^{35}Cl-NMR STUDIES OF Co^{2+} CARBONIC ANHYDRASES*

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Summary: ^{35}Cl NMR studies of Co^{2+} substituted carbonic anhydrase (CA) reveal a difference in the pK of hydrolysis for the high and low specific activity forms of the enzyme in agreement with studies on the zinc enzymes. A time dependence was observed for the reaction of Co^{2+} with bovine apo CA but not with human B apo CA. A CN^- titration of the Co^{2+} CA indicates only one cobalt-chloride binding site. This work indicates that ^{35}Cl NMR can be used to monitor the interaction of Co^{2+} ions with proteins.

Substitution of Co^{2+} for Zn^{2+} in carbonic anhydrase results in an active enzyme with properties which are very similar to those of the native Zn^{2+} enzyme. The optical properties of the Co^{2+} enzyme have made it a highly suitable system for studies of the active site of CA. Recent ^{35}Cl NMR studies of the Zn^{2+} isozymes of CA have indicated an appreciable difference in the Zn^{2+} environments as measured by the pK's for hydrolysis of the metal ion.[1] It is of interest therefore to know whether these differences also pertain to the Co^{2+} system.

^{35}Cl NMR studies of the Zn^{2+} ion in low and high specific activity forms of CA reveal that the pK's for hydrolysis of the metal ion differ by approximately two units. Because the observed pK is a function of the total chloride concentration, it was necessary to plot the observed

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pK vs. log $(1 + Cl/K_i)$ to determine the pK at zero chloride. Upon extrapolation to zero chloride, values of 8.19 and 6.4 were obtained for human B and C CA respectively. In general, however, the value of the pK determined in 0.5 M NaCl can be used for comparative purposes. The pK's for human B and C in 0.5M NaCl are 9.22 and 7.42 while the value for the bovine enzyme is 7.3. These values are good to 0.1 of a unit.[1]

Bovine and Human B apo CA were prepared by dialysis against 0.01M o-phenanthroline at pH 5.5. Addition of 1 mole of Zn^{2+} per protein molecule produced a fully active enzyme. The assay used was the hydrolysis of p-nitrophenylacetate.

A Co^{2+} titration of bovine apo CA appears in Figure 1. Each point results from the addition of one μl of 10^{-3}M $CoCl_2$ to a solution of apo CA in 0.5M NaCl and buffered at pH 6.15. Each point is the average of eight line width measurements. The end point is reasonably well defined at a molar ratio of one Co^{2+} per apo CA molecule. Similar results were obtained with human CA-B.

^{35}Cl NMR line width measurements indicate that the rate of formation of the final Co^{2+}-bovine CA complex is slow. If Co^{2+} is added to a solution of bovine apo CA at a pH of ~6 at a molar ratio of 1:1 the ^{35}Cl NMR line width increases for a number of hours until a maximum value is reached. If Zn^{2+} is added, however, no time lag is observed in obtaining a maximum ^{35}Cl line broadening. No such time lag was observed upon the addition of Co^{2+} or Zn^{2+} to human apo CA-B. These observations are in agreement with the reactivation of the enzymes as determined by kinetic means.[2]

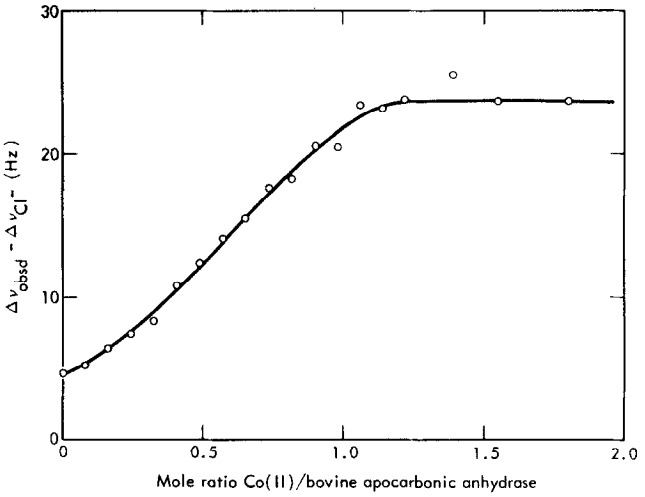

Fig. 1. Line width, $\Delta\nu_{obsd}-\Delta\nu_{Cl^-}$(Hz), vs. mole ratio Co^{2+}/apo bovine carbonic anhydrase. The solution was 0.5M NaCl, 0.54 mg per ml of apo bovine carbonic anhydrase and 0.05M Bis-Tris, pH 6.15.

