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In vitro inhibitory effects of some heavy metals on human erythrocyte carbonic anhydrases

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Abstract

The inhibition of two human carbonic anhydrase (HCA, EC 4.2.1.1) isozymes, the cytosolic HCA I and II, with heavy metal salts of Pb(II), Co(II) and Hg(II)has been investigated. Human erythrocyte CA-I isozyme was purified with a specific activity of 920 $EUmg^{-1}$ and a yield of 30% and CA-II isozyme was purified with a specific activity of 8000 $EUmg^{-1}$ and a yield of 40% using Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography. The overall purification was approximately 104-fold for HCA-I and 900-fold for HCA-II. The inhibitory effects of different heavy metals (lead, cobalt and mercury) on CA activity were determined at low concentrations using the esterase method under *in vitro* conditions. K_i values for these metals were calculated from Lineweaver-Burk graphs as 1.0, 3.22 and 1.45 mM for HCA-I and 0.059, 1.382 and 0.32 mM for HCA-II respectively. Lead was a noncompetitive inhibitor for HCA-I and competitive for HCA-II, cobalt was competitive for HCA-I and noncompetitive for HCA-II and mercury was uncompetitive for both HCA-I and HCA-II. Lead was the best inhibitor for both HCA-I and HCA-II.

Keywords: *Carbonic anhydrase, heavy metals, inhibition, HCA-1, HCA-II*

Abbreviations: *CA, carbonic anhydrase; HCA-I, human carbonic anhydrase I; HCA-II, human carbonic anhydrase II*

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in higher vertebrates including humans [1]. These metalloproteins catalyze the equilibration between CO_2 and HCO_3^- using zinc as co-factor.

$$CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$$

Sixteen isozymes of the zinc binding enzyme have been described that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI). Hilvo et al. (2005) have reported that CA XV isoform is not expressed in humans or in other primates but it is abundant in rodents and other higher vertebrates [2]. There are also three acatalytic forms called CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI [1].

It is known that carbonic anhydrase isozymes are present in various tissues of living organisms. In humans CAs exist in the gastrointestinal tract, the reproductive tract, the nervous system, kidneys, lungs, skin and eyes, among others [1].

CA isozymes are involved in important physiological and pathological functions, such as pH and CO_2 homeostasis, respiration and transport of CO_2/HCO_3^- between metabolizing tissues and the lungs, electrolyte secretion in different tissues/organs and biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis). The two major CA isozymes (CAI and CAII) are present at high concentrations in the

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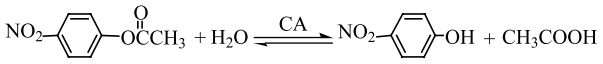
cytosol in erythrocytes, and CAII has the highest turnover rate of all the CAs [3].

As well as physiological functions, the carbonic anhydrase enzymes also catalyze some non-physiological reactions [4], for instance, it was observed that the purified enzyme has esterase activity in *in vitro* conditions [5].



The enzyme has been purified from many tissues including human erythrocytes and its kinetic properties have been examined in human erythrocytes [6], fish gills [7] and erythrocytes [8], rat saliva and erythrocytes [9], *Plasmodium falciparum* [10], insect [11], bovine bone [12], bovine leucocytes [13]. Morover, it has been reported that CA has been partially characterized from plants, yeast and bacteria [10].

Exposure to heavy metals is an important problem of environmental toxicology. Most heavy metals are toxic to humans, animals and plants. Man is at great risk of suffering from health hazards associated with toxic metals because of bioaccumulation [14].

Hura and Hura 2005 has evaluated the heavy metal content in the food of the Eastern Romania area. They have found that meat, vegetables, bakery products and diets included lead and cadmium. Radwan and Salama 2005 carried out a survey to assess the levels of lead, cadmium, copper and zinc in various fruits and vegetables sold in Egyptian markets. They detected the highest mean levels of Pb, Cd, Cu and Zn in strawberries, cucumber, dates and spinach, respectively [15]. Sivaperumal et al. 2006 evaluated the concentration of heavy metals in commercially important species of fish, shellfish and fish products from fish markets in and around the Cochin area. The study showed that different metals (Cd, Pb, Hg, Cr, As, Zn, Cu, Co, Mn, Ni, and Se) were present in the samples at different levels [16]. In another study the concentrations of nickel and cobalt in refined beet sugar samples were evaluated [17].

