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BOVINE SERUM ALBUMIN–DYE BINDING

*J. Sereikaite**, *Z. Bumeliene*, and *V.-A. Bumelis*

Department of Chemistry and Bioengineering, Faculty of Fundamental Sciences, Vilnius Gediminas Technical University, Sauletekio al. 11, LT-2040, Vilnius, Lithuania

SUMMARY

The interaction of the dye reactive bright red 6C with bovine serum albumin has been investigated. Formation of a complex between reactive bright red 6C and bovine serum albumin was detected by use of absorption difference spectroscopy. The curves obtained by titration of the protein against the dye were recorded at different pH and ionic strength of the solution. An unusual form of titration curve was obtained.

INTRODUCTION

Dye–ligand chromatography is a powerful technique for protein purification. The anthraquinone dye cibacron blue F3G-A (CB) is widely used in affinity chromatography. The dye is commercially available, inexpensive, and can be easily immobilized [1]. Several nucleotide-binding enzymes were purified on adsorbents with the immobilized dye [2–4]. CB as a ligand can bind to other proteins such as phospholipase A_2 [5], human interferon α-2b [6], lysozyme, γ-globulin [7], alkaline phosphatase [8] and serum albumin [9–11]. Chromatography on immobilized CB is a useful step in serum albumin purification [12]. The CB–human serum albumin complex is the best yet investigated. This complex was isolated by use of gel-permeation chromatography on Sephadex G-100. A dye/protein molar ratio of 3:1 was obtained over the pH range 6.5–10 [13]. Three gene segments that code three domains of human serum albumin (following amino acids: I 1–197; II 189–385; III 381–585) were cloned separately. Each of the recombinant domains obtained had a CB binding site [14]. Despite extensive applications, the dye–protein interaction is not fully understood.

Investigation of dyes of different structures binding to protein could help in the collection of new data enabling better understanding of dye–protein interaction mechanisms. In this paper we report results obtained

from binding of the dye reactive bright red 6C (BR) to bovine serum albumin as model protein.

EXPERIMENTAL

Materials

Bovine serum albumin was from Sigma (essentially fatty acid free) and from Serva (analytical grade). Buffer salts were of the highest purity (Reachim). BR was a kind gift from Dr O. Sudziuviene (Institute of Biotechnology, Lithuania). The dye was purified according to a method described elsewhere [15].