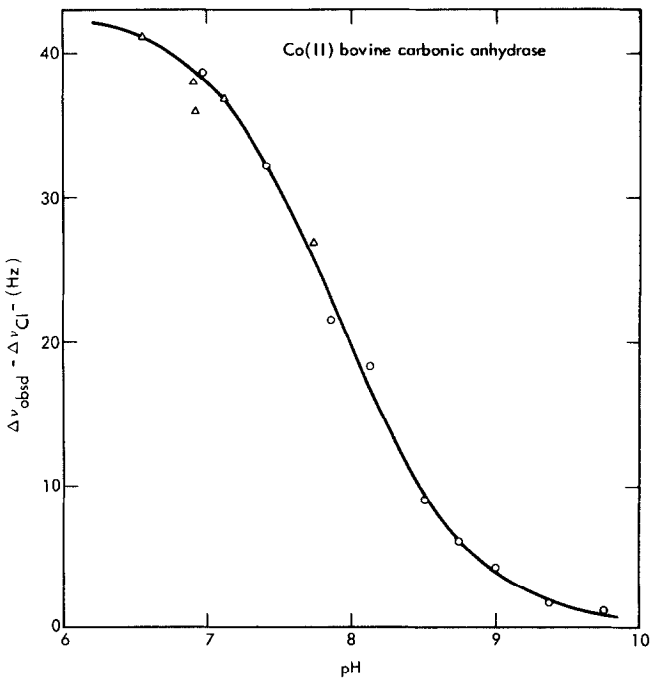

Fig. 2. Line width, $\Delta\nu_{obsd}-\Delta\nu_{Cl^-}$(Hz), vs. pH for a solution containing 0.86 mg per ml of apo bovine carbonic anhydrase and 2.86×10^{-5}M Co^{2+} in 0.5M NaCl. The pK value is 7.94.

The pH titrations of Co^{2+} substituted bovine CA and human CA-B in 0.5M NaCl appear in Figures 2 and 3. In both cases the solutions were prepared and stored overnight at 4^{o} before the pH titration was carried out. The pK for the Co^{2+} substituted bovine enzyme is 7.94 while that for human B is 9.06. The pK for Co^{2+} human CA-B agrees with that of the zinc protein, whereas the Co^{2+} substituted bovine enzyme is ~0.5 of a unit higher. The difference in pK's for the two Co^{2+} inzymes is in good agreement with the similar difference in the Zn^{2+} proteins.

An interesting question about the Co^{2+} enzyme concerns the number of metal coordination sites occupied by the protein and available for solvent interaction. Dennard and Williams[3] have discussed this point

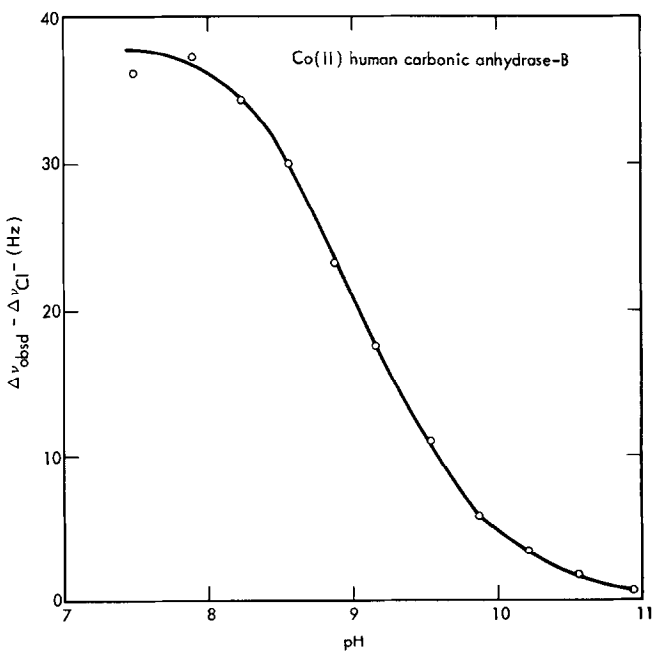

Fig. 3. Line width, $\Delta\nu_{obsd}-\Delta\nu_{Cl^-}$ (Hz), vs. pH for a solution containing 0.92 mg per ml of apo human carbonic anhydrase B and 3.1×10^{-5}M Co^{2+} in 0.5 M NaCl. The pK value is 9.06.

at length. In a manner analogous to studies with the Zn^{2+} enzymes[1] Co^{2+} human CA-B was titrated with KCN at pH 8. The addition of KCN reduces the ^{35}Cl line width to that of aqueous Cl^-. The titration is reasonably sharp with an endpoint at a molar ratio of one CN^- to one Co^{2+}. This data indicates only one available Cl^- coordination site and presumably one H_2O site on the Co. If the protein supplied the same number of ligands to the Co^{2+} as it does to the Zn^{2+} this would indicate a four coordinate Co^{2+} ion. It should be noted, however, that whereas the optical spectrum of the cyanide substituted Co^{2+} enzyme agrees well with that expected for a tetahedral Co^{2+} the spectrum of aquo Co^{2+} enzyme is not as well understood. The possibility exists that CN^- produces a conformational change which results in a tetrahedral species.[3]

References

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