proteins to ion exchangers on the "wrong" side of their isoelectric point (Himmelhoch, 1972).

An interesting observation reported in the present paper deals with a comparison between the ω-aminoalkylagarose chosen (Seph-C_6-NH_2) and DEAE-cellulose; Seph-C_6-NH_2 gave a better purification, removing protein impurities which were still present after passage through the DEAE-cellulose column. This is presumably due to the fact that in selecting Seph-C_6-NH_2 we adjusted the contribution of hydrophobic interactions to the optimal level.

Finally, this paper illustrates the usefulness of homologous series of ω-aminoalkylagaroses in protein purification as compared with affinity chromatography. It provides an example of how these two techniques can be combined to achieve efficient purification of an enzyme.

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Electron Paramagnetic Resonance Studies of the Structure and Metal Ion Exchange Kinetics of Vanadyl(IV) Bovine Carbonic Anhydrase†

John J. Fitzgerald‡ and N. Dennis Chasteen*

ABSTRACT: Detailed electron paramagnetic resonance (epr) studies of powder, room temperature, and frozen solution samples of vanadyl(IV) bovine carbonic anhydrase B (VO^{2+}-BCA), in which VO^{2+} has been substituted for Zn^{2+} in the native enzyme, are reported. The modified enzyme is inactive toward *p*-nitrophenyl acetate. In solution, high and low pH forms of the metal site exist with a functional group(s) having an apparent pK_a = 7.1 involved. The functional group does not appear to be an OH^- group bound to the metal as has been suggested for the Zn^{2+} and Co^{2+} enzymes by other workers. Epr parameters suggest that the high pH form is structurally similar to that found in powder samples, *i.e.*, the VO^{2+} is coordinated to at least two imidazole groups of histidyl residues. In the low pH form, which does not exist in powder samples, the enzyme has undergone an important conformational rearrangement. One possible interpretation is an increase in the number of coordinating protein ligands at low pH. The stability constant for the VO^{2+}-BCA complex was measured as a function of pH and is approximately 10^{11} at pH 8.0. At pH 7.9 and 25° the "spontaneous" dissociation rate of the VO^{2+}-BCA complex, as measured by epr, follows first-order kinetics in [VO^{2+}-BCA] with a rate constant of 6.8×10^{-4} sec^{-1}. Under the same conditions, the Zn^{2+}-promoted VO^{2+} dissociation is first order in both [Zn^{2+}] and [VO^{2+}-BCA] with a second-order rate constant of 1.4 sec^{-1} M^{-1}. This study further demonstrates the applicability of VO^{2+} epr spectroscopy as a metal probe capable of providing structural and kinetic information about metal binding sites in proteins.

Electron paramagnetic resonance spectroscopy (epr)[1] yields information which is often difficult to obtain by other means. The method is particularly well suited for biological investigations because of its applicability to dilute aqueous samples. The analyses of epr spectra of polycrystalline vanadyl (VO^{2+}) insulin and carboxypeptidase A have demonstrated the utility of VO^{2+} epr measurements in probing metal binding sites in crystalline proteins (Chasteen *et al.*, 1973; DeKoch *et al.*, 1974). Use of this site-specific epr probe in correlating structural information such as that obtained by X-ray crystallography with *in vitro* room temperature solution studies is heretofore unexplored.

In the past, extrapolation of X-ray structural results to

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[1] Abbreviations used are: epr, electron paramagnetic resonance; VO^{2+}-BCA, vanadyl(IV) bovine carbonic anhydrase B; NphOAc, *p*-nitrophenyl acetate.

problems of enzyme mechanism in solution was based on the hypothesis that minimal protein structural changes occur from solution to the solid state. A body of recent evidence, however, suggests that small conformational changes and structural irregularities, particularly near active-site regions of enzymes, are significant in terms of catalysis. For example, discrepancies between metal stability constants, inhibitor constants, and activities of carboxypeptidase A in solution and cross-linked carboxypeptidase A crystals indicate structural modifications of the active-site region (Bishop *et al.*, 1966; Quiocho and Richards, 1966; Quiocho *et al.*, 1967). Johansen and Vallee (1971) have recently suggested that a tyrosine residue not coordinated to zinc metal in carboxypeptidase A crystalline state (Lipscomb, 1970) must undergo a 12-Å movement to coordinate to the metal ion in carboxypeptidase A solutions.

In view of these recent insights, we have examined the use of VO^{2+} epr as a bridge between structural studies in the solid state and solution studies for the vanadyl analog of the zinc metalloenzyme bovine carbonic anhydrase B (BCA). In addition, we have explored the utility of VO^{2+} epr for investigating the kinetics of metal–protein dissociation and metal substitution processes.

The structural features at the active site of human carbonic anhydrase C have been determined by X-ray diffraction to 2.0-Å resolution (Liljas *et al.*, 1972). Human and bovine carbonic anhydrases have molecular weights of *ca.* 31,000 and contain one tightly bound Zn^{2+} which is essential for activity. Zn^{2+} can be replaced with other metals, *i.e.* Co^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, and VO^{2+} (*vide infra*), with only the Zn^{2+} and Co^{2+} derivatives exhibiting esterase and CO_2 hydration activity (Lindskog, 1963, 1966; Lindskog and Malmström, 1962; Lindskog *et al.*, 1971).

Experimental Section

Precautions were taken against trace metal contamination from containers, syringes, and reagents by acid washing and extraction procedures as described previously (Chasteen *et al.*, 1973). All solutions used in vanadyl epr and kinetic experiments were purged with prepurified nitrogen gas to minimize oxidation of the vanadyl ion. Above pH 7, the reducing agent, sodium dithionite ($Na_2S_2O_4$, *ca.* 6×10^{-4} M), was occasionally added to vanadyl solutions in a 2:1 molar ratio to prevent rapid oxidation under alkaline conditions. Epr samples protected in this manner gave spectra identical with those of samples not containing $Na_2S_2O_4$; however, the reducing agent often improves the intensity of the epr signal. Dithionite is particularly helpful when used during experiments requiring several hours. Stock solutions of $VOSO_4$ (Alfa Inorganics) were standardized spectrophotometrically as described previously (molar decadic extinction coefficient of 18.0^{-1} M^{-1} cm^{-1} at 750 nm; Fitzgerald and Chasteen, 1974).

Bovine carbonic anhydrase (BCA, EC 4.2.1.1) was purchased from Sigma Chemical (lot no. 33C-8000, 32C-8150) and the B enzyme was isolated by the method of Lindskog (1960). The zinc-free apoenzyme was prepared by dialysis against 10^{-2} M *o*-phenanthroline in 0.1 M acetate buffer (pH 5.0) at 4° as reported previously (Lindskog and Malmström, 1962). After two 3-day dialyses against *o*-phenanthroline followed by two 3-day dialyses against distilled–deionized water, only 0.01–0.05 g-atom of Zn^{2+}/mol of enzyme remained as determined by atomic absorption spectroscopy. Protein concentrations were determined spectrophotometrically at 280 nm, using $\epsilon_{280} = 5.6 \times 10^4$ M^{-1} cm^{-1} (Lindskog and Malmström, 1962). The dialysis tubing was purchased from two sources, Spectrapor membrane tubing no. 1, mol wt cutoff 6000–8000 (National Scientific), or cellulose dialysis sacks, mol wt cutoff 15,000 (Sigma Chemical), and was soaked in 10^{-2} M *o*-phenanthroline and deionized–distilled water prior to its use.

