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Determination of driving forces for bovine serum albumin-Ponceau4R binding using surface plasmon resonance and fluorescence spectroscopy: A comparative study

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ABSTRACT

Ponceau 4R (P4R) and bovine serum albumin (BSA) may interact changing food properties. We compared fluorescence spectroscopy and surface plasmon resonance (SPR) for studying, *in vitro*, the interactions between BSA and P4R at pH 7.4 and 3.5 in different temperatures. Fluorescence data pointed to the formation of a complex where P4R was bound on site I or II of BSA, with a stoichiometry around one and a binding constant (K_b) ranging from 1.37×10^5 to 20.15×10^6 L mol^{-1}. The complex formation at both pH was enthalpically driven (standard enthalpy change, $\Delta H^\circ_F = -60.69$ and -63.06 kJ mol^{-1}, for pH 7.4 and 3.5, respectively). Using SPR, we also found the formation of 1:1 BSA-P4R complexes, but the calculated K_b values were much smaller, on the order of 10^3 L mol^{-1}. Again, we found that the formation of BSA-P4R complex was driven by enthalpy decreasing; however the standard enthalpy change was less negative than that found by fluorescence ($\Delta H^\circ_{SPR} = -15.05$ and -40.55 kJ mol^{-1}, at pH 7.4 and 3.5, respectively). Our results show that these distinct techniques provided different thermodynamic binding parameters for the BSA-P4R interaction, especially regarding ΔH° values, indicating that BSA-P4R binding was a multisite phenomenon, and that sites far from tryptophan residues were the main responsible by electrostatic interaction. Thus, this work clearly shows the importance of using complementary techniques for a complete thermodynamic characterization of complexes formed between azo-colorants and proteins; which is directly related to physicochemical properties of systems containing both molecules together.

1. Introduction

Synthetic dyes and proteins are widely used in drinks, food, medicines, and cosmetics. Ponceau 4R (E124), a well-known negatively charged and water-soluble azo dye (Datta, Mahapatra, & Halder, 2013; Tanaka, 2006), is used in foods, such as cakes, candies, ice creams and dairy desserts to make them more attractive to consumers (Tikhomirova, Ramazanova, & Apyari, 2017; Zhang et al., 2017). Bovine serum albumin (BSA) is also present and/or widely applied in these foods as gelling (Prata & Sgarbieri, 2008; Semasaka, Katopo, Buckow, & Kasapis, 2017), emulsifying (Al-Malah, Azzam, & Omari, 2000; Liu et al., 2015) and/or transport agent (Zhu, Sun, Wang, Xu, & Wang, 2017).

The binding of serum albumins, like BSA, with small molecules such as Ponceau 4R is of great interest in food science because proteins and food additives present together in a food formulation may form complexes, which will modify properties of both colorant (Brantom, Stevenson, & Wright, 1987; Tanaka, 2006) and protein

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(Lelis et al., 2017); and consequently alter some food properties. Thus, studying the binding process of BSA and Ponceau 4R could help to optimize the physicochemical properties of food formulations containing both molecules.

Many techniques have been used to study intermolecular interactions involving proteins and small molecules, such as nanocalorimetry (isothermal titration nanocalorimetry and differential scanning nanocalorimetry) (Bou-Abdallah & Terpstra, 2012; Pathak et al., 2016), nuclear magnetic resonance (He et al., 2016), equilibrium dialysis (Uddin, Saffoon, & Alam, 2012), infrared and Raman spectroscopies (Mandal, Hossain, Devi, Suresh, & Chaudhuri, 2013; Zhang, Ma, Wang, Zhang, & Zhou, 2012), ultracentrifugation (Perozzo, Folkers, & Scapozza, 2004), surface plasmon resonance (Surwase, Patil, Srinivas, & Jadhav, 2016), and fluorescence spectroscopy (Moeiniafshari, Zarrabi, & Bordbar, 2015).

Fluorescence spectroscopy is a powerful technique for investigating small molecule-protein binding, because it is sensitive, reproducible, and convenient (Zhang et al., 2012). Through fluorescence quenching, it is possible to obtain information on protein conformational changes as well as on fluorophores vicinity of the protein (He, Xu, Zeng, Qin, & Chen, 2016). However, fluorescence is limited to interactions occurring near the fluorophore surroundings; therefore, to completely characterize the binding thermodynamics, it is necessary to use an additional technique that determines interactions with other sites.

Surface plasmon resonance (SPR) has also attracted attention for studying protein binding (Surwase et al., 2016). It provides thermodynamic and kinetic binding parameters, associated with the interaction between small molecules and any protein site, by monitoring changes in the refractive index at gold surfaces on which ligands are immobilized (Kobayashi et al., 2011), and uses a very small quantity of reagents.

