Tr. J. of Chemistry
21 (1997) , 134 – 138.

Electron Spin Resonance Studies on Cobalt Carbonic Anhydrase-Substrate Complexes

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Received 3.9.1996

The native zinc atom of bovine erythrocyte carbonic anhydrase purified by affinity choromatography was removed by dialysis against pyridine 2,6-dicarboxylic acid. Cobalt carbonic anhydrase was prepared from the zinc-free apoenzyme. The binding conditions of CO_2 and p-nitrophenylacetate to cobalt carbonic anhydrase were investigated by electron spin resonance at different pH levels.

Key words: Bovine carbonic anhydrase, ESR spectroscopy, substrate complexes.

Introduction

Bovine carbonic anhydrase (BCA) is a monomeric zinc(II) containing an enzyme moleculer weight of 29.000, which catalyzes the reversible hydration of CO_2 to H_2CO_3, the hydration of aldehydes and the hydrolysis of esters[1,2]. Cobalt (II) is the only paramagnetic ion that can substitute for zinc and retain substantially the same activity as the parent enzyme[3]. Many physical and spectroscopic measurements have been carried out in order to elucidate the binding and the stereochemistry at the metal site. The electron spin resonance (ESR) spectra of Cu(II) and Co(II) derivatives of human, monkey, and bovine carbonic anhydrases and their inhibitor complexes have been reported at different pH levels[4,5,6]. The effects of temperature and solvent on the enzyme have been also studied by ESR[7]. These ESR studies and studies of anionic derivatives of Co(II)CA by a pH-dependent visible absorption spectrum[8,9] show a characteristic four or five coordinate environments about the metal ion. The zinc ion is bonded to its three histidine ligands and a fourth OH^- ligand in a tetrahedral configuration. The active site is amphiphilic; one wall is dominated by hydrophobic residues and the other by hydrophilic residues[10]. Although the crystal structures of this enzyme alone [11] and with bound inhibitors[12] have been solved, the substrate binding sites have not yet been clearly elucidated. NMR studies[13] suggest that bicarbonate is directly related to zinc, while both NMR[13] and IR[14] studies suggest that CO_2 is not covalently bound to zinc. Molecular dynamics and free energy perturbation simulations carried out in two laboratories[15,16] suggest several possible CO_2 binding sites. Since CO_2 and p-nitrophenylacetate being substrates of BCA have not been used in analysis of the enzyme structure by ESR, the purpose of this study was to gain more information about this analysis by investigating the ESR spectra of the low and high pH forms of Co(II) BCA-substrate complexes.

Materials and Methods

Purification of Bovine Carbonic Anhydrase: Erythrocytes were purified from bovine blood obtained from the Erzurum Slaughterhouse. The blood was centrifuged at 1500 rpm for 15 minutes and the plasma and buffy coat were removed. The erythrocytes were washed with NaCl (0.9 %) twice and hemolyzed with cold water. The erythrocyte membranes were removed by spining down for 15 min at 4000 rpm. The pH of the hemolysate reached 8.7 with solid Tris. The hemolysate was applied to an affinity column having a structure of Sepharose-4B-L-tyrosine sulfanylamide and equilibrated with 25 mM Tris-HCl/0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with a solution of 25 mM Tris HCl/22 mM Na_2SO_4 (pH 8.7). The carbonic anhydrase was eluted with a solution of 0.1M $NaCH_3COOO$/ 0.5M $NaClO_4$ (pH 5.6)[17]. Specific activities for hemolysate and purified enzyme solutions were calculated with the methods of Wilbur and Anderson[18] as modified by Rickli et al.[19] for the enzyme activity determination and Coomassie-blue[20] for protein determination, yielding a purification of $\sim$ 400 folds (Table 1).