The vanadyl carbonic anhydrase derivatives were prepared as powders or aqueous solution samples. Powder samples were prepared by the crystal soaking method described previously (Chasteen *et al.*, 1973). The powders were prepared by soaking 20 mg of enzyme in 2.0 ml of 0.05 M Na_2EDTA and 3 M $(NH_4)_2SO_4$ (pH 6.05) followed by 0.01–0.1 M $VOSO_4$–3 M $(NH_4)_2SO_4$–0.05 M citrate buffer (pH 3.0–7.0). For most samples less than 5% of the original Zn^{2+} remained.

Epr spectra were recorded on Varian E-4 and E-9 spectrometers, both instruments operating at *ca.* 9.5 GHz (X-band) with 100-kHz field modulation. The g values were determined relative to DPPH, $g = 2.0036$, or Varian strong pitch, $g = 2.0028$. Most quantitative intensity measurements were obtained with the E-9 spectrometer equipped with a dual cavity and strong pitch as a reference. Of particular importance in the solution experiments are the careful tuning procedures (Fitzgerald and Chasteen, 1974) necessary to obtain reliable intensity data throughout a series of sample measurements. In this manner, the signal height of various features of the VO^{2+}-BCA signal is proportional to the concentration of the reconstituted enzyme.

In the solution epr experiments, the sample was prepared directly in the quartz flat cell in order to minimize the quantity of enzyme required. Typically, 200–500 μl of nitrogen-purged unbuffered apoenzyme solution was added with a microliter syringe to a serum stoppered flat cell filled with nitrogen, followed by the addition of a known amount of VO^{2+} ion. Mixing of the cell contents was performed by drawing off the solution from the bottom of the cell into a nitrogen-purged 3.0-ml disposable syringe with a minimum pressure of nitrogen flowing into the cell from the top. The syringe was never removed from the cell (except when measuring the spectrum) but only served as a reservoir to shake or mix the cell contents. The solution was then returned to the cell and this procedure repeated two or three times for every addition to the enzyme solution. In this manner, one may add buffer, VO^{2+}, Zn^{2+}, or other metals, inhibitors, and substrates to the enzyme solution in the cell successively, thereby efficiently using a small sample of apoenzyme for a series of epr measurements. For example, when the titration of the apoenzyme active site with VO^{2+} ion was performed, only a 500-μl sample was required to titrate the solution from 0.2 to 10 g-atoms of VO^{2+} ion per mol of enzyme in acetate buffer. It should be noted that the most intense signal was obtained when the VO^{2+} ion was added to an unbuffered apoenzyme solution followed by addition of buffer, rather than to an apoenzyme solution previously buffered.

The titration of the VO^{2+}-BCA derivative over the pH range 5.35–9.50 was performed in a specially adapted 2.0-ml test tube equipped with a 5-mm diameter side arm fitted with a serum stopper to permit removal of samples during the experiment. The pH of a typical 1.25-ml sample was determined with a Radiometer pH M26 meter equipped with a GK2321C calomel glass combination electrode which was sealed to the top of the titration cell with a Pasteur pipet bulb cut to form rubber tubing of the proper dimensions. The cell could be conveniently purged with nitrogen gas

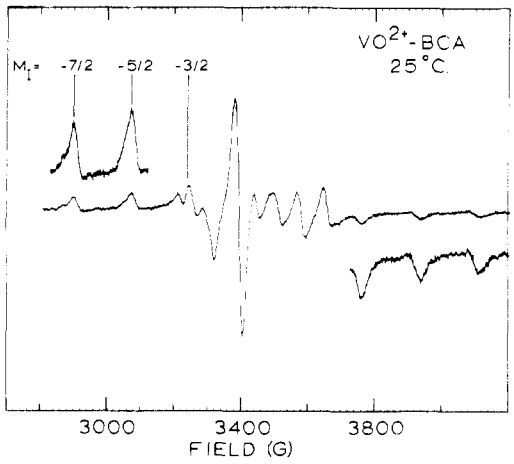


FIGURE 1: Electron paramagnetic resonance spectrum of vanadyl bovine carbonic anhydrase (VO^{2+}-BCA) in aqueous solution. Conditions: 4.92×10^{-4} M apo-BCA-4.85×10^{-4} M VO^{2+}-9×10^{-4} M $Na_2S_2O_4$, pH 5.35, 25°. The amplitudes of the parallel low-field ^{51}V nuclear hyperfine lines, labeled $M_I = -7/2$, $-5/2$, and $-3/2$, were monitored throughout solution experiments; X-band.

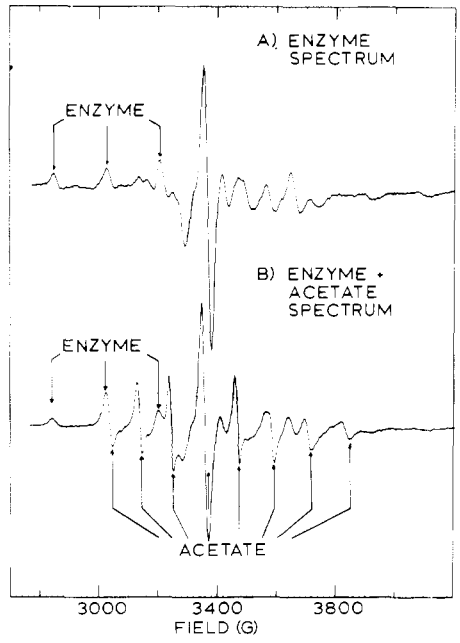


FIGURE 2: Epr spectra taken during the course of the titration of apo-BCA with VO^{2+} ion: (A) spectrum of VO^{2+}-BCA after addition of 0.84 g-atom of VO^{2+}/mol of apoenzyme; (B) combined spectra of VO^{2+}-BCA and VO^{2+}-acetate complex. Mixture formed by addition of 2.1 equiv of VO^{2+}/mol of apo-BCA. Resonance peaks corresponding to VO^{2+}-BCA and VO^{2+}-acetate are labeled in A and B. Conditions: 4.18×10^{-4} M apo-BCA-5×10^{-2} M acetate buffer, pH 5.0, 25°, X-band. Instrument gain setting of A is twice that of B.

throughout the titration by using hypodermic needles. The VO^{2+}-BCA enzyme was reconstituted by addition of a purged sample of the apoenzyme solution to the nitrogen-purged cell, followed by addition of an aliquot of VO^{2+} sufficient to completely reconstitute the apoenzyme. Aliquots of 1 or 0.1 N NaOH solution were syringed into the cell *via* the side arm and the sample was mixed using a magnetic "spinfin" contained in the bottom of the cell. At appropriate pH values, unbuffered VO^{2+}-BCA samples (250 μl) were withdrawn from the cell and their epr spectrum recorded.

