Activatory Effects of Oxytetracycline on Some α-Carbonic Anhydrases

Bazı Alfa-Karbonik Anhidrazlarda Oksitetrasiklinin Aktivatör Etkileri

Research Article / Araştırma Makalesi

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ABSTRACT

Three α-carbonic anhydrases, including human carbonic anhydrase I and II (hCA I, II) and sheep kidney carbonic anhydrase (sCA) were purified and kinetic interactions between the enzymes and oxytetracycline were investigated. The purification procedure was composed of preparation of homogenate (or hemolysate) and affinity chromatography on Sepharose 4B-tyrosine-sulfanilamide. Oxytetracycline exhibited *in vitro* activatory effects on hCA I, II and sCA enzymes activity. Activity % values for this antibiotic were determined by plotting activity % vs. [A].

Key Words

Carbonic anhydrase, Human, Sheep, Kidney, Activator, Oxytetracycline.

ÖZET

Üç alfa-karbonik anhidraz, insan karbonik anhidraz I, II (hCA I, II) ve koyun böbrek karbonik anhidraz (sCA) saflaştırıldı ve enzimlerle oksitetrasiklinin kinetik etkileşmeleri incelendi. Saflaştırma prosedürü homojenat hazırlama ve Sepharose 4B-trizonsüllfanilamid afinite kramatografisinden oluştu. Oksitetrasiklin hCA I, II ve sCA enzim aktivitelerinde in vitro aktivatör etkisi gösterdi. Bu antibiyotik için % aktivite-[A] grafiği kullanılarak % aktivite değerleri belirlendi.

Anahtar Kelimeler

Karbonik anhidraz, insan, koyun, böbrek, aktivatör, oksitetrasiklin.

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INTRODUCTION

Oxytetracycline was the second of the broad-spectrum tetracycline group of antibiotics to be discovered. Oxytetracycline is a broad spectrum antibiotic that is active against a wide variety of bacteria. However, some strains of bacteria have developed resistance to this antibiotic, which has reduced its effectiveness for treating some types of infection [1].

Carbonic anhydrase (CA; Carbonate hydrolyase, EC 4.2.1.1) has been a well characterized pH regulatory enzyme in most tissues including erythrocytes. CA catalyses the reversible hydration of CO_2 to HCO_3^- and H^+, therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis. In addition, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system and inner ear [2]. The CAs are ubiquitous zinc enzymes, present in Archaea, prokaryotes and eukariotes, being encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, eubacteria, algae and cytoplasm of green plants), the α-CAs (predominantly in eubacteria, algae and chloroplasts of both mono- as well as dicotyledons) and the α-CAs (mainly in Archaea and some eubacteria), respectively [3,4]. The α-CA family (mainly present in but not exclusive to mammals) has been thoroughly investigated from the drug design viewpoint [5].

CA isoenzymes play an important role in the animal, vegetal and bacterial kingdoms, in processes such as photosynthesis, respiration, homeostasis and pH regulation [6]. Only recently the X-ray crystallographic structures of the first adducts of the physiologically relevant hCA II with the activators histamine and phenylalanine (Figure 1) have been reported by Supuran's group. Some of these compounds might be used in the treatment of the CA deficiency syndrome, a genetic disease of bone, brain and kidney affecting a large enough number of patients [6,7]. CA activators are also important for understanding the CA catalytic and inhibition mechanisms [6-9].

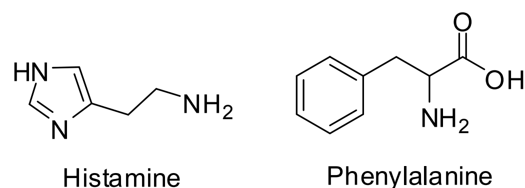

Figure 1. Structures of some carbonic anhydrase activators.

Up to now, CA has been purified from many different tissues including human and animals [10,11]. The interactions between specific enzyme systems and different drugs, metal ions and chemicals have been extensively studied in the recent years [8-11]. These studies are very important in terms of drug design and elicit of mechanisms of the enzymes.

Taking into account above information, the current study aims in purification of carbonic anhydrase enzymes from human erythrocytes, sheep kidney and investigation of impacts of oxytetracycline on these enzyme activities.