Table 1. Purification of carbonic anhydrase by affinity chromatography

Enzyme	Fraction	Volume (ml)	Activity (EU/ml)	Total Activity (EU	%)	Protein (mg/ml)	Specific Activity (EU/mg)	Purification (n-Fold)
	Hemolysate	50	0.749	37.45	100	143.8	0.00521	-
BCA								
	Affinity Column	50	0.608	30.4	81	0.286	2.13	408.8

Preparation of Bovine Apocarbonic Anhydrase: The purified bovine carbonic anhydrase was concentrated with Sephadex G-25 in a batchwise process and was dialyzed first against distilled water and then against Tris-SO_4 (0.05M, pH 7.4). The resulting enzyme ($\sim$ 100 mg) was dialyzed against 0.075 M pyridine 2,6-dicarboxylic acid in 0,2 M phosphate buffer (pH 7.4) for 5 hours in order to remove the Zn^{2+} from the enzyme[21]. The resulting apoenzyme measured by esterase activity[22] was prepared with a yield of 100 %.

Preparation of Bovine Cobalt Carbonic Anhydrase: The mixed solution of 1 ml 3.6×10^{-5} M apocarbonic anhydrase and 4 ml 2.85×10^{-5} M $CoSO_4.7H_2O$ was dialyzed first against the same $CoSO_4.7H_2O$ solution for 24 hours in order to place the Co^{2+} and then against distilled water for 24 hours in order to remove unbonded Co^{2+}.

ESR Spectra: The spectra at 19°C were obtained on a Varian E-104 Spectrometer using a 100 kHz modulation frequency. The ESR spectrometer settings were accumulated: microwave power, 100 mW; frequency, 9.51 GHz; modulation amplitude, 40 G; time constant, 1.0 sn; scan time, 2 min with 32 scans. The pH values of Co(II)BCA medium were changed by 1 M NaOH and 1 M HCl. 3 mM p-nitrophenylacetate and cold CO_2-saturated solutions were used to make Co(II)BCA-substrate complexes.

Results and Discussion

The native (zinc II) carbonic anhydrase was purified from bovine erythrocytes by affinity chromatography, yielding a purification of $\sim$ 400 folds. Then zinc-free apocarbonic anhydrase with a yield of 100% was prepared from the purified native carbonic anhydrase by dialysis against pyridine 2,6-dicarboxylic acid[21]. Since cobalt(II) is the only paramagnetic ion that can substitute for zinc and retain substantially the same

activity as the parent enzyme[3], cobalt (II) bovine carbonic anhydrase was prepared from the zinc-free apocarbonic anhydrase.

As the Co(II)BCA was used in analysis of the enzyme structure by different techniques, the Co(II)BCA was also used in our study to obtain more information about Co (II)BCA-substrate complexes by ESR. First, for comparison with other ESR spectra, the ESR spectrum of a solution of $CoSO_4.7H_2O$ was obtained at pH 7 (Figure 1). The spectrum consists of a width intensity line with g=2.192 at 3100 G and six lines with $g \cong 2$ and A = 95.26 centered at 3400 G (shown with arrows in Figure 1). Secondly, the ESR spectra of Co(II)BCA at different pH are given in Figure 2, along with the ESR parameters obtained from these spectra. The g values obtained for the with intensity line at low field given in Table 2 are almost same as those (g exp = 2.278 and gcal = 2.340) obtained for Co^{2+} in CdF_2 [22]. Therefore, this line was attributed to Co^{2+}, which has the electronic configuration $3d^7$. Therefore, Co^{2+} has S = 3/2 and I = 7/2. Because of linewidth, the hyperfine in the spectra was not observed[23]. The six lines in the spectra belong to Mn^{2+}, which has the electronic configuration $3d^5$ and a total spin of S = 5/2 and a nuclear spin of I = 5/2. That gives the usual sextet of lines in the ESR solution spectra at room temperature. When the ESR spectrum of Co(II)BCA at pH 7 is compared with the spectrum of the solution of $CoSO_4.7H_2O$, it is seen that g values increase because of Co^{2+} in the active side of enzyme. While the intensity of the ESR line of Co^{2+} decreases at pH 5, it almost disappears at pH 8. These results are also valid for an impurity of Mn^{2+}. These results showed that the spectra of Co(II)BCA are pH-dependent and agree with the ESR spectra of Co(II)BCA obtained at low temperature[24], and the visible absorption spectra of Co(II)BCA[8,9]. It can be expressed in our ESR studies, as in previous studies[8,9], that the acidic form of the enzyme maintains an equilibrium distribution of 4 and 5 coordinate forms, and the alkaline form of enzyme maintains a tetrahedron form, because of an environment of a Co^{2+} active site with changed pH.