The esterase activity of Zn^{2+}- and VO^{2+}-BCA preparations was determined at pH 7.55 using a Tris buffer (ionic strength 0.09 made up of 0.009 M Tris-HCl and 0.081 M NaCl) and the ester, *p*-nitrophenyl acetate (NphOAc), obtained from Sigma Chemical (lot no. 42C-2480). The assay method of Pocker and Stone (1967) was modified because of the oxidation precautions required for the VO^{2+}-BCA enzyme. An approximately 3×10^{-3} M NphOAc solution was prepared by dissolving 30 mg of NphOAc in 5.0 ml of acetone and diluting to 50 ml with deionized-distilled water. This is in contrast to Pocker and Stone, where the NphOAc assay solution was 10% in acetonitrile. The procedure used here resulted in an assay solution *ca.* 0.1% in acetone. Stock apoenzyme solutions varied from 2 to 4×10^{-4} M. The analysis of rate data was performed as described by Pocker and Stone (1967).

In a typical kinetic run 3.00 ml of this buffer was injected into a 1-cm quartz cell, followed by 30 μl of apoenzyme, native enzyme, or reconstituted metal-apoenzymes and 30 μl of NphOAc. Quartz cells were serum stoppered and had previously been purged with nitrogen gas as had all the assay solutions. The VO^{2+} enzyme was reconstituted in a separate test tube by addition of apo-BCA solution and appropriate concentration of vanadyl ion. The final assay mixtures contained $2\text{-}3 \times 10^{-5}$ M NphOAc and $2\text{-}4 \times 10^{-6}$ M enzyme in Tris buffer.

The carbonic anhydrase inhibitors, NaCN, KCN, and NaN_3 (Fisher Scientific), and the sulfonamide, ethoxyzolamide, were added in successive aliquots up to 100-fold molar excess to the VO^{2+}-BCA in 0.025 M Tris-HCl buffer (pH 8.5) or 0.005 M acetate buffer (pH 5.0).

Kinetics of Zn^{2+} displacement of VO^{2+} from VO^{2+}-BCA were carried out at 25° under two sets of conditions: (1) 0.025 M Tris-HCl (pH 8.5), 1.38×10^{-4} M VO^{2+}-BCA, 2.8×10^{-4} M $Na_2S_2O_4$, and 2.96×10^{-4} $ZnCl_2$; or (2) 0.002 M Tris-HCl (pH 7.9), 1.57×10^{-4} M VO^{2+}, 3.1×10^{-4} M $Na_2S_2O_4$, and 1.86×10^{-4} M $ZnCl_2$. Under condition 1 the reaction was followed by monitoring the disappearance of $M_I = -7/2$ and $-5/2$ parallel lines of the VO^{2+}-BCA complex. These were the only lines with sufficient intensity which did not overlap with those due to a VO^{2+}-Tris complex formed during the reaction. Under condition 2, at the low Tris concentration, the most intense $M_I = -1/2$ line of the VO^{2+}-BCA spectrum could be monitored without VO^{2+}-Tris complex interference. The kinetics of the dissociation of VO^{2+} from VO^{2+}-BCA was conducted under condition 2 except that 1.57×10^{-4} M Na_2EDTA was substituted for $ZnCl_2$; the intensity of the $M_I = -5/2$ line (second from low field) of the generated VO^{2+}-EDTA was followed. There is little overlap between this line and those of VO^{2+}-BCA. Spectra were measured before addition of Zn^{2+} or EDTA and repetitive spectral scans were made within 5 min after the addition. Metal analyses and pH measurements were performed on solutions before and after each kinetic run.

Results and Discussion

Stoichiometry of VO^{2+} Binding to BCA. The epr spectrum of a room temperature solution sample of vanadyl bovine carbonic anhydrase (VO^{2+}-BCA) (pH 5.35) is shown in Figure 1. The spectrum is composed of resonance signals arising from one vanadyl ion bound to carbonic anhydrase at the site previously occupied by zinc. The intensity and spacing between the anisotropic "parallel" resonance lines, labeled $M_I = -7/2$, $-5/2$, and $-3/2$ in Figure 1 (assuming a negative hyperfine coupling constant), were monitored throughout the solution experiments. The spectrum shape,

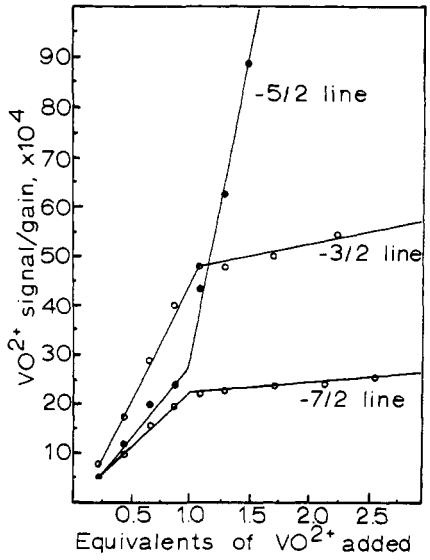


FIGURE 3: Plot of signal height/receiver gain *vs.* equivalents of VO^{2+} ion added to an apoenzyme solution. The signal height/receiver gain ratio, measured for the low-field parallel resonance lines, $M_I = -7/2$, $-5/2$, and $-3/2$, is proportional to the VO^{2+}-BCA concentration. Conditions are as in Figure 2.

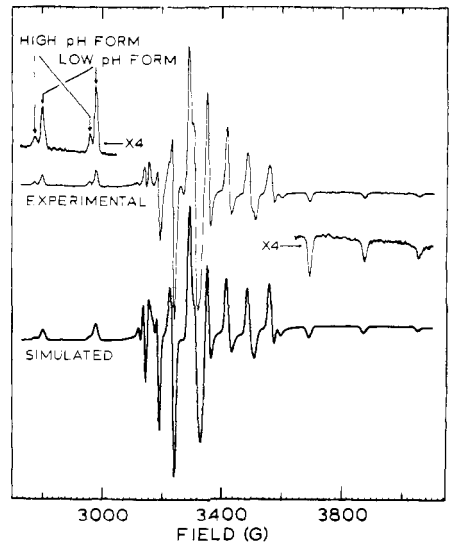


FIGURE 4: Experimental and simulated first-derivative X-band epr frozen solution spectrum of VO^{2+}-BCA. Experimental spectrum conditions: pH 5.35, 77°K, 3.96 × 10^{-4} M VO^{2+}-BCA. Spectra represent epr signals arising from two VO^{2+}-BCA species designated "high and low pH forms."

i.e., the appearance of parallel and perpendicular regions, is usually found with polycrystalline or frozen solution samples, and in this case reflects the binding of the VO^{2+} ion to and "immobilized" region of the protein molecule. The molecular motion of VO^{2+} ion is apparently largely governed by the slow molecular tumbling of the protein molecule in room temperature solutions; the rotational correlation time of the protein is ~2.9 × 10^{-8} sec (Chen and Kernohan, 1967).

Evidence for binding of the vanadyl ion to carbonic anhydrase at the site previously occupied by zinc is threefold. First, representative epr solution samples contained less than 0.05 g-atom of zinc per molecule of protein. Secondly, the bindings of VO^{2+} and Zn^{2+} are mutually exclusive since no binding of VO^{2+} to the native Zn^{2+} enzyme is observed in solution. Thirdly, titration of the apoenzyme with vanadyl indicates that only one VO^{2+} binds to the apoenzyme (as is the case with Zn^{2+}).