METHODS AND MATERIALS

Chemicals

Oxytetracycline, Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

Purification of carbonic anhydrase from human erythrocytes and sheep kidney 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 4ºC. The pH of the hemolysate was adjusted to 8.7 with solid Tris [11]. Sheep kidney was purified from frozen sheep tissues obtained from a local slaughterhouse. Tissue samples were centrifuged at 10000 rpm for 30 min and the plasma and precipitate were removed. The pH of the homogenate was adjusted to 7.5 with solid Tris. The homogenate was applied to the prepared

Sepharose 4B L-tyrosine sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 7.5). The affinity gel was washed with 100 mM Tris-HCl/22 mM Na_2SO_4 (pH 7.5). The sheep kidney carbonic anhydrase (sCA) enzyme was eluted with 1.25 M NaCl/25 mM Na_2HPO_4 (pH 6.5). Human erythrocytes hemolysate was applied to the prepared Sepharose 4B L-tryosine-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 hCA-II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4°C [10,11].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [12]. CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (t_o-t_c/t_c) where t_o and tc are the times for pH change of the non-enzymatic 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 (NPA) to 4-nitrophenylate ion over a period of 3 min at 25ºC using a spectrophotometer (CHEBIOS UV-VIS) according to the method described by Verpoorte et al. [13]. 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. The activatory effect of oxytetracycline was examined. Oxytetracycline was tested in triplicate at each concentration used. Different activator concentrations were used. Human erythrocytes CA I, II, and sCA enzyme activities were measured for oxytetracycline (0.0125-0.0625 mM), at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For oxytetracycline an Activity (%)-[Activator] graphs were drawn. In these experiments, 4-nitrophenylacetate was used as substrate [14].

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595nm according to the Bradford method, using bovine serum albumin as the standard [15].

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 procedure. 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 [16].

In Vitro Effects of Oxytetracycline

In order to determine the effects of oxytetracycline on hCA I, II and SCA, different concentrations of activator was added into the reaction medium. The enzyme activity was measured, and an experiment in the absence of activator was used as control (100% activity). The % Activity values were obtained from activity (%) vs. activator concentration plots (Table 1).

Table 1. % Activity ration for some hCA I, hCA II and sCA ([a]Ref 6., [b]Ref 19.).

Activator	[Activator]	HCA I	HCA II	SCA
Oxytetracycline	0.025 mM	220	188	165.1
Dipyrone[a]	150 mM	143	352	-
1-Ethyl-1H-imidazole[b]	0.010 mM	-	203	-

RESULTS AND DISCUSSION

Carbon dioxide, produced in human and sheep tissues, is hydrated rapidly by carbonic anhydrase enzyme, converted into bicarbonate, and transported in the blood. Approximately 98% of the transported and stored carbon dioxide is in bicarbonate form. At the respiratory epithelium, erythrocytic CA catalyses the rapid dehydration of HCO_3^- to molecular CO_2, which then diffuses passively into the ventilatory water stream. Moreover, the CO_2/HCO_3^- system constitutes one of the most important physiological buffers for acid–base regulation [1,9-11,17-19].

Oxytetracycline behaved as CA activator similar to dipyrone, 1-ethyl-1H-imidazole derivatives, against all three enzymes. Similarly to all CA activators reported up to now, oxytetracycline presumably intervene in the catalytic cycle, leading to the formation of an enzyme-activator complex (similarly to the enzyme-inhibitor adducts, but without substitution of the metal bound solvent molecule), in which the activator bound within the active site facilitates proton transfer processes (which represent the rate-limiting step in catalysis) [6-9]. The driving force of this effect might be the fact that intramolecular reactions are more rapid than intermolecular ones [6].

Obviously, oxytetracycline possess the hydroxybenzamide and hydroxyl moiety which can participate in the proton transfer processes between the active site and the environment (similarly to histamine) (Figure 2). Oxytetracycline can bind more effectively to the enzyme, allowing thus for more efficient activation processes as compared to dipyrone. Indeed, the active site edge of all three CA isozymes investigated by us contain a high proportion of polar amino acid residues which might interfere with polar groups such as ROH or RNH_2 fact such amino acid residues might explain the different catalytic properties of the diverse isozymes, as well as their diverse susceptibility to be inhibited/activated by modulators of activity [6,8,17].

