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Binding of angiogenesis inhibitor kringle 5 to its specific ligands by frontal affinity chromatography

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ABSTRACT

The interactions between angiogenesis inhibitor Kringle 5 and its five specific ligands were investigated by frontal affinity chromatography in combination with fluorescence spectra and site-directed molecular docking. The binding constants of trans-4-(aminomethyl) cyclohexane carboxylic acid (AMCHA), epsilon-aminocaproic acid (EACA), benzylamine, 7-aminoheptanoic acid (7-AHA) and L-lysine to Kringle 5 were 19.0×10^3, 7.97×10^3, 6.45×10^3, 6.07×10^3 and 4.04×10^3 L/mol, respectively. The five ligands bound to Kringle 5 on the lysine binding site in equimolar amounts, which was pushed mainly by hydrogen bond and Van der Waals force. This binding affinity was believed to be dependent on the functional group and flexible feature in ligands. This study will provide an important insight into the binding mechanism of angiogenesis inhibitor Kringle 5 to its specific ligands.

1. Introduction

The study on protein–ligand and protein–protein interactions is crucial to the design and discovery for drugs. In particular, serum albumins, G-protein-coupled receptors, some specific enzymes and ion channels, represent a large fraction of current drug targets for new drug development [1]. Understanding and predicting the interactions between these proteins and their specific ligands can accordingly offer useful help in the design and discovery of lead compounds.

To date, two types of approaches have already been developed to explore the interactions between proteins and their specific ligands. The first one was often processed in solution, including nuclear magnetic resonance [2], ultraviolet and visible spectra [3], circular dichroism [4], fluorescence spectroscopy [5] and isothermal titration calorimetry [6]. The second one included surface plasma resonance (SPR) [7], enzyme linked immunosorbent assay [8] and frontal affinity chromatography (FAC) [9,10].

FAC has provided a very useful tool for the analysis of drug-protein binding [11]. This method was often performed by flushing the column containing the immobilized proteins using different concentrations of the ligands to produce series of breakthrough curves. The association constant and the number of binding sites distributed over the surface of the immobilized proteins can be obtained through the analysis of these curves by linear [12] and nonlinear approaches [13]. The main issue of the two mathematical assays [13] is that one need to select a desired model describing a certain adsorption mechanism just based upon statistics (i.e. fitting). Therefore the ultimate methodology was proposed comprising of calculating the adsorption/affinity energy distribution (AED) a priori to any model assumption which means one will have the energy of interactions and number of adsorption sites without assuming any model [14–21]. Moreover, the AED-approach was recently expanded and successfully applied also for experimental protein-ligand data coming from modern biosensors based on surface plasmon resonance [15] and quartz crystal microbalance technologies [19–21], respectively. Although nonlinear and AED-approaches were more accurate to describe the protein–ligand interaction, the complex calculations were often simplified by linearization [22].

Angiogenesis inhibitor Kringle 5 (Kringle 5) is an individual triple-looped kringles formed by three pairs of disulfide bonds in plasminogen. It was reported that Kringle 5 was the most potent endogenous molecule for the suppression of angiogenesis and tumor growth compared with the other angiogenesis inhibitor Kringles [23]. This work is to realize the binding of Kringle 5 with its five specific ligands including L-lysine, epsilon-aminocaproic acid (EACA), 7-aminoheptanoic acid (7-AHA), trans-4-(aminomethyl) cyclohexane carboxylic acid (AMCHA) and benzylamine [24–28], as shown in Fig. 1, using frontal affinity chromatography in combination with fluorescence spectra and site-directed molecular docking.

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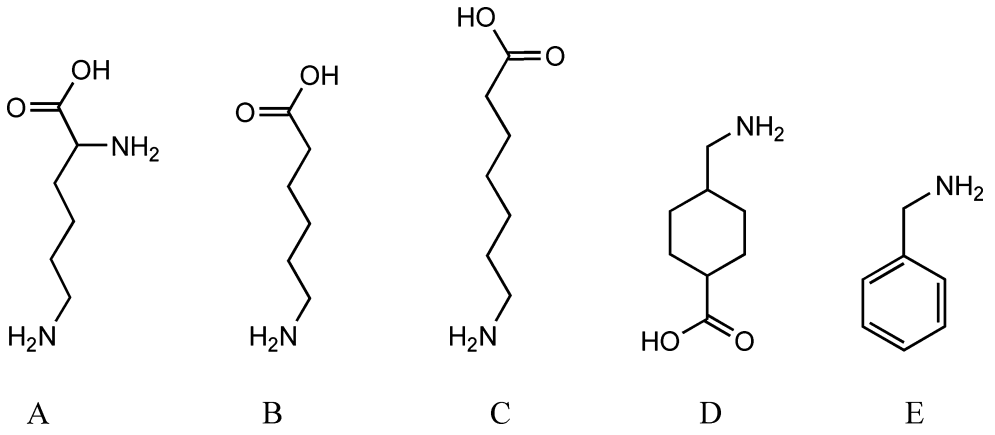

Fig. 1. Five specific ligands to angiogenesis inhibitor Kringle 5. A: L-lysine; B: EACA; C: 7-AHA; D: AMCHA; E: Benzylamine