The apoenzyme in 0.025 M acetate buffer (pH 5.0) was titrated with VO^{2+} up to 10 equiv and the epr spectrum monitored. In Figures 2A and 2B the epr spectra of solutions containing 0.84 and 2.0 equiv, respectively, of VO^{2+} are illustrated. Vanadyl acetate lines appear beyond 1 equiv (Figure 2B). In Figure 3, plots of epr signal height divided by the instrument gain *vs.* equivalents of VO^{2+} added show breaks at 1 equiv, thus establishing a 1:1 complex.

pH Dependence of Solutions of VO^{2+}-BCA; High and Low pH Forms of the Metal Site. Solution spectra of VO^{2+}-BCA are markedly pH dependent, characterized by epr parameter variations in addition to intensity changes. Variations in intensity, g values, and the hyperfine splitting parameters $A_\parallel$ and $A_\perp$ were examined while the enzyme was titrated between pH 5.3 and 9.5. In the frozen solution, particularly the parallel $-7/2$ and $-5/2$ hyperfine lines, changes in VO^{2+} coordination are evident from the appearance of a doubling of the resonance signals (Figure 4). The doubling effect arises from overlapping spectra from two chemical forms of the metal site labeled "high and low pH forms" in accord with previous identification of acid and base forms for Zn^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} carbonic anhydrase (Lindskog, 1963; Lindskog *et al.*, 1971; Koenig and Brown, 1972; Lanir *et al.*, 1973; Fabry *et al.*, 1970; Mushak and Coleman, 1972). The low pH form dominates the spectra below pH 7, whereas the high pH form is most abundant at alkaline pH. A plot of log [acid]/[base] *vs.* pH is linear over the pH range 5–9 and yields an apparent pK_a' of 7.7 for frozen solutions (Figure 5).

However, the slope of the curve in Figure 5 is only 0.22 as opposed to 1.0 expected from the Henderson–Hasselbalch equation for a single-ionizing group. In an attempt to find an explanation for this, we carefully examined room temperature solution spectra over the pH range 5–9, and only were able to observe the presence of both high and low pH forms near pH 7 as would be expected from relative in-

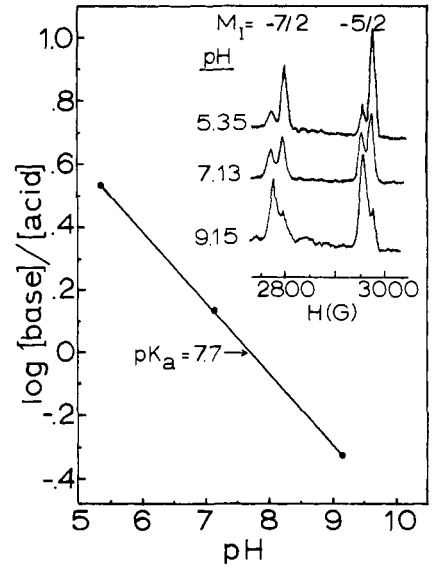


FIGURE 5: Plot of log [base]/[acid] *vs.* pH. Log [base]/[acid] calculated from intensity measurements of the $M_I = -5/2$ parallel resonance line of the high and low pH forms. Data obtained from frozen solution spectra at pH 5.35, 7.13, and 9.15; the corresponding resonance peaks at these pH values are shown in the insert. A pK_a of 7.7 was obtained at log [base]/[acid] = 0. No buffer was employed.

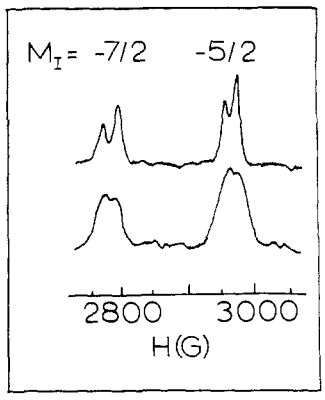


FIGURE 6: Comparison of low-field parallel lines of 77°K frozen (upper curve) and 25°C room temperature (lower curve) solution of VO^{2+}-BCA (3.96×10^{-4} M VO^{2+}-BCA, pH 7.13). A change in the relative amounts of high and low pH forms upon freezing the solution can be seen. No buffer was employed.

tensities of the lines of the high and low pH forms according to the Henderson–Hasselbalch equation. By assuming a single ionizing group, we calculate an apparent $pK_a' = 7.1$ for room temperature solutions. This value is similar to that found for other metal derivatives of the enzyme.

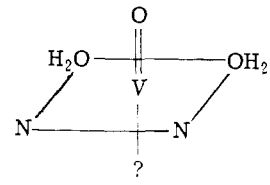
Obviously, freezing of the sample in liquid nitrogen markedly perturbs the acid–base equilibrium as shown in Figure 6. The deviation from unity in the slope of the curve in Figure 5 may be a consequence of a nonequilibrium situation in frozen solutions.

It is noteworthy that the pK_a of imidazole is very temperature dependent and, in fact, increases from 7.0 to 7.6 when the temperature is lowered from 25 to 0° (Sober, 1968). Pocker and Stone (1968b) investigated the temperature dependence of the pK_a of the native zinc enzyme and their results suggest that an imidazole group is involved. The large temperature effect on the acid–base equilibrium of vanadyl enzyme could be interpreted in this way.

Perhaps a word of caution is appropriate here. Generally, epr spectra of transition metals bound to proteins are obtained at 77 or 4°K. From our results, it is apparent that significant spectral changes, associated with a conformational change in the protein, can take place in the freezing process. Such changes could conceivably be large enough that the results obtained on frozen solutions would be misleading.

Epr Parameters and the Nature of the VO^{2+}-BCA Binding Site. (A) EQUIVALENCE OF POWDER SAMPLES AND HIGH pH FORM IN FROZEN SOLUTION. The coordination geometry and ligand environment of the zinc site in the bovine B enzyme are probably structurally similar to the site of human carbonic anhydrase C where three imidazole and one water molecule are ligated to the zinc ion in an approximate tetrahedral arrangement (Liljas *et al.*, 1972). This analogy between BCA and human carbonic anhydrase C is based on similar structural and kinetic investigative results of their metal-substituted derivatives (Lindskog *et al.*, 1971; Thorslund and Lindskog, 1967).

Epr parameters for VO^{2+}-BCA powder, frozen solution, and aqueous room temperature solution samples are given Table I, together with additional parameters reported previously for polycrystalline VO^{2+} insulin (Chasteen *et al.*, 1973). The epr parameters for powder VO^{2+}-BCA samples (Table I) are identical with those parameters reported for polycrystalline VO^{2+}-insulin. VO^{2+} coordination in the two proteins, therefore, appears to be essentially the same. Both proteins have three histidyl groups coordinated to zinc. Two histidine and two water molecules likely comprise the equatorial coordination positions of the VO^{2+} ion, with an additional histidine residue possibly in the axial position, *viz.*[2]



Powder epr spectra of VO^{2+}-BCA do not vary in A and g values with pH 3–7, as likewise found for the major metal site in VO^{2+}-insulin (Chasteen *et al.*, 1973) and VO^{2+}-carboxypeptidase A (DeKoch *et al.*, 1974). Presumably, the effects of pH on conformation and structural properties of the metal site are relatively minor in the solid state.