It is clear that different food may contain heavy metals at different levels. Determination of these substances in food is important in environmental monitoring for the prevention, control and reduction of pollution as well as for health.

Many chemical susbstances and synthesized drugs affect metabolisms by changing enzyme activities. Chemicals are generally known to activate or inhibit several body enzymes *in vivo* [18] and affect the metabolic pathways.

In the present study we have purified carbonic anhydrase I and II from human erythrocytes and examined the *in vitro* inhibition effects of some heavy metals on these important enzymes.

Materials and methods

Chemicals

CNBr-activated Sepharose 4B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzenesulfonamide and L-tyrosine were from E. Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt Germany). All other chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20 000 rpm for 30 min at 4°C. The pH of the hemolysate was adjusted to 8.7 with solid Tris. The hemolysate was applied to the prepared Sepharose 4B-L-tyrosine-sulfanylamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (HCA-I and CA-II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3-COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4°C [19].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson (1976). CO_2-Hydratase activity as an enzyme unit (*EU*) was calculated by using the equation $\left(\frac{t_0 - t_c}{t_c}\right)$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBİOS UV-VIS) according to the method described by Verpoorte

et al. [5]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [21].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli. A 20 μg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye. The electrophoretic pattern was photographed (see Figure 1).

In vitro inhibition studies with heavy metals

The inhibitory effects of lead, cobalt and mercury salts (Merck) were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. HCA-I enzyme activities were measured for $Pb(NO_3)_2$ (2×10^{-4}–1×10^{-3}M), $CoCl_2 \times 6H_2O$ (1×10^{-3}–5×10^{-3} M), $HgCl_2$ (1×10^{-3}–5×10^{-3}M) at cuvette concentrations and HCA-II enzyme activities were measured for $Pb(NO_3)_2$ (2×10^{-4}–1×10^{-3} M), $CoCl_2 \times 6H_2O$ (2×10^{-5}–3×10^{-3} M), $HgCl_2$ (1×10^{-3}–5×10^{-3}M) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity%-[Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested; $Pb(NO_3)_2$: 2×10^{-4}–1×10^{-3} M for HCA-I; 2×10^{-4}–5×10^{-4} M for HCA-II, $CoCl_2 \times 6H_2O$: 1×10^{-3}–5×10^{-3}M for HCA-I; 2×10^{-5}–2×10^{-3} for HCA-II, $HgCl_2$: 1×10^{-3}–4×10^{-3}M for HCA-I; 2×10^{-3}–5×10^{-3} M for HCA-II. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.3–0.9 mM). The Lineweaver–Burk curves obtained were used for the determination of K_i and the inhibitor type.

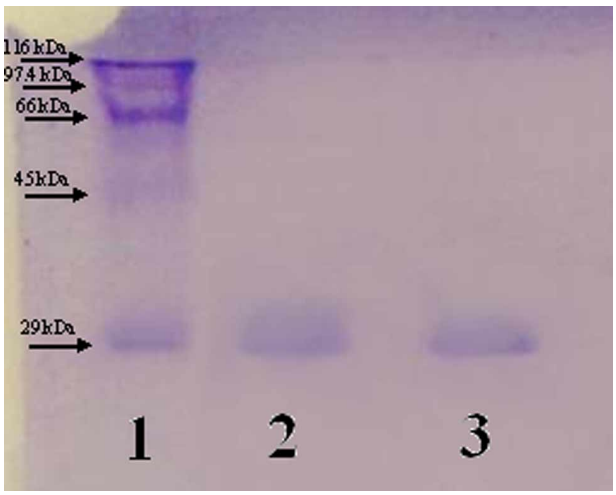


Figure 1. SDS-PAGE analysis of purified HCA-I and HCA-II. Lane (1) standard proteins (*E.Coli* β-galactosidase (116 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa)). Lane (2) HCA-I. Lane (3) HCA-II.

