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Full Length Research Paper

A thermodynamic investigation of bovine carbonic anhydrase II interaction with cobalt ion at 300 and 310K

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A thermodynamic study on the interaction of bovine carbonic anhydrase II, CAII, with cobalt ions was studied by using isothermal titration calorimetry (ITC) at 27 and 37°C in tris buffer solution at pH = 7.5. The heats of Co^{2+}+CAII interaction are reported and analysed in terms of the new solvation theory. It was indicated that there are three identical and non-cooperative sites for Co^{2+}. The binding of a cobalt ion is exothermic with dissociation equilibrium constants of 81.306 and 99.126 µM at 27 and 37°C respectively. The binding of cobalt ions can cause some changes in structure of enzyme, which results in a decrease in the activity and stability of the enzyme.

Key words: Bovine carbonic anhydrase, cobalt ion, isothermal titration calorimetry.

INTRODUCTION

The carbonic anhydrase, CA, is ubiquitous zinc enzymes, presents in Archaea, prokaryotes and eukaryotes, being encoded by three distinct, evolutionarily unrelated gene families: the α–CA, β-CA and the γ-CA (Sly and Hu, 1995; Lyer et al., 2006; Sarraf et al., 2004; Supuran et al., 2001). CA is one of the fastest enzymes known, with a maximal turnover rate for CO_2 hydration of ~10^6 s^{-1} at 25°C, which catalyze the reversible hydration of CO_2 to form HCO_3^- and protons according to the following reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. The first reaction catalyst by carbonic anhydrase and second reaction occurs in stantaneously, which is probably the reason why the activation of CA has not been much studied. In contrast, inhibition of CA has been widely investigated and several crystal structures of CA complexes with inhibitor molecules have been reported (Sly and Hu, 1995; Lyer et al., 2006; Sarraf et al., 2004; Supuran et al., 2001; Bertini et al., 1983). Seven distinct isozymes are presently known in higher vertebrates, though their physiological function is not completely known.

CAII is novel as a metal protein due to its unusually high affinity for zinc, so that the CAII+Zn^{2+} dissociation constant is 1 - 10 pM (Sly and Hu, 1995). The role of highly conserved aromatic residues surrounding the zinc binding site of human carbonic anhydrase II (CAII) in determining the metal ion binding specificity of this enzyme has been previously examined by mutagenesis (Lindskog and Nyman, 1964; Sarraf et al., 2005). Residues F93, F95, and W97 are located along a β-strand containing two residues that coordinate zinc, H94 and H96, and these aromatic amino acids contribute to the high zinc affinity and slow zinc dissociation rate constant of CAII. Substitutions of these aromatic amino acids with smaller side chains enhance the copper affinity (up to 100-fold) while decreasing the affinity of both cobalt and zinc, thereby altering the metal binding specificity up to 10^4-fold. Furthermore, the free energy of the stability of native CAII, determined by solvent-induced denaturation, correlates positively with increased hydrophobicity of the amino acids at positions 93, 95, and 97 as well as with cobalt and zinc affinity (Stadie and O'Brien, 1933; Nishino et al., 1999). Conversely, increased copper affinity correlates with decreased protein stability. Although CAII is loaded with zinc in its physiologically relevant format, it can bind a number of other metal ions in the zinc binding site, such as Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+} with various affinities (Supuran et al., 2001; Sarraf et al., 2005).

Some Zn (II) and Cu (II) metal complexes of sulfonamides

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incorporating polyaminopolycarboxylated tails have also been reported, which indeed showed very good in vitro CA inhibitory activity against isoforms CA I, II, and IV. Rami et al reported the preparation and inhibition assay of some Cu (II) complexes of aromatic/heterocyclic sulfonamides incorporating EDTA and DTPA tails. In addition, such copper (II) derivatives with potent CA IX/XII inhibitory activity might also be important for developing positron emission tomography (PET) imaging agents for tumor hypoxia (Winum et al., 2007; Alzuet et al., 1998). In this paper the effect of the cobalt ion on the structure and stability of the CAII, in addition to some investigations on the binding parameters of Co^{2+} to the enzyme has been considered.