The room temperature solution and frozen solution spectra of VO^{2+}-BCA, on the other hand, are pH dependent. The presence of a well-defined acidic and basic species has already been noted in the previous section. The epr parameters for the species labeled "high pH form" in frozen solution are very similar to the epr parameters for powder samples of VO^{2+}-BCA (Table I). This suggests complementary ligand environments about the VO^{2+} ion[3] for these two states of the protein.

(B) LOW pH FORM OF VO^{2+}-BCA. The epr parameters for the "low pH form" of VO^{2+}-BCA in room temperature and frozen solutions indicate that the coordinating ligands and/or metal site geometry about the VO^{2+} ion are quite different from the "high pH form." The most sensitive parameters, the hyperfine splittings $A_\parallel$ and $A_\perp$, decrease by *ca.* 4.5×10^{-4} and 4.0×10^{-4} cm^{-1}, respectively, in going from high pH to low pH frozen solutions.

Spectroscopic changes with pH have also been observed for the Mn^{2+}, Co^{2+}, and Cu^{2+} derivatives of BCA (Koenig and Brown, 1972; Lindskog and Nyman, 1964; Taylor and Coleman, 1971; Lanir *et al.*, 1973; Fabry *et al.*, 1970). In each case, these changes involve some transformation at the metal site with a pH dependence characterized by an apparent pK_a' of 7.1. This feature of the metal site environment appears to be common to both the active Zn^{2+} and Co^{2+} (Lindskog *et al.*, 1971; Prince and Woolley, 1972) and inactive Cu^{2+}, Mn^{2+}, and VO^{2+} derivatives. It has been proposed that the functional group responsible for this transformation and the concomitant spectral changes in a water molecule bound to the metal which is titrated to a metal-bound hydroxide group at alkaline pH (Lindskog *et al.*, 1970; Lindskog and Coleman, 1973). This interpretation has recently been challenged by Koenig and Brown (1972), who suggest that an ionization on the protein is in-

[2] A reasonable assignment of the equatorially coordinating ligands of the VO^{2+}-BCA binding site can be obtained using the "rule of average environment" based upon the isotopic nuclear hyperfine coupling constant A_0 for the vanadyl ion (Chasteen *et al.*, 1973). For two nitrogens and two water molecules coordinated equatorially one predicts $A_0 = 97.3 \times 10^{-4}$ cm^{-1} in agreement with 95.8×10^{-4} and 97.3×10^{-4} cm^{-1} for frozen solution and powder samples, respectively.

[3] The hyperfine splittings are slightly smaller in the frozen solution than in the polycrystalline state. This phenomenon was also found for VO^{2+}-carboxypeptidase and probably reflects a temperature variation in the epr parameters in going from 25°C to 77°K (liquid N_2). Similar temperature dependence has been observed for vanadyl acetylacetonate (Wilson and Kivelson, 1966).

TABLE I: Epr Parameters of Vanadyl-Labeled Bovine Carbonic Anhydrase (BCA) and Bovine Insulin.

Protein	Exptl Condition[b]	$g_{\|\|}$ (±0.001)	$g_\perp$ (±0.002)	g_0[c] (±0.002)	$A_{\|\|}$[d] (±0.7)	$A_\perp$[d] (±1.1)	A_0[d,e] (±1.1)
BCA	Powder	1.943	1.977	1.965	167.7	62.1	97.3
Insulin[a]	Polycrystalline	1.943	1.977	1.965	167.1	61.7	96.8
BCA, high pH	Frozen soln	1.944	1.979	1.966	167.1	60.2	95.8
BCA, high pH	RT soln[f]	"1.945"	"1.976"	1.965	"162.1"	"62.6"	95.8
BCA, low pH	Frozen soln	1.941	1.980	1.967	162.6	56.0	91.6
BCA, low pH	RT soln[f]	"1.943"	"1.978"	1.966	"157.3"	"58.4"	90.6

[a] Data from Chasteen *et al.* (1973). [b] Polycrystalline and powder spectra recorded at room temperature on samples prepared by crystal soaking; frozen solution (77°K) spectra recorded on samples also used in room temperature solution epr experiments. [c] Calculated from $g_0 = (g_{\|} + 2g_\perp)/3$. [d] Units of 10^{-4} cm^{-1}. [e] Calculated from $A_0 = (A_{\|} + 2A_\perp)/3$. [f] Values of $g_{\|}$, $g_\perp$, $A_{\|}$, and $A_\perp$ for the room temperature (RT) solution samples are placed in quotations since they are apparent parameters derived from partially rotationally averaged spectra. However, the values of the isotropic parameters, g_0 and A_0, calculated from the apparent "parallel" and "perpendicular" parameters are probably close to the actual values.

volved. Pocker and Stone (1967, 1968a,b) have proposed a histidyl residue.

The epr data on VO^{2+}-BCA are inconsistent with hydroxide binding to the vanadium at high pH. For the limited number of mixed ligand–hydroxide complexes of vanadyl reported in the literature (Wüthrich, 1965) OH^- binding always reduces the hyperfine splitting, as expected for a π-bonding ligand, the opposite of what is observed for VO^{2+}-BCA in going from the low pH to the high pH form.

The reduction in hyperfine splitting upon lowering the pH might be due to a marked conformational change about the VO^{2+} group or alternatively to the displacement of a water molecule from the first coordination sphere by a protein ligand.[4] Substitution of an aromatic nitrogen ligand for an equatorial water molecule in vanadyl complexes is expected to reduce the isotropic hyperfine splitting, A_0, by about 4.5×10^{-4} cm^{-1}.[5] This compares with a difference in A_0 values for the high and low pH forms of 4.2×10^{-4} cm^{-1} which is suggestive of the displacement of an equatorial water molecule by an imidazole group at low pH.

While this conclusion is at best only tentative, it is consistent with the results of Lanir *et al.* (1973) and Koenig and Brown (1972), based on proton relaxation measurements, that water does not coordinate to the metal ion at low pH in the Mn^{2+} and Co^{2+} enzymes. Their results could be rationalized by an increase in the number of coordinating protein ligands at low pH with a concomitant loss of water as we are proposing for the vanadyl derivative.

Normally one would not except an increase in the number of coordinating protein functional groups with decreasing pH as proposed above. It is possible that some type of cooperative phenomenon governed by the overall stability of the protein is responsible for this unexpected behavior. It is generally agreed that the metal site in this protein is quite unusual Lindskog *et al.*, 1971).

Finally, it should be noted that VO^{2+}-BCA is inactive and fails to bind common inhibitors of carbonic anhydrase (*vide infra*). The extent to which conclusions based on the pH dependence of vanadyl epr spectrum can legitimately be applied to other metal derivatives has not been established here. Rather, the vanadyl results suggest a possible interpretation of existing data on other metallocarbonic anhydrases which has not been considered previously.