In this study, hCA I, hCA II and sCA enzymes were isolated. Purification procedure was carried out by the preparation of the homogenate and affinity chromatography on Sepharose 4B tyrosine-sulfanilamide. As a result of the two consecutive steps, hCA-I, hCA-II and sCA enzymes were purified up to 89, 476 and 85–fold with recovery ratios of 41%, 36% and 47% compared to homogenate, respectively. After the sample had completely passed through, the column was washed with 25 mM Tris-HCl/0.1 M Na_2SO_4 buffer whose pH was 7.5. While washing was being done, absorbencies of fractions were spectrophotometerically measured at 280 nm and 348 nm. These values of the absorbance showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, sCA enzyme was eluted with 1.25 M NaCl/25 mM Na_2HPO_4 pH 6.5. At the end of the last step, highly purified enzymes were obtained exhibiting a single band on SDS-PAGE. We used only two chromatographic techniques, Sepharose 4B tyrosine-sulfanilamide affinity chromatography by modification of washing and elution conditions. These results mean that the procedure used in the purification is good enough to be used in further studies, and also has an advantage of an experimental period as short as a day.

In this study, oxytetracycline was chosen to investigate their activatory effects on hCA I, hCA II and sCA. %Activity-[Activator] graphs show that oxytetracycline activated these CAs (Figures 3-5).

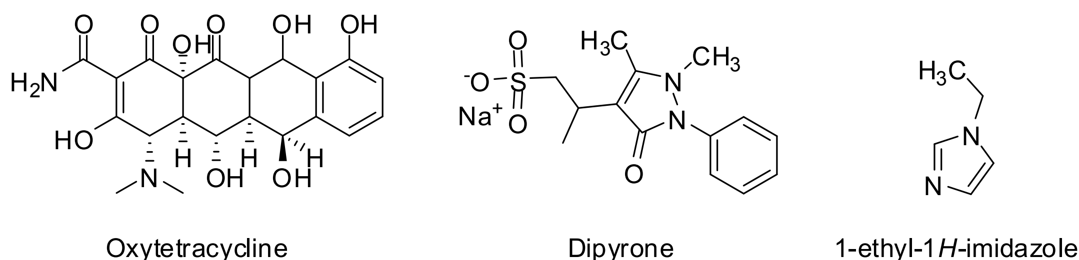

Figure 2. Structures of some carbonic anhydrase activators.

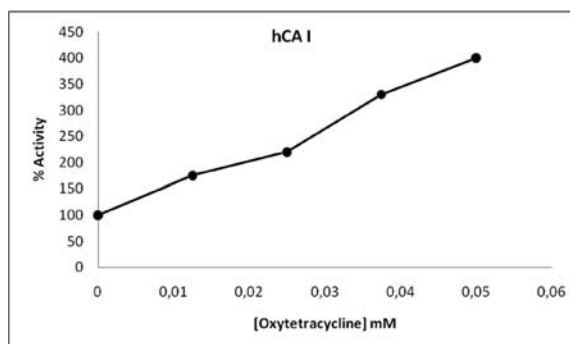

Figure 3. The effects of four different concentrations of oxytetracycline on human erythrocyte carbonic anhydrase I.

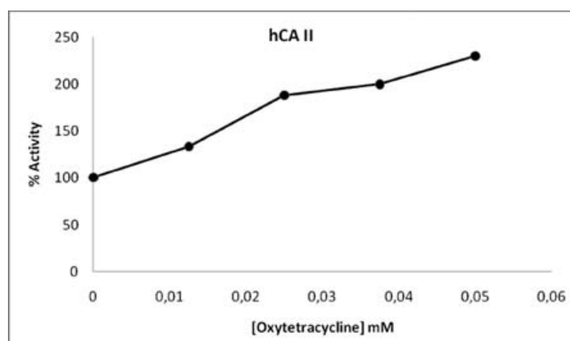

Figure 4. The effects of four different concentrations of oxytetracycline on human erythrocyte carbonic anhydrase II.

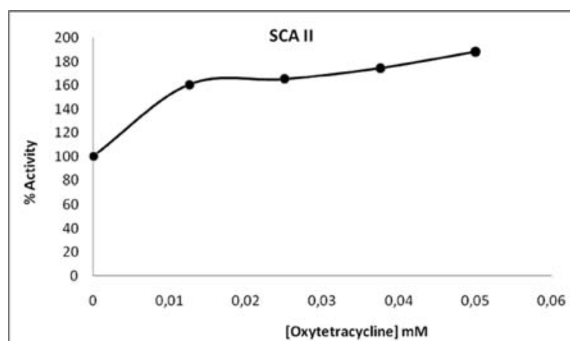

Figure 5. The effects of four different concentrations of oxytetracycline on sheep kidney carbonic anhydrase.

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