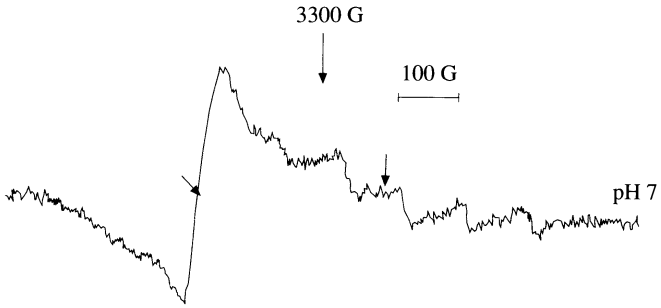

Figure 1. ESR spectrum of solution of $CoSO_4.7H_2O$ at pH 7 obtained at 19 °C.

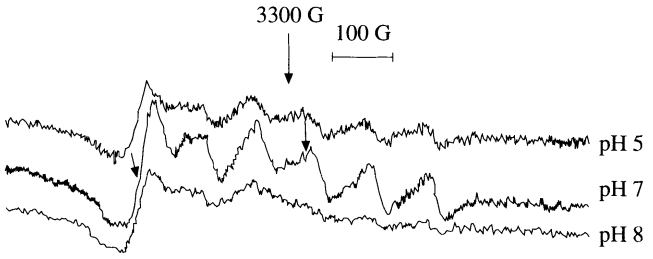

Figure 2. ESR spectra of Co(II)BCA obtained at different pH levels at 19 °C.

In order to analyze Co(II)BCA-substrate complexes, which have not been investigated by ESR, CO_2 and p-nitrophenylacetate were used as substrates of Co(II)BCA. The ESR spectra of Co(II)BCA-CO_2, and p-nitrophenylacetate complexes were obtained at different pH levels. Although the spectra of the complex with CO_2 at pH 2 and 5 (Figure 3) are almost the same as those of Co(II)BCA at pH 5 and 7 (Figure 2), the line of Co^{2+} in the spectra of the complex with CO_2 almost disappeared at pH 9 (Figure 3). These comments are also valid for an impurity of Mn^{2+}. These results showed that the spectra of the Co(II)BCA-CO_2 complex are also pH-dependent. In addition, the spectrum of acidic complexes, particularly with CO_2 at pH 2, exhibited that the environment of cobalt at the active site of the enzyme is almost stable because interaction of CO_2 with all active site residues is important for stabilizing different intermediate states during the binding process[15]. Finally, the 5 coordinate form of the enzyme becomes stable with CO_2 at an acidic pH, at which the OH^- coming from water and bound Co^{2+} nucleophilically attacks CO_2[15]. But the spectrum of the complex with CO_2 at pH 9 exhibited that CO_2 bound to the active site is converted

to HCO_3^- as a result of nucleophilically attacking OH^- and being removed from the complex[15]. Thus, Co(II)BCA is converted to a tetrahedral structure.