Analysis of Room Temperature Solution Spectra. In contrast to most other paramagnetic metal ions, room temperature epr spectra of VO^{2+} bound to proteins are reasonably well resolved. As mentioned earlier, VO^{2+}-BCA exhibits room temperature solution spectra similar in shape to those of polycrystalline and frozen solution samples of vanadyl complexes. However, the apparent epr parameters measured directly off the room temperature solution spectrum reflect some motional averaging. For example, with the high pH form in frozen solution, one obtains $A_{\|} = 167.1 \times 10^{-4}$ and $A_\perp = 60.2 \times 10^{-4}$ cm^{-1}, whereas the corresponding room temperature values are 162.1×10^{-4} and 62.2×10^{-4} cm^{-1}. A reduction in $A_{\|}$ and an increase in $A_\perp$ are expected for a partially averaged spectrum. Other room temperature spectral features can be interpreted similarly.

It should be noted, however, that the isotropically averaged parameters, A_0 and g_0, derived from anisotropic data for the room temperature solution spectra are quite compatible with the frozen solution and polycrystalline parameters.[6] Thus, structural assignments for the two forms of the VO^{2+} metal site in the frozen solutions are probably also valid for room temperature solutions, although as noted earlier equilibrium between the two forms is changed upon freezing the sample.

Kinetics of VO^{2+}-BCA. (A) ESTERASE ACTIVITY. The

[4] The A_0 values of nearly all VO^{2+} complexes are lower than that for $VO(H_2O)_5^{2+}$. Displacement of H_2O from an equatorial position in the first coordination sphere by a protein ligand, whatever the protein ligand may be, should result in a reduction in A_0. This is consistent with a larger protein ligand coordination number for the "low pH form" relative to the "high pH form"; see footnote 2.

[5] Here we are using the reasonably well established "rule of average environment" for vanadyl complexes in which the A_0 value is an empirical measure of ligand type (Wüthrich, 1965; Chasteen *et al.*, 1973; Boucher *et al.*, 1969). For several vanadyl complexes $g_{\|}$ increases with decreasing A_0 although there are a number of exceptions (see Table II of Boucher *et al.*, 1969). VO^{2+}-BCA is also an exception. For the low pH form $g_{\|}$ is slightly smaller than for the high pH form (Table I).

[6] A_0 and g_0 for the room temperature solution spectrum are calculated from $A_0 = \frac{1}{3}A_{\|} + \frac{2}{3}A_\perp$ and $g_0 = \frac{1}{3}g_{\|} + \frac{2}{3}g_\perp$, where these equations are strictly valid when $A_{\|}$, $A_\perp$, $g_{\|}$, and $g_\perp$ are obtained from a completely anisotropic spectrum. However, since the "apparent" anisotropic parameters from the room temperature spectrum are not very different from the frozen solution values, application of these equations is probably reasonable.

TABLE II: Results of Hydrolysis Kinetics[a] of *p*-Nitrophenyl Acetate as Catalyzed by Zn^{2+} and VO^{2+} Bovine Carbonic Anhydrase Derivatives at 25°, pH 7.55.

Enzyme	[Enzyme][b] $\times 10^6$ M	[NphOAc],[c] $\times 10^5$ M	$10^{-3}k_{obsd}$ (min^{-1})[d]	$10^{-4}k_{enz}$ (min^{-1} M^{-1})[d]	% Act.[e]
apo-BCA	4.56	2.10	5.27	0.12	3.5
Native Zn^{2+}-BCA	1.85	2.25	58.2	3.24	100
Zn^{2+}-BCA (reconstituted)	4.56	2.04	138.5	3.04	94
VO^{2+}-BCA (reconstituted)	4.56	2.12	4.33	0.09	2.9

[a] Experimental conditions: [BCA] = 1–5 $\times 10^{-6}$ M, [NphOAc] = 2–2.5 $\times 10^{-5}$ M, pH 7.55 Tris buffer, μ = 0.09, 25°, 0.1% acetone. [b] Concentration of assay solution. Determined with enzyme stock solution prior to dilution ($\epsilon_{280\ nm} = 5.6 \times 10^4$ cm^{-1} M^{-1}; Lindskog and Malström, 1962). See Experimental Section. [c] Determined spectrophotometrically using $\epsilon_{400\ nm} = 18{,}400$ cm^{-1} M^{-1} at $t = \infty$. [d] Calculated according to Pocker and Stone (1967). [e] Relative to the k_{enz} for native BCA.

major reaction catalyzed by carbonic anhydrase is the reversible hydration and dehydration of carbon dioxide. Because of the inherent experimental complication involved in assaying BCA for CO_2 activity, *e.g.*, stopped-flow methods and uncatalyzed reaction corrections, measurement of the esterase activity is frequently employed (Pocker and Stone, 1965, 1967, 1968a,b). Work by Thorslund and Lindskog (1967) points to a similar metal specificity and mechanistic scheme for ester hydrolysis and CO_2 hydration for carbonic anhydrase derivatives.

We have examined the esterase activity of apo-BCA, nave BCA, and the reconstituted Zn^{2+}- and VO^{2+}-BCA metal derivatives at 25° to ascertain the presence of any esterase activity for the vanadyl derivative. The results of a series of assays on these derivatives are given in Table II. From a comparison of the enzymatic rate constants, k_{enz}, and per cent activity relative to native BCA, we conclude that the vanadyl-BCA derivative is inactive. To date, only the Co^{2+} and Zn^{2+} metal derivatives display significant esterase and CO_2 hydration activity.

(B) INHIBITION STUDIES. Titration of VO^{2+}-BCA with inhibitors known to bind to the native Zn^{2+} metal or the active-site region of Co^{2+}- and Cu^{2+}-BCA metal derivatives was performed as described in the Experimental Section. The inhibitors studied include typical poisons such as CN^- and N_3^- as well as a specific carbonic anhydrase sulfonamide inhibitor, ethoxyzolamide. Although it was anticipated that binding of inhibitor molecules would significantly alter g and hyperfine parameters of the VO^{2+}-BCA spectrum, we found no effects for any of the inhibitors. Titration of the VO^{2+}-BCA derivative up to a 100-fold excess of CN^-, N_3^-, and ethoxyzolamide at pH 8.5 and N_3^- at pH 5.0 resulted in epr spectra identical with the VO^{2+}-BCA pectra in the absence of these inhibitors. Evidently these inhibitors do not enter the first coordination of the VO^{2+}; however, they may still bind to the enzyme. These results are significant in that we do observe binding of CN^- and N_3^- to VO^{2+} in the absence of enzyme. Possibly ligand sites of protein-bound VO^{2+} ion are no longer accessible to the bulk solvent due to the vanadyl oxygen which is absent in other metal derivatives. It is also possible that coordination takes place in the axial position to which epr is not as sensitive.

Ethoxyzolamide binding to VO^{2+} might not be expected since the inactive Hg(II) and Cd(II) derivatives bind sulfonamides more weakly than the active Zn(II) and Co(II) derivatives (Coleman, 1967; Mushak and Coleman, 1972).

