CPA, we have investigated their competitive binding with VO^{2+}. When either Ag^+ or PMB is added in a 1:1 ratio to a solution of VO^{2+}-CPA in Tris buffer at pH 7.5, or unbuffered solution adjusted to pH 8.5, surprisingly, the solution spectrum of VO^{2+} bound at the active site (Figure 6) is abolished. A reduction in intensity to ~25% of the original is observed when $HgCl_2$ is used. In the case of Ag^+, a redox reaction takes place and metallic Ag and V(V) are formed. By contrast $HgCl_2$ and PMB displace VO^{2+} from the active site. (To ensure that VO^{2+} was not likewise being oxidized by Hg^{2+} or PMB, excess EDTA was added to the solution at the end of the experiment.[8] The intensity of the epr spectrum of the generated VO^{2+}-EDTA complex indicated that oxidation did not occur.)

This result is significant in two respects. First, these reagents are obviously not as site specific as generally believed; this had already been noted by Vallee and Wacker (1970). Second, the toxicity of alkyl mercury compounds may, in part, be due to their competition with native metals for binding sites in metalloproteins, even when these sites do not involve coordination of sulfhydryl groups. It is possible that the reactivity of these reagents toward the CPA apoenzyme is enhanced due to the intrinsic nature of the active site (Vallee *et al.*, 1970). On the other hand, the binding of Hg^{2+} and PMB may simply reflect the metal requirement of the protein for overall stability; mono-, di-, and trivalent metals are all known to bind to the apoenzyme.

Additional VO^{2+} Binding in Solution. When buffered or unbuffered solutions of VO^{2+}-CPA are allowed to stand at room temperature for an hour or more, a new eight-line isotropic spectrum of hyperfine splitting $(85.7 \pm 0.1) \times 10^{-4}$ cm^{-1} and g_0 of 1.971 ± 0.001 often begins to appear superimposed on the spectrum of the active site. The change in the spectrum is accompanied at times by some precipitation of the protein. The same eight-line spectrum can sometimes be obtained without the superimposed active site spectrum by adding 1 mol of VO^{2+} to the native enzyme containing one Zn atom per enzyme molecule. Two moles of VO^{2+} cause no further increase in intensity, thus establishing that one binding site is responsible for the eight-line spectrum. We have also observed the spectrum by adding 1 mol of Zn^{2+} to the apoenzyme followed by 1 mol of VO^{2+}. When Hg^{2+} or PMB are added to VO^{2+}-CPA solutions, this spectrum disappears along with that of the active site.

To determine if VO^{2+} was reducing the disulfide linkage in CPA enzyme solutions were tested using 5,5′-dithiobis(2-nitrobenzoic acid) both before and after addition of VO^{2+} (Ellman, 1959). No color change was observed (and furthermore, addition of EDTA produced a VO^{2+}-EDTA spectrum of an intensity consistent with no oxidation of the vanadyl ion). As a control, bovine serum albumin which contains -SH group(s) gave a pronounced yellow color indicative of the presence of a sulfhydryl group.

The rotational correlation time for this site, as estimated from line widths (Chasteen and Hanna, 1972), is $\simeq 5 \times 10^{-10}$ sec as compared with $\simeq 10^{-8}$ sec expected for the enzyme as a whole. This suggests that we may be observing monodentate coordination to the enzyme.

The rather small A_0 ($\simeq 85.7 \times 10^{-4}$ cm^{-1}) value is probably indicative of considerable in-plane π bonding about the VO^{2+} group. A π-bonding group such as tyrosine could not be involved; however, it alone cannot account for the small hyperfine splitting. Inspection of A_0 values for vanadyl complexes (Kuska and Rogers, 1958; Goodman and Raynor, 1970) and application of the rule of average environment (Wüthrich, 1965a; Chasteen *et al.*, 1973) lead one to the foregoing conclusion. If indeed the protein is coordinated monodentate to the VO^{2+} ion as suggested by the fast rotational correlation time, then OH^- ion which has orbitals available for π bonding probably is also bound to VO^{2+} group. Mixed hydroxide-ligand complexes of VO^{2+} are known to exist (Wüthrich, 1965b; Wüthrich and Connick, 1968). This site may be the same as the C site observed in the polycrystalline samples. With the present data, it is difficult to say more.

Conclusion

The VO^{2+} ion has been shown to be a useful probe of metal–protein interactions. The vanadyl-labeling method shows promise as a valuable link between the nature of the metal binding in room temperature solutions, frozen solutions, and polycrystalline samples. While binding of VO^{2+} to the active site in carboxypeptidase A appears to be essentially the same in all three physical states of the protein, this is not the case with vanadyl carbonic anhydrase in which two forms of the metal site are observed in solution but only one form in powder samples (Fitzgerald and Chasteen, 1974b). Obviously, generalizations cannot be made and each protein must be considered individually.

Only a handful of metalloproteins can be cited where the specific structure and function of the metal site have been elucidated, and in many of these cases important questions remain unanswered. It is apparent from the work with vanadyl derivatives of the zinc metalloproteins carboxypeptidase (reported here), carbonic anhydrase (Fitzgerald and Chasteen, 1974b) and insulin (Chasteen *et al.*, 1973), that VO^{2+} epr is a sensitive technique for detecting multiple binding sites in the protein structure and for deciphering which of these sites is vital to the protein function. Furthermore, often reasonable assignments can be made as to the

[8] The V(IV) species which exists in solution at pH >6 in the absence of a ligand such as EDTA or CPA does not exhibit a room temperature epr spectrum and thus cannot be distinguished from oxidized V(V) species.

identity of some of the protein functional groups involved in coordination.

Much work is needed to further define the scope and limitations of the vanadyl-labeling technique. Toward this end, investigation of VO^{2+} binding to a variety of enzymatic and nonenzymatic proteins are under way in this laboratory. The recent discovery of vanadium as an essential trace element (Schwarz and Milne, 1971) adds further impetus to this work.

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