Journal of Photochemistry and Photobiology B: Biology 97 (2009) 161–168

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Spectroscopic study on the interaction of celecoxib with human carbonic anhydrase II: Thermodynamic characterization of the binding process

Masomeh Mehrabi [a], Sirous Ghobadi [a,*], Reza Khodarahmi [b,c]

[a] Department of Biology, Faculty of Science, Razi University, 67149-67346 Kermanshah, Iran
[b] Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy 67145-1673, Iran
[c] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

ARTICLE INFO

Article history:
Received 13 March 2009
Received in revised form 6 September 2009
Accepted 16 September 2009
Available online 19 September 2009

Keywords:
Human carbonic anhydrase II
Celecoxib
Competitive inhibition
Fluorescence quenching
Binding study
Protein surface hydrophobicity

ABSTRACT

The effect of celecoxib, a sulfonamide drug, on the structure and function of human carbonic anhydrase II (hCA II) was investigated by various spectroscopic techniques such as UV–Vis, fluorescence and circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC), in 20 mM Tris, pH 7.75 at 27 °C. Kinetic results revealed that celecoxib inhibits the esterase activity of hCA II in a linear competitive manner with K_i = 61.61 ± 3.05 nM. DSC data indicated that the thermal stability of the enzyme has a minor increment in the presence of celecoxib. Fluorescence measurements showed that the celecoxib acts as a quencher of the enzyme fluorescence and calculation of the protein surface hydrophobicity (PSH), using 1-anilinonaphthalene-8-sulfonate (ANS), revealed the decrement of its PSH upon interaction with the drug. Acrylamide quenching experiments indicated the less accessibility of the tryptophan residues to the acrylamide due to the presence of celecoxib. Stern–Volmer analysis of quenching data at different temperatures elucidated that the quenching of intrinsic fluorescence of hCA II is occurred through a static quenching mechanism. Analysis of the thermodynamic parameters of binding showed that hydrogen bonding and hydrophobic interactions play the major role in stabilization of the enzyme–drug complex. The Job's plot confirmed the existence of one binding site for celecoxib in hCA II. The far- and near-UV CD experiments indicated that celecoxib causes a little increment in α-helicity content of hCA II whereas its flexibility is decreased somewhat upon celecoxib binding.

1. Introduction

Human CA II (EC 4.2.1.1) is a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide to bicarbonate and hydrogen ions [1,2]. At least 14 different carbonic anhydrase isoforms were isolated in higher vertebrates. These isozymes have diversified tissue distribution and subcellular positions and they exist in archaea, eubacteria, animals and plants [3]. Many of these isozymes are significant targets for inhibitors with clinical applications [4,5]. Human CA II has a single polypeptide chain of 259 amino acid residues with a molecular mass of about 29 kDa [6]. This enzyme is known as a cytoplasmic isozyme and has a high catalytic activity and very high affinity for sulfonamides. Most of the CAs can be inhibited by aromatic and heterocyclic sulfonamides which are specific and strong inhibitors of CAs. A terminal sulfonamide of the most potent inhibitors of CAs acts as an anchoring group to coordinate the catalytic zinc [3,4]. Celecoxib (4-[5-(4-methyphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide), with the empirical formula of $C_{17}H_{14}F_3N_3O_2S$, belongs to the family of sulfonamide drugs (Fig. 1) [7,8]. This drug has an aryl sulfonamide moiety in its structure and is a selective inhibitor of cyclooxygenase-2 (COX-2). This nonsteroidal anti-inflammatory drug (NSAID) also acts as a strong inhibitor of carbonic anhydrase activity [7]. The crystal structure of the hCA II–celecoxib complex (PDB code: 1oq5) shows similar binding mode compared to typical sulfonamide CA inhibitors [8]. High resolution X-ray crystallographic investigations indicated that celecoxib perfectly binds to the hCA II active site with the aid of its trifluoromethyl group in the hydrophobic part of the active site and *p*-tolyl moiety in the hydrophilic one [8,9]. Recent data has shown nanomolar inhibition of carbonic anhydrase activity by celecoxib [7,8] however, the detailed study of the inhibitory effect of drug on the structure and function of hCA II has not been reported. In this study, we investigated the effect of celecoxib on the structure and function of hCA II. The UV–Vis spectroscopy, intrinsic and extrinsic fluorimetry, differential scanning calorimetry and circular dichroism techniques were used for elucidating the mode of binding and probable structural alterations of hCA II upon the binding process.

* Corresponding author. Tel.: +98 831 4274545; fax: +98 831 4274545.
E-mail addresses: ghobadi@sci.razi.ac.ir, sirousghobadi@yahoo.com (S. Ghobadi).

1011-1344/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotobiol.2009.09.005

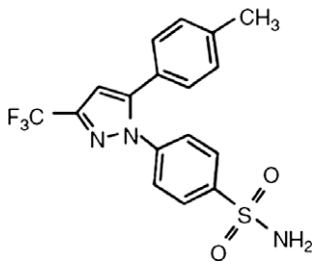

Fig. 1. The chemical structure of celecoxib.

2. Materials and methods

2.1. Chemicals

Pure celecoxib was obtained as a generous gift from Tehran Darou Co. (Tehran, Iran). *p*-Nitrophenyl acetate (*p*-NPA) and 1-anilinonaphthalene-8-sulfonate (ANS), were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The other reagents were of analytical grade from Merck (Darmstadt, Germany). All the solutions were prepared in doubly-distilled water and all of the experiments were carried out in 20 mM Tris–sulfate, pH 7.75 as the buffer at 27 °C, except CD experiments which were carried out in 20 mM Tris–HCl, pH 7.75 in order to decrease the amount of noise on CD spectrum. All of the measurements were done in triplicate.