MATERIALS AND METHODS

Erythrocyte bovine carbonic anhydrase was obtained from Sigma. Copper sulfate was obtained from Merck. The buffer solution used in the experiments was 50 mM Tris, pH = 7.5, which was obtained from Merck. All the experiments were carried out in 300 and 310 K. The experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. Each channel is twin heat conduction calorimeter (multijuction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. Both sample and reference vessels were made from stainless steel. The limited sensitivity for the calorimeter is 0.1 μ cal. cobalt nitrate solution (5 mM) was injected by use of a Hamilton syringe into the calorimetric titration vessel, which contained 1.8 mL CA, 30 µM, in Tris buffer (30 mM), pH = 7.5. Thin (0.15mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of copper nitrate solution into the perfusion vessel was repeated 30 times and each injection included 20µL cobalt nitrate solution. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the cobalt solution was measured as described above except CAII was excluded. Also, the heat of dilution of the protein solution was measured as described above except that the buffer solution was injected to the protein solution in the sample cell. The enthalpies of copper and protein solutions dilution were subtracted from the enthalpies of cobalt nitrate solutions in CAII solutions. The determined enthalpies for Co^{2+}+CAII interactions, were listed in Table 1 (in µJ). The micro-calorimeter was frequently calibrated electrically during the course of the study.

RESULTS AND DISCUSSION

It has been shown previously (Rezaei et al., 2006; Rezaei and Saboury, 2007; Rezaei et al., 2008; Rezaei and Saboury, 2008; Rezaei et al., 2008) that the enthalpies of interactions of biopolymers with ligands (Co^{2+}+CAII in this case) in the aqueous solvent (Co^{2+}+water in the present case) mixtures, can be reproduced via the following equation:

$$q = q_{\max} x'_B - \delta^{\theta}_A (x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

The parameters δ^{θ}_A and δ^{θ}_B are the indexes of the CAII stability as a result of interaction with Co^{2+} in the low and high Co^{2+} concentrations respectively. If the binding of ligand at one site increases the affinity for ligand at another site, the macromolecule exhibits positive co-operativity. Conversely, if the binding of ligand at one site lowers the affinity for ligand at another site, the protein exhibits negative cooperativity. If the ligand binds at each site independently, the binding is non-cooperative. $p < 1$ or $p > 1$ indicate positive or negative cooperativity of macromolecule for binding with ligand respectively; $p = 1$ indicates that the binding is non-cooperative. x'_B can be expressed as follows:

$$x'_B = \frac{p x_B}{x_A + p x_B} \quad (2)$$

x_B is the fraction of the Co^{2+} needed for saturation of the binding sites, and $x_A = 1 - x_B$ is the fraction of unbounded Co^{2+}. We can express x_B fractions, as the total Co^{2+} concentrations divided by the maximum concentration of the Co^{2+} upon saturation of all CAII as follows:

$$x_B = \frac{[Co^{2+}]_T}{[Co^{2+}]_{\max}} \qquad x_A = 1 - x_B \quad (3)$$

$[Co^{2+}]_T$ is the total concentration of cobalt and $[Co^{2+}]_{max}$ is the maximum concentration of the cobalt upon saturation of all CAII. In general, there will be "*g*" sites for binding of Co^{2+} per CAII molecule. L_A and L_B are the relative contributions of unbounded and bounded Co^{2+} to the enthalpies of dilution with the exclusion of CAII and can be calculated from the enthalpies of dilution of Co^{2+} in buffer, q_{dilut}, as follow:

$$L_A = q_{dilut} + x_B \left(\frac{\partial q_{dilut}}{\partial x_B} \right) \quad ,$$

$$L_B = q_{dilut} - x_A \left(\frac{\partial q_{dilut}}{\partial x_B} \right) \quad (4)$$

The heats of Co^{2+}+CAII interactions, q, were fitted to Equation 1 over the whole range of Co^{2+} compositions. In this procedure, the only adjustable parameter (*p*) was changed until the best agreement between the experimental and calculated data was approached (Figure 1).