Results

The purification of the enzymes was performed with a simple one step method by a Sepharose-4B-L tyrosine-sulfanilamide affinity column. HCA-I enzyme was purifed ~104-fold with a specific activity of 920 $EUmg^{-1}$ and overall yield of 30% and the HCA-II enzyme was purifed ~900-fold with a specific activity of 8000 $EUmg^{-1}$ and overall yield of 40% (Table I). Figure 1 shows the SDS-PAGE obtained for determining the purity of the enzymes.

Inhibitory effects of heavy metals on enzyme activities were tested under *in vitro* conditions and K_i values were calculated from Lineweaver–Burk graphs and are given in Table II and the representative graphs for lead are shown in Figures 2 and 3.

Discussion

Carbonic anhydrase which is a widespread metalloenzyme has previously been purified and characterized from many living organisms including animals [10,24–26]. The isozymes of CA play important roles in different tissues [27,28]. The similarities of CAs from various sources have been determined from their crystal structures [29]. It is known that carbonic anhydrase has been purified many times from different organisms and the effects of various chemicals, pesticides and drugs on its activity have been investigated [30–32]. Hundreds of pollutants in the form of metals, acids, bases and other toxic compounds are being added to rivers, seas and the atmosphere a situation which has resulted in the destruction of the natural balance [30].

In this study, CA-I and II isoenzymes from human eyrtrocytes were purified by a simple one step procedure by using Sephrarose 4B-L-tirozin-sulfanilamide affinity column. The activity of the effluents were determined

Table I. Summary of purification procedure for HCA-I and HCA-II.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	135	90	15.7	1368	12150	8.88	100	1
HCA-I	368	10	0.4	4	3680	920	30	104
HCA-II	800	6	0.1	0.6	4800	8000	40	900

Table II. K_i values and inhibition types for 3 inhibitors of HCA-I and HCA-II.

	K_i		Average values of K_i (mM)		Type of inhibition	
Inhibitors	HCA-I	HCA-II	HCA-I	HCA-II	HCA-I	HCA-II
$Pb(NO_3)_2$	1.33	0.0543	0.99	0.056	Noncompetitive	Uncompetitive
	0.64	0.0553				
	1.00	0.0590				
$CoCl_2 \cdot 6H_2O$	3.17	1.10	3.91	1.70	Competitive	Noncompetitive
	4.15	1.30				
	4.41	2.70				
$HgCl_2$	2.12	0.404	1.42	0.312	Uncompetitive	Uncompetitive
	1.35	0.274				
	0.81	0.259				

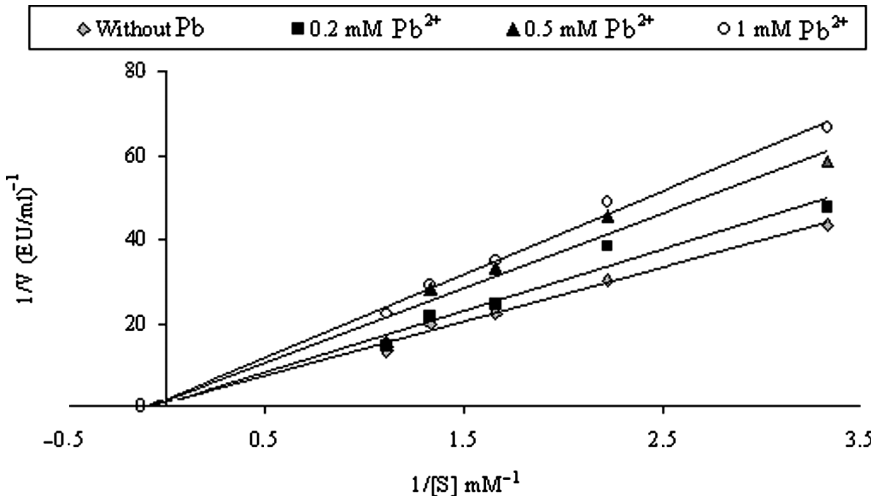


Figure 2. Lineweaver-Burk graph using 5 different substrate (4-nitrophenylacetate) concentrations and 3 different Pb^{+2} concentrations for determination of K_i for HCA-I.