Stability Constant Measurements. In addition to these characteristic changes in the doubling of the resonance lines as a function of pH, we observed a decrease in intensity of the VO^{2+}-BCA epr signal as the solution was titrated toward the alkaline pH range. The amplitude of the most intense first-derivative resonance signal[7] at 3400g (Figure 1) in the spectrum of VO^{2+}-BCA was found to decrease as one titrates the enzyme derivative from pH 5.30 to 9.58.

A plot of relative intensity of the VO^{2+}-BCA signal *vs.* pH is illustrated in Figure 7. Spectra were not recorded below pH 5 because of the reported initiation of acid denaturation of the bovine enzyme (Rosenberg, 1966; Beychok *et al.*, 1966). Approximately 1 hr elapsed between measurement of each point in Figure 7.

The origin of the intensity decrease of the VO^{2+}-BCA signal with increasing pH can be attributed to the competition between the protein and hydroxide ion for free vanadyl ion. The appearance of a faint $VO(OH)_2$ precipitate was observed as the pH was increased. Since definitive hydroxide equilibria data are not available for VO^{2+} solutions above pH 5, we assume that the free VO^{2+} concentration is governed by the solubility of $VO(OH)_2$,[8] $K_{sp} = (1.08 \pm 0.05) \times 10^{-22}$ (Francavilla and Chasteen, 1974).

By using the K_{sp} equation as the equilibrium expression governing the free VO^{2+} concentration, we have estimated the association constant for VO^{2+}-BCA as a function of pH from data of the pH titration experiment.

$$VO^{2+} + \text{apo-BCA} \rightleftharpoons VO^{2+}\text{-BCA}$$

$$K_{ass} = [VO^{2+}\text{-BCA}]/[VO^{2+}]_{free}[\text{apo-BCA}]$$

The VO^{2+}-BCA concentration was estimated from the intensity of the VO^{2+}-BCA signal. By using this method to calculate pK_{ass}, one obtains the pH dependence as exhibited in Figure 7. This plot is similar but with a different slope from that obtained for Zn^{2+}-BCA as reported by Thorslund and Lindskog (1967) using equilibrium dialysis. The log K_{ass} for VO^{2+}-BCA at pH 8.5 is 11.4 ± 0.5 from Figure 7 as compared with 13.2 for the native zinc enzyme (Thorslund and Lindskog, 1967).

[7] Its large amplitude relative to the rest of the signal is a consequence of the near coincidence of the $M_I = -1/2$ parallel and perpendicular principal resonance fields at only 22-G separation. The reduction in intensity with increasing pH is not due to oxidation of VO^{2+} since acidification of the solution produces a $VO(H_2O)_5^{2+}$ epr signal of full intensity. Also 5 $\times 10^{-4}$ M of the reducing agent dithionite was employed.

[8] Calculations using equilibrium values reported by Rossotti and Rossotti (1955) suggest that the concentrations of species such as $VO(OH)^+$ do not control the free VO^{2+} concentration above pH 5.

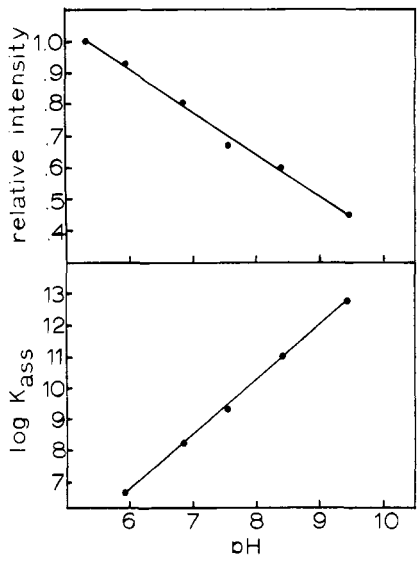


FIGURE 7: Data obtained from the pH titration of VO^{2+}-BCA at 25°, solution. (Upper) Plot of epr spectrum relative intensity of VO^{2+}-BCA complex *vs.* pH. Note that relative intensity refers to amplitude of the first-derivative line. (lower) Log K_{ass} *vs.* pH. Conditions: 2.5×10^{-4} M VO^{2+}-BCA and 5.0×10^{-4} M $Na_2S_2O_4$ in unbuffered solution. Description of experiment, details of calculations, and interpretation are described in the text.

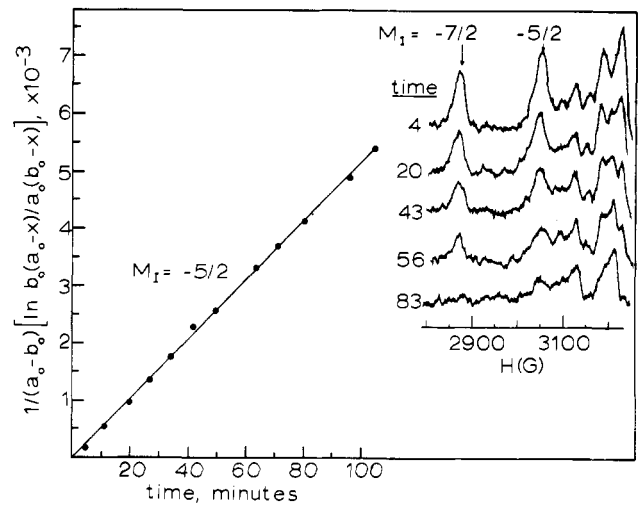


FIGURE 8: Second-order kinetic data obtained from the metal replacement reaction between VO^{2+}-BCA and Zn^{2+}. Plot of $1/(a_0 - b_0)[\ln b_0(a_0 - X)/a_0(b_0 - X)]$ *vs.* time (minutes). Kinetic data obtained by intensity measurements of the $M_I = -5/2$ line at pH 8.5 as shown in the insert. See Experimental Section for details. Definition of symbols given under Results and Discussion, Metal Exchange Kinetics section.

Metal Exchange Kinetics. The kinetics of metal exchange reactions in active sites of metalloenzymes has been little explored (Wilkins, 1973). Since the epr spectrum of the VO^{2+}-BCA complex can be readily observed with room temperature solutions, it occurred to us that the kinetics of the exchange of Zn^{2+} for VO^{2+} in VO^{2+}-BCA might be conveniently followed by epr spectroscopy.

The addition of an aliquot of Zn^{2+} ion to VO^{2+}-BCA in Tris buffer solution, pH 7.9 or 8.5 and 25°, results in displacement of VO^{2+} from the metal site according to the overall reaction $Zn^{2+} + VO^{2+}$-BCA $\rightarrow VO^{2+} + Zn^{2+}$-BCA (see Experimental Section for details).

Linear plots of $1/(a_0 - b_0)[\ln b_0(a_0 - x)/a_0(b_0 - x)]$ *vs.* time are obtained (Figure 8), where a_0 is the initial Zn^{2+} concentration, b_0 is the initial VO^{2+}-BCA concentration, and x is the concentration of Zn^{2+}-BCA formed. Thus, the metal replacement reaction follows second-order kinetics, *i.e.*

$$-d[VO^{2+}\text{-BCA}]/dt = k_2[VO^{2+}\text{-BCA}][Zn^{2+}]$$

At pH 8.5, 2.5×10^{-2} M Tris-HCl, the rate constant k_2 is 0.86 ± 0.01 and 1.02 ± 0.02 sec^{-1} M^{-1} from a linear regression on the data for the −5/2 and −7/2 "parallel" lines, respectively. At pH 7.9, 2×10^{-3} M Tris-HCl, K_2 is 1.38 ± 0.05 sec^{-1} M^{-1} from data on the $M_I = -1/2$ line.