The optimized δ^{θ}_A and δ^{θ}_B values are recovered from the coefficients of the second and third terms of equation

Table 1. The heats of Co^{2+}+CAII interactions, q, at 300 K (O) and 310 K (ϒ). qdilut are the heats of dilution of $Co(NO_3)_2$ with water. Precision is ±0.100 µJ or better.

$[Co^{2+}]$ / mM	[CAII] / µM	q (O) / µJ	q_{dilut} (O)/ µJ	q (ϒ) / µJ	q_{dilut} (ϒ)/ µJ
0.055	29.670	-635.3	-424.8	-563.5	-398.3
0.109	29.348	-1065.4	-790.4	-954.2	-742
0.161	29.032	-1350.6	-1089.6	-1222.6	-1021.5
0.213	28.723	-1543.7	-1344	-1410.7	-1260
0.263	28.421	-1679.4	-1562.4	-1546.7	-1464.7
0.312	28.125	-1778.5	-1740	-1648.3	-1631.2
0.361	27.835	-1853.4	-1895.2	-1726.5	-1776.7
0.408	27.551	-1911.7	-2028.8	-1788.2	-1902
0.454	27.273	-1958.2	-2140.8	-1838	-2008
0.500	27.000	-1996.1	-2232	-1879	-2092.5
0.544	26.733	-2027.6	-2316.5	-1913.2	-2171.3
0.588	26.470	-2054.1	-2390.1	-1942.2	-2240.3
0.631	26.214	-2076.7	-2456.5	-1967.1	-2302.5
0.673	25.962	-2096.2	-2510.9	-1988.6	-2353.5
0.714	25.714	-2113.2	-2561.8	-2007.4	-2401.7
0.755	25.472	-2128.1	-2605	-2024	-2442.2
0.794	25.233	-2141.3	-2644.8	-2038.8	-2479.7
0.833	25.000	-2153.1	-2681.6	-2052	-2513.2
0.872	24.771	-2163.7	-2715.2	-2063.9	-2544.7
0.909	24.545	-2173.2	-2745	-2074.6	-2572.4
0.946	24.324	-2181.8	-2771.4	-2084.3	-2597.2
0.982	24.107	-2189.7	-2794.8	-2093.2	-2618.9
1.018	23.894	-2196.9	-2816.4	-2101.3	-2640.2
1.053	23.684	-2203.5	-2836.2	-2108.8	-2658.9
1.087	23.478	-2209.6	-2854.6	-2115.7	-2676.6
1.121	23.276	-2215.2	-2870.4	-2122.1	-2691.6