To the best of our knowledge, there is no data regarding the thermodynamic parameters of BSA-P4R binding; thus, we propose to completely characterize the formation of the BSA-P4R complex by simultaneous fluorescence spectroscopy and SPR measurements, through the determination of the following parameters: binding constant (K_b), complex stoichiometry (n), standard Gibbs free energy change (ΔG°), standard enthalpy change (ΔH°), and standard entropy change (ΔS°).

2. Materials and methods

2.1. Materials

BSA (>99% wt.), warfarin (reagent grade), ibuprofen (>98% wt.), digitoxin (>92% wt.), bibasic sodium phosphate ($NaHPO_4$), monohydrated sodium phosphate ($NaH_2PO_4 \cdot H_2O$), citric acid ($C_6H_8O_7$), and sodium citrate ($Na_3C_6H_5O_7$) (all reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The colorant Ponceau 4R (P4R, >85% wt.) was kindly provided by Gemacom (Juiz de Fora, Brazil).

Bibasic sodium phosphate (0.077 mol L^{-1}), monohydrated sodium phosphate (0.023 mol L^{-1}), citric acid (0.080 mol L^{-1}), sodium citrate (0.020 mol L^{-1}), and deionized water were used to prepare buffer solutions (pH 7.4 and 3.5), without the addition of any acid or alkali.

2.2. Steady state fluorescence experiment

Fluorescence spectra of BSA were obtained with an LS55 spectrofluorimeter equipped with a thermostat bath (Perkin Elmer, Waltham, MA, USA). For measurements, 3.0 mL of pH 7.4 or 3.5 BSA solution (3.01×10^{-5} mol L^{-1}) containing different concentrations of P4R (3.0×10^{-6} to 5×10^{-5} mol L^{-1}), was added to a 1.0 cm quartz cell. The fluorescence emission spectra were then measured at five different temperatures (293.15, 298.15, 308.15, 318.15, and 328.15 K) over a 281–450 nm range (excitation wavelength = 280 nm).

2.3. Competitive binding experiment

Competitive binding studies were carried out using warfarin, ibuprofen, and digitoxin, which are markers for sites I, II, and III of BSA, respectively. A solution of BSA and each marker, both at 3.01×10^{-5} mol L^{-1}, was titrated with an increasing concentration of P4R, and fluorescence quenching experiments were performed as described previously. Thus, the binding parameters for the P4R–BSA interactions were determined in the presence of each site marker.

2.4. Surface plasmon resonance

Surface plasmon resonance analyses were performed using a BIACORE ×100 instrument equipped with a research-grade CM5 sensor chip from GE Healthcare, controlled by Biacore ×100 Control Software.

2.4.1. Immobilization of protein

BSA was immobilized on the CM5 sensor chip using amine-coupling chemistry in accordance with the recommended protocol in the Biacore ×100 Handbook BR-1008-10 Edition AC. The flow cell, formed when the sensor chip is docked in the instrument, was activated for 7 min with a 1:1 mixture of 0.1 mol L^{-1} 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) and 0.1 mol L^{-1} *N*-hydroxysuccinimide (NHS) at a flow rate of 20 μL min^{-1} and at 25 °C. Then, 30 μg mL^{-1} BSA in 10 mmol L^{-1} sodium acetate, at pH 4.0, was injected for 7 min, resulting in immobilized densities around 7000 Resonance Units (RU). A 7 min pulse of 1 mol L^{-1} ethanolamine hydrochloride, pH 8.5, was then used to deactivate the excess hydroxysuccinimidyl groups on the surface that did not react with the protein.

2.4.2. BSA-P4R interaction followed by SPR

BSA-P4R adsorption titration experiments were performed at 288.15, 293.15, 298.15 and 303.15 K, and pH 7.4 and 3.5. P4R solution at working concentration (15.66×10^{-6} to 20.04×10^{-5} mol L^{-1}) was prepared in running buffer (pH 7.4 or pH 3.5). For each binding experiment, the P4R solution at a specific concentration was injected in order to increasing colorant concentration over both the ligand (immobilized BSA) and the reference (without BSA) surfaces, which was used to correct for systematic noise and instrument drift. Before each P4R binding cycle, buffer was injected to obtain the baseline.

2.5. Statistical analysis

All assays were performed in triplicate. Mean values and standard deviations were evaluated using analysis of variance (ANOVA). All statistical data were processed using Statistical Analysis System (SAS) version 9.1.

3. Results and discussion

3.1. BSA-P4R binding parameters obtained by fluorescence

Fluorescence is considered a convenient tool to investigate binding between small molecules and proteins because it provides enough information to characterize the binding mechanism, thermodynamic binding parameters, and conformational protein