The ESR spectra of Co(II)BCA-p-nitrophenylacetate complexes were obtained at different pH levels (Figure 4). While the spectra of complexes with p-nitrophenylacetate at pH 5 and 7 (Figure 4) were the same as those of Co(II)BCA at pH 5 and 7 (Figure 2), the spectrum of this complex almost disappeared at pH 2 (Figure 4). These results indicate that the binding of p-nitrophenylacetate is different than that of CO_2 because of the required pH for stable binding. When the g values of Co^{2+} in Co(II)BCA-CO_2 and Co(II) BCA-p-nitrophenylacetate are compared with each other against the g values of Co^{2+} in Co(II)BCA as a reference, they are seen to be different (Table 2). Since substrates of BCA are different, their catalytic mechanisms should be different as well. The efficiency must arise from the specific physical conditions at enzyme catalytic sites. The most obvious effects are proximity and orientation. Reactants must come together with the proper spatial relationship for a reaction to occur[25]. g and A values obtained for impurity Mn^{2+} are also given in Table 2.

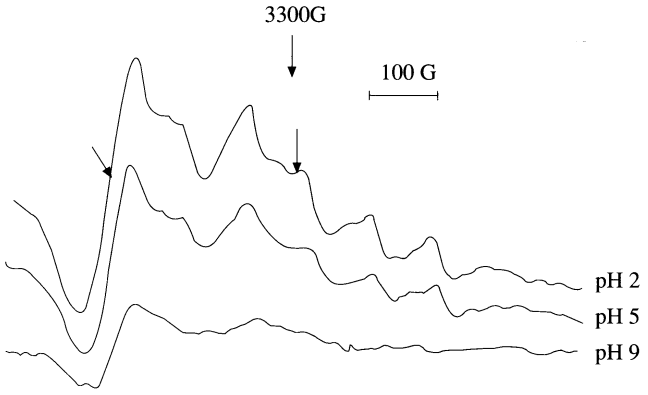

Figure 3. ESR spectra of Co(II)BCA-CO_2 complex obtained at different pH levels at 19 ° C.

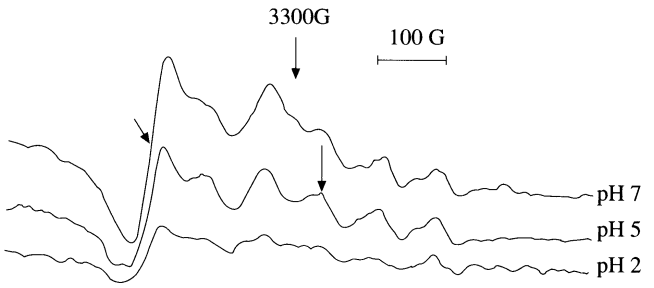

Figure 4. ESR spectra of Co(II)BCA-p-nitrophenylacetate complex obtained at different pH levels at 19 ° C.

Table 2. ESR spectral parameters for substrate complexes of Co(II)BCA as a function of pH at 19 ° C (The values of the spectra were determined by graphical methods).

		Co^{2+}	Mn^{2+}	
Complex	pH	g	g	A(G)
Co(II)BCA	5	2.228	2.043	98.20
	7	2.228	2.043	98.20
	8	2.228	not-observed	not-observed
Co(II)BCA-CO_2	2	2.280	2.053	109.20
	5	2.280	2.053	111.80
	9	2.280	not-observed	not-observed
Co(II)BCA-p-nitro -phenylacetate	2	2.233	2.028	97.40
	5	2.233	2.028	96.90
	7	2.233	2.028	96.30

When all of the spectra obtained under different conditions are compared, interpretation of Co(II)BCA-substrate complexes by ESR studies can be made. First, both Co(II)BCA and Co(II)BCA-substrate complexes are pH-dependent. Secondly, while Co(II)BCA-CO_2 complex requires an acidic pH, Co(II)BCA-p-nitrophenylacetate complex requires a neutral pH because of the orientation of substrates. Thirdly, different

residues are involved in the binding of CO_2 and p-nitrophenylacetate. These results may help to explain the CA-substrate complex structure.

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