2.2. Protein purification

Human CA II was purified from human erythrocytes according to the method described by Nyman [10]. The enzyme purity was confirmed by SDS–PAGE. The protein concentration was measured by the method of Lowry et al. [11].

2.3. Carbonic anhydrase assay

Activity of hCA II was assayed based on *p*-NPA esterase activity of the enzyme according to Pocker and Stone [12] in the absence and the presence of celecoxib. The catalytic reaction was monitored at 400 nm (due to liberation of *p*-nitrophenol as the hydrolysis product) using a Cary-100 Bio (Varian) UV–Vis spectrophotometer in quartz cells with 1 cm pathlength. The stock solution of *p*-NPA (138 mM) was made by dissolving *p*-NPA in dry acetonitrile. Stock solution of celecoxib (1 mM) was prepared by dissolving the drug in dry acetonitrile. In order to determine the mode of inhibition and the inhibitory constant, hCA II activity was assayed in a variety of *p*-NPA concentrations in the presence of increasing concentrations of celecoxib (0, 25, 50, 75 and 100 nM). The initial velocity, V_0, was determined as the slope of the absorbance changes at 400 nm during the linear phase of the catalytic reaction [13]. The enzyme concentration was calculated using its extinction coefficient (ε_{280} = 54 000 M^{-1} cm^{-1}) [14]. Final enzyme concentration in the assay buffer (20 mM Tris–sulfate buffer pH 7.75) was 0.05 mg/ml. The celecoxib and hCA II were allowed to reach an equilibrium for 30 s in the assay solution before adding *p*-NPA as the substrate.

2.4. Calorimetric study and determination of T_m

Thermal denaturation of hCA II (0.56 mg/ml) was performed in the absence and the presence of 400 nM celecoxib using a Nano-DSC II differential scanning microcalorimeter (Setaram, USA). All the measurements were carried out in 20 mM Tris–sulfate, pH 7.75 as the buffer. The protein solution (with or without drug) was heated at the constant rate of 2 K min^{-1} from 45 to 70 °C and under the constant pressure of 2 atm over 0.342 ml capillary glass cells. The reversibility of the thermal transition of hCA II was tested by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling. In spite of the fact that no aggregation was formed after heating (as evidenced by the absence of an exothermic peak after unfolding and clarity of solution across 350–800 nM), the thermal unfolding of protein was irreversible. The calorimetric data and the temperature dependence of the excess heat capacity, C_p, was further analyzed and finally plotted as a function of temperature by Cpextract software. The T_m of the denaturation process was calculated as the temperature with the maximal excess C_p.

2.5. Fluorescence measurements

2.5.1. Intrinsic fluorescence measurements

Fluorescence emission spectra of the enzyme in the absence and the presence of different concentrations of celecoxib (100, 200, 300 and 400 nM) were recorded on a Cary Eclipse (Varian) spectrofluorimeter with jacketed cell holders in which temperature was adjusted by an external thermostated water circulation. The excitation and emission wavelengths were set at 291 and 300–400 nm, respectively and the excitation and emission slit widths were 5 and 10 nm, respectively. The protein concentration was 0.02 mg/ml.

2.5.2. Determination of PSH in the presence and the absence of celecoxib

Titration of enzyme solutions in the presence of increasing concentrations of ANS provides information about differences in the ANS binding properties of carbonic anhydrase by determining the F_{max} and K_d^{app} of the carbonic anhydrase–ANS complex, in the absence and the presence of celecoxib. F_{max} is the maximum fluorescence intensity at the saturated ANS concentration which indicates the number of surface hydrophobic sites of the protein. K_d^{app} is the apparent dissociation constant of the fluorescent ANS–hCA II complex and $1/K_d^{app}$ is the binding affinity of ANS to the enzyme in the absence and presence of celecoxib [15]. The values of $1/K_d^{app}$ and F_{max} were obtained from the slope and the *x*-intercept of Scatchard plot, respectively (in this case, Scatchard plot is the plot of fluorescence intensity/$[ANS]_{free}$ versus fluorescence intensity). Determination of free ANS concentration needs plotting of fluorescence intensity versus total ANS concentration. There is a linear relationship between fluorescence intensity (F) and ANS concentration (c) only for dilute solutions of ANS ($F = Bc$, B is the proportionality coefficient between fluorescence intensity and ANS concentration). ANS was added from a stock solution (1 mM) to a final concentration range from 2 to 110 μM. The enzyme concentration was 0.3 mg/ml. Considering hCA II has several binding sites for ANS, it is assumed that within 0–10 μM ANS concentration (~10 μM hCA II) all ANS molecules are bound to hCA II, and so the proportionality coefficient between fluorescence intensity and ANS concentration (B) can be calculated from the slope of linear portion of the plot of F versus [ANS]. The concentration of the bound ANS molecules (for each of the different total concentrations of ANS used) was calculated using the following equation: $[ANS]_{bound} = F/B$, and the concentration of the free ANS molecules was calculated from the difference between total and bound ANS concentrations ($[ANS]_{free} = [ANS]_{Total} - [ANS]_{bound}$) [16].

From the Scatchard plot, the protein surface hydrophobicity index in the absence and presence of the drug can be determined using the following equation [15]:

$$PSH = F_{max}/[\text{hCA II}]K_d^{app} \quad (1)$$