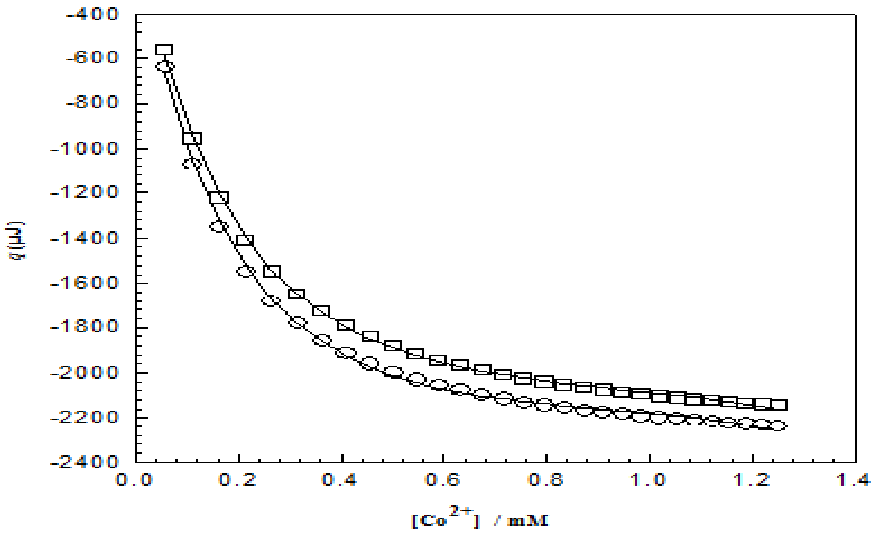

Figure 1. Comparison between the experimental heats for Co^{2+}+CAII interactions at 300 K (O) and 310 K (ϒ) and calculated data (lines) via Equation 1.

coefficients of the second and third terms of Equation 1. The agreement between the calculated and experimental results (Figure 1) is excellent, and gives considerable support to the use of Equation 1.

Φ is the fraction of CAII molecule undergoing complexation with Co^{2+} which can be expressed as follows:

$$\Phi = \frac{q}{q_{\max}} \quad (5)$$

$q_{\max}$ represents the heat value upon saturation of all CAII. The appearance equilibrium constant values, K_a, as a function of free concentration of Co^{2+}, $\left[Co^{2+}\right]_F$, can be calculated as follows:

$$K_a = \frac{\Phi}{(1-\Phi)[Co^{2+}]_F} = \frac{\Phi}{(1-\Phi)[Co^{2+}]_T (1-x_B)} \quad (6)$$

The standard Gibbs free energies as a function of Co^{2+} concentrations can be obtained as follows:

$$\Delta G^o = -RTLnK_a \quad (7)$$

The standard Gibbs energies, ΔG^o, at different temperatures calculated from Equation 7 have shown graphically in Figure 2. $T\Delta S$ values were calculated using ΔG^o values at different temperatures and have shown in Figure 3.

Consider a solution containing a ligand (Co^{2+}) and a macromolecule (CAII) that contains "*g*" sites capable of binding the ligand. If the multiple binding sites on a macromolecule are identical and independent, the ligand binding sites can be reproduced by a model system of monovalent molecules $[(CAII)_g \rightarrow g(CAII)]$ with the same set of dissociation equilibrium constant, K_d, values. Thus, the reaction under consideration can be written:

$$M + L \Leftrightarrow ML \qquad K_d = \frac{[M][L]}{[ML]} \quad (8)$$

If α is defined as the fraction of free binding sites on the biomacromolecule, M_0 is the total biomacromolecule concentration, and L_0 is the total ligand concentration, then the free concentrations of monovalent molecule [M] and ligand [L] as well as the concentration of bound ligand [ML] can be deduced as follows:

$$[ML] = g(1-\alpha)M_0 \quad (9)$$

$$[L] = L_0 - [ML] = L_0 - g(1-\alpha)M_0 \quad (10)$$

$$[M] = gM_0 - [ML] = gM_0 - g(1-\alpha)M_0 = \alpha g M_0 \quad (11)$$

Substitution of free concentrations of all these components in Equation (8) gives:

$$K_d = (\frac{\alpha}{1-\alpha})L_0 - \alpha g M_0 \quad (12)$$

or

$$\alpha M_0 = (\frac{\alpha}{1-\alpha})\frac{1}{g}L_0 - \frac{K_d}{g} \quad (13)$$

The value of 1 – α as the fraction of occupied binding sites on the biomacromolecule:

$$1-\alpha = \frac{q}{q_{\max}} \quad (14)$$

Where *q* represents the heat value at a certain L_0 and q_{max} represents the heat value upon saturation of all biomacromolecules. If *q* and q_{max} are calculated per mole of biomacromolecule then the molar enthalpy of binding for each binding site (ΔH_{bin}) will be:

$$\Delta H_{bin} = \frac{q_{\max}}{g}$$

The combination of Equations 13 and 14 yields:

$$\frac{\Delta q}{q_{\max}} M_0 = (\frac{\Delta q}{q})L_0 \ \frac{1}{g} - \frac{K_d}{g} \quad (15)$$

Where $\Delta q = q_{\max} - q$. Therefore, the plot of $\frac{\Delta q}{q_{\max}} M_0$ versus $\frac{\Delta q}{q} L_0$ should be a linear plot with a slope of $\frac{1}{g}$ and a vertical-intercept of $\frac{K_d}{g}$.