In the only other metal exchange reaction of a metal derivative of BCA studied to date, Wilkins and Williams (1974) obtained a value of $k_2 = 1.30$ sec^{-1} M^{-1} for the Zn^{2+} displacement of Mn^{2+} from Mn^{2+}-BCA at pH 7.65. Interestingly enough, their k_2 value is close to that found for VO^{2+} carbonic anhydrase B at pH 7.9 reported here. The agreement might be coincidental. Metal exchange studies on other metal derivatives of BCA are needed to determine whether a common rate determining step is involved.

Spontaneous Dissociation Rate of VO^{2+}-BCA. The reduction in the intensity of the VO^{2+}-BCA epr signal with increasing pH (Figure 7) suggests that the "spontaneous" dissociation of VO^{2+} from the enzyme is rapid, at least on the order of minutes. We measured the rate of this reaction by adding a stoichiometric amount of Na_2EDTA to a VO^{2+}-BCA solution in 2×10^{-3} M Tris-HCl, pH 7.9. The stability constant of VO^{2+} binding with EDTA is 10^{18} (Sillen and Martell, 1964) *vs.* 10^{11} with the enzyme.

The appearance of the epr signal of VO^{2+}-EDTA was measured as a function of time (Figure 9). The reaction is first order in VO^{2+}-BCA concentration with a rate constant $K_d = 6.78 \times 10^{-4}$ sec^{-1} and a corresponding half-life of 17 min. This is quite fast relative to the Zn^{2+} enzyme which has a dissociation half-life of 5–6 years (Henkens and Sturtevant, 1968).

Addition of a 100-fold excess of EDTA to the VO^{2+}-BCA solution increases the first-order half-life of the dissociation from 17 to 25 ± 1 min. The reason for this is not clear.

We can estimate the second-order rate constant k_f for the recombination of VO^{2+} with the apoenzyme, *i.e.*, K_{ass} =

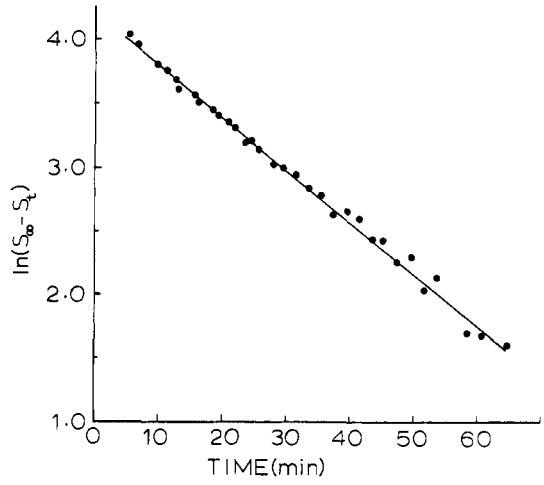


FIGURE 9: Plot of ln $(S_\infty - S_t)$ *vs.* time (minutes). S_∞ is the epr signal height in arbitrary units of the $M_I = -5/2$ line of VO^{2+}-EDTA at completion of the reaction. S_t is the signal height at any time t. The first-order rate constant k_d for the "spontaneous" dissociation of VO^{2+} from the protein is the negative of the slope.

K_f/k_d. With $K_{ass} \simeq 10^{11}$ M^{-1} and $k_d \simeq 10^{-3}$ sec^{-1}, one obtains a rather high value of $k_f \simeq 10^8$ sec^{-1} M^{-1}. The high stability of the VO^{2+}-BCA complex apparently resides in its rapid formation. This contrasts with Zn^{2+}-BCA and Mn^{2+}-BCA in which the K_{ass} is mainly governed by the "spontaneous" dissociation rate (Wilkins and Williams, 1974).

The k_f value for the reaction of VO^{2+} with apo-BCA is much larger than that observed for several other metal derivatives (Wilkins, 1973); k_f for Zn^{2+}-BCA is only 10^4 sec^{-1} M^{-1} (Henkens and Sturtevant, 1968). The slower rate of reaction of Zn^{2+} with the apoenzyme relative to VO^{2+} is nicely demonstrated by the fact that the VO^{2+}-BCA epr spectrum is generated when an equimolar mixture of VO^{2+} and Zn^{2+} is mixed with a stoichiometric amount of apoenzyme (1:1 Zn^{2+} and VO^{2+}–apo-BCA) at pH 8.5. The epr spectrum subsequently decays as Zn^{2+} displaces VO^{2+} from the enzyme according to the second-order rate law discussed in the previous section.

The rate for simple metal complex formation reactions is largely governed by the loss of water molecule from the first coordination sphere of the hydrated metal ion (Eigen and Wilkins, 1965), although this mechanism may not apply to complex proteins. The rate of exchange of equatorial water in $VO(H_2O)_5^{2+}$ is $\sim 5 \times 10^2$ sec^{-1} (Reuben and Fiat, 1967; Wüthrich and Connick, 1967, 1968) which is much too slow to account for the rapid reaction of VO^{2+} with the apoenzyme, $k_f \sim 10^8$ sec^{-1} M^{-1}. However, the rate of exchange of the axially coordinated water molecule is at least 10^8 sec^{-1} (Wüthrich and Connick, 1968) and could account for the large value of k_f. In the associative process, a coordinating imidazole group presumably binds first in an axial position.

Conclusion

The development of site-specific probes, whether spin labels, metal ion probes, or enzyme modification techniques, to investigate conformational, structural, and kinetic properties or enzymes has progressed rapidly in recent years. These advances have aided researchers in answering numerous questions concerning structure-activity relationships. The application of the vanadyl ion (VO^{2+}) as a site-specific epr probe in the metalloenzyme carbonic anhydrase has demonstrated its utility as (1) a metal ion probe capable of providing structural information about metal ions at the active sites of enzymes, (2) a technique enabling investigators to compare structural environments of metal sites in crystalline proteins to the often inconclusive structural data obtained under *in vitro* solution conditions, and (3) a kinetic probe useful in measuring metal replacement and equilibrium reactions.

In terms of structural detail, the VO^{2+} epr spectroscopic results of this study suggest that the ligand environment about the metal ion in the metal site of BCA probably consists of at least two histidines residues and two water molecules in equatorial positions at high pH in solution and over the total pH range in the powder state. Data on the "low pH form" of VO^{2+}-BCA suggest an additional protein residue, possibly histidine, is coordinated to the metal.

Correlations of solution, frozen solution, and powder data are consistent with the interpretation that the state of the VO^{2+} metal site in the crystalline enzyme is identical with that of the high pH form in frozen and room temperature solutions but that at low ph the metal site has undergone important conformational rearrangement. The pH profile of VO^{2+}-BCA epr parameters in solution (room temperature or frozen) is consistent with the results of other site-specific probe investigations, *e.g.*, Co^{2+} visible absorption, and Cu^{2+} epr spectroscopy, where an acid–base type equilibrium in carbonic anhydrase is also observed.

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

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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.