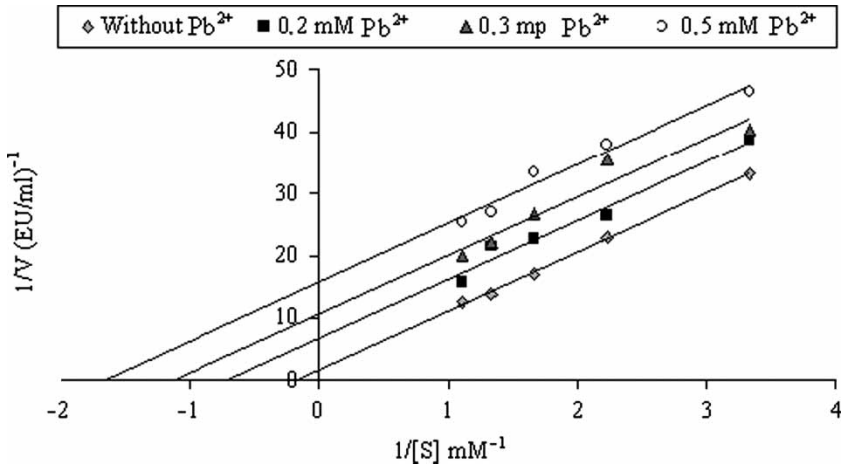


Figure 3. Lineweaver-Burk graph using 5 different substrate (4-nitrophenylacetate) concentrations and 3 different Pb^{+2} concentrations for determination of K_i for HCA-II.

by the hydratase method [20] and other further kinetic studies were performed using the esterase activity method [5]. HCA-I was purified 104-fold with specific activity (920 $EUmg^{-1}$) and yield (30%). Similarly, HCA-II was purified 900-fold with specific activity (8000 $EUmg^{-1}$) and yield (40%). SDS-PAGE of the enzymes showed a single polypeptide band (Figure 1).

Generally, the hydratase activity method is used in kinetic studies on CA and, we have not encountered any studies on the inhibitory effects of heavy metals on CA esterase activity. In fact, most heavy metals inhibit CA I/II in the micromolar range as determined by the CO_2 hydratase method. However the esterase method usually gives much higher K_i values with all inhibitors. Vitale et al. investigated the *in vitro* effects of some heavy metals on estuarine crab CA using the CO_2 hydration reaction and calculated the IC_{50} values [31]. When these results are compared with those here obtained using the esterase method, it is clearly seen that the inhibition values obtained from the CO_2 hydration reaction are lower. This shows the importance of the use of the physiological substrate in kinetic studies. For example, Gervais and Tufts used CO_2 as a substrate of CA under physiological conditions to determine the K_{cat} value [32]. However, using the esterase method for the same reason in another study found that the K_{cat} value from the hydratse method was higher than that of the esterase method (Unpublished Data). However, the advantage of the esterase activity determination is that it is a spectrophotometric and easy method.

Inhibition effects of many substances such as medical drugs, various metals, anions and pesticides have been reported in the literature [27,33–36]. Many chemicals affect metabolism by changing normal enzyme activity, particularly inhibition of a specific enzyme [37,38], and the effects can be dramatic and systemic [39]. Hence, heavy metals have various toxicological effects on living organisms. For example, it has been reported that heavy metals, such as mercury and cadmium, exert a toxic action in a synergistic fashion with salinity [31,37,39]. Also, it has been expressed that the binding of heavy metals with membrane transport ligands can alter their catalytic function [31,42]. § Lead, cobalt and mercury salts were chosen for investigation of their inhibitory effects on CA in this study and it was important that heavy metals inhibited the enzyme activity at low concentrations. K_i parameters of these metals for HCA-I and HCA-II were determined and it was found that Pb^{2+},Co^{2+} and Hg^{2+} were potent inhibitors of CA. Lineweaver-Burk plots showed that Pb^{2+} inhibited HCA-I in a noncompetitive manner; HCA-II in an uncompetitive manner (Figures 2, 3, Table II), Co^{2+} inhibits HCA-I in a competitive manner; HCA-II in a noncompetitive manner (Table II) and Hg^{2+} inhibits both HCA-I and HCA-II in an uncompetitive manner (Table II). As can be clearly seen from the K_i values, the best inhibitor for both HCA-I and HCA-II was lead.

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