The linearity of the plot has been examined by different estimated values for q_{max} to reach the best value for the correlation coefficient. The best linear plot with the correlation coefficient value ($r^2 \approx 1$) was obtained using -2398 µJ and -2333 µJ (equal to -22.204 kJ/mol and -21.602 kJ/mol at 27 and 37°C respectively). The values of *g* and

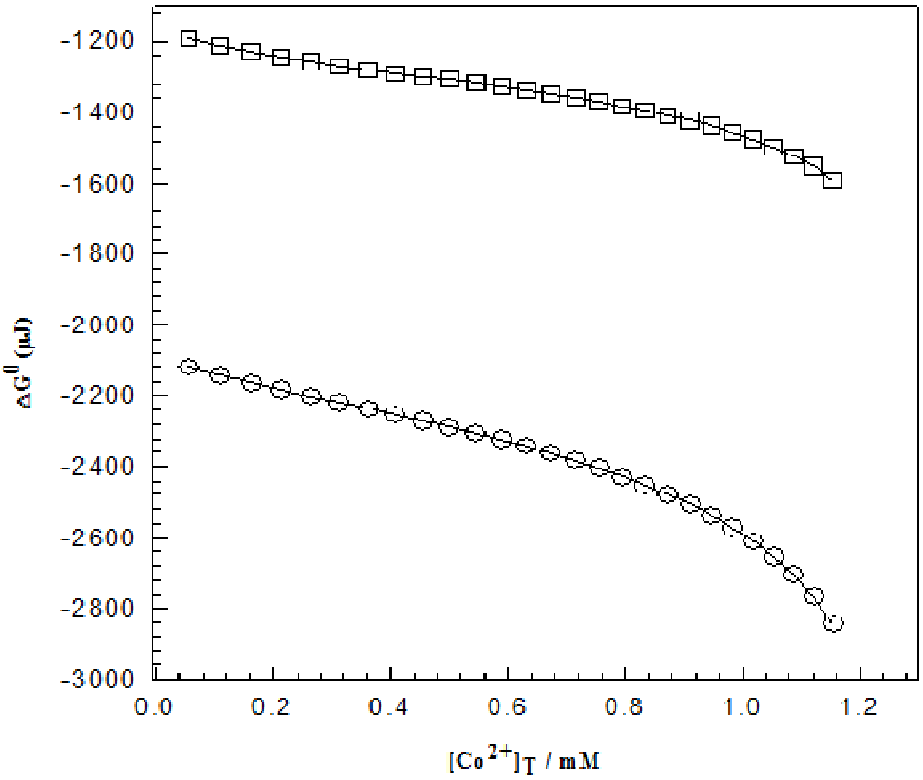

Figure 2. Comparison between the experimental Gibbs free energies at 300 K (O) and 310 K (ϒ) for Co^{2+}+CAII interactions in and calculated data (lines) via Equations 7 and 8.