2. Experimental

2.1. Materials and equipment

Kringle 5 was isolated and purified according to the method established previously (purity > 98%) [29]. Specific ligands including L-lysine, EACA, 7-AHA, AMCHA and benzylamine were purchased from Sigma (St. Louis, MO, USA) and used without further purification. The silica gel (particle size 7.0 μm, pore size 30.0 nm, specific surface area 480.0 m^2/g) was purchased from the Separation Group (Hesperia, CA, USA). The other chemicals were analytical grade reagents and doubly distilled water was used throughout. The stock solutions of Kringle 5 (1.0×10^{-4} mol/L) and ligands (1.0×10^{-2} mol/L) were prepared by exactly weighing and dissolving their solid powders in the buffer containing 10.0 mmol/L Tris-HCl, 0.5 mol/L EDTA and 1.0 mol/L $MgCl_2$ (pH 7.4). The stock solutions were kept at dark in a refrigerator for further use.

All the frontal analyses were carried out with a Shimadzu LC-10A high performance liquid chromatograph (Kyoto, Japan) and the experimental temperature was automatically controlled with a CTO-10AS vp column oven by an SCL-10A vp system controller. The chromatographic columns were packed with a ZZXT-A filling machine (Dalian Elite Analytical Instruments Co., Dalian, China). Fluorescence measurements were performed with a Hitachi Model F-4500 spectrofluorimeter (Tokyo, Japan) equipped with a 150 W xenon lamp and a thermostat bath, using a 1.0 cm quartz cell. Molecular docking was performed using AutoDock 4.2 software.

2.2. Oriented immobilization of Kringle 5

Kringle 5 was oriented immobilized on the surface of silica gel according to the method reported previously [30,31] (Fig. 2). In brief, the macroporous silica gel was first activated through immersing in methanol-hydrochloric acid solution at a ratio of 1:1 (V:V) for 30 min followed by an additional 30.0 min incubation. The activated gel was then dried under a stream of nitrogen prior to subsequent modification. Then, aminopropyl silica gel was obtained by stirring the dried gel in anhydrous toluene containing 3.0% (V/V) of γ-aminopropyl triethoxysilane under nitrogen for 12.0 h at 110 °C. Following three rinses in fresh toluene, the aminopropyl silica gel was dried under nitrogen stream and stored in an airtight container at 4.0 °C for further use. After the formation of amino silicane layer, the gel was suspended in 100.0 mL methanol solution containing 1.5 mmol/L *p*-nitrobenzaldehyde by stirring for about 42.0 h at 60 °C. Following the filtration and cleaning procedure by methanol, *p*-nitrobenzaldehyde-modified gel was further treated for about 16.0 h by 0.2 mmol/L sodium borohydride aqueous solution at room temperature. The product was filtrated, rinsed three times by water, and dried at 110 °C under vacuum until the weight remains stable. The nitro group in the gel was then reduced to amino group by mixing the gel in 100.0 mL 42.0 μmol/L stannous chloride ethanol solution and keeping the reaction for about 6.0 h at 90 °C. Then the suspension was alkalified using 50.0 mL of 5% sodium bicarbonate, followed by a sequential saturated NaCl and distilled water cleaning.

Before reacting with Kringle 5, the amino group coated surface was converted to the diazobenzyl form by treatment with a solution containing 30.0 mL 20.0 mmol/L hydrochloric acid, and distribution droplets of 20.0 mmol/L freshly prepared $NaNO_2$ solution until the turning point of potassium iodide starch paper changing to blue. The diazotized surfaces were then washed three times, each for about 5.0 min, with ice-cold sodium acetate buffer (50.0 mmol/L, pH 4.7) followed by cleaning with ice-cold deionized water. Immediately, the diazotized gel was utilized to attach his-tagged proteins by suspending the mixture of purified Kringle 5 (10.0 mmol/L, 10.0 mL) and the gel in 100.0 mL ice-cold deionized water at 4.0 °C. Two hours later, the gel with immobilized Kringle 5 was collected through filtration and washed three times by equilibration buffer (10.0 mmol/L Tris-HCl, 0.5 mol/L EDTA, 1.0 mol/L $MgCl_2$, pH 7.4).

2.3. Packing and characterization of Kringle 5-oriented column

The Kringle 5 coated silica gel was packed into a stainless steel column (4.6 mm × 50.0 mm) using phosphate buffer (20.0 mmol/L, pH 7.4) as the slurry and replacing agent at the pressure of 4.0×10^7 Pa. The Kringle 5-oriented column was characterized by frontal analyses of five ligands and 2% $NaNO_2$. The column was first flushed by equilibration buffer, then the feed solutions of the five ligands and sodium nitrite were separately brought into the column and their breakthrough curves were recorded. The raw data of each breakthrough curve was exported to an Excel worksheet as a set of two columns, videlicet, retention time and signal intensity to obtain the breakthrough time through differentiation by the software of Origin 8.0. In addition, a control column containing aminopropyl silica gel was used to evaluate the non-specific adsorption of immobilized Kringle 5.

2.4. Determination of breakthrough times

Under different feed concentrations, the breakthrough curves and breakthrough times of five ligands on the Kringle 5-oriented column were determined through frontal analysis. The column was first balanced with equilibration buffer, subsequently a given concentration of ligand solution was passed through the column