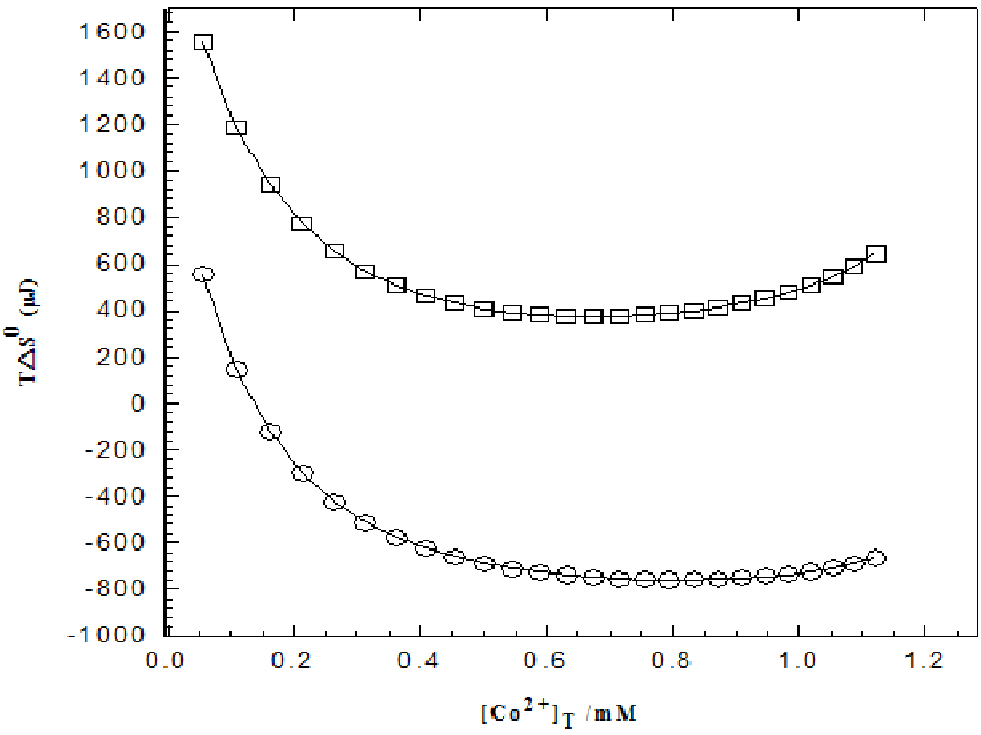

Figure 3. Comparison between the experimental entropies at 300 K (O) and 310 K (ϒ) for Co^{2+}+CAII interactions in and calculated data (lines) via equations 7 and 8.

values of *g* and K_d, obtained from the slope and vertical-intercept plot, are listed in Table 2. The calorimetric method described recently allows obtaining the number of binding sites (g), the molar enthalpy of binding site (ΔH_{bin}) and the dissociation equilibrium constant (K_d) for a set of biomacromolecule binding sites. The lack of a suitable value for q_{max} to obtain a linear plot of □ ($\Delta q/q_{max}$) *M0 vs.* (□$\Delta q/q$) *L0* may be attributed to the existence of non-identical binding sites or the interaction between them. Using this method shows that there is a set of three identical and non-interacting binding sites for cobalt ions. Binding parameters for Co^{2+}+CAII interactions using the new model are listed in Table 2.

It is possible to introduce a correlation between change in δ_A^θ and increase in the stability of proteins. The δ_A^θ value reflects the hydrophobic property of CAII, leading to the enhancement of water structure. The small δ_A^θ values recovered from Equation 1 suggest that there are no significant changes in CAII structure as a result of it interaction with Co^{2+} ion. In the high concentration of Co^{2+}, the positive value of δ_B^θ (3.680) reflects stabilization of the CAII structure in the high concentration of Co^{2+} ion. P = 1 value shows the non-cooperativity for the interaction of Co^{2+} ions with CAII including specific interactions with native CAII structure. These results are consistent with the association equilibrium constants due to specific interactions in the certain sites on CAII ($K_1 = K_2 = K_3 = 87.150$ mM^{-1}), underlying the existence of some partially unfolded intermediate forms of CAII, which form Co^{2+}+CAII complexes. The positive values of δ_B^θ suggest that specific interactions, defined here as preferential interactions between Co^{2+} ion and the native folded state of CAII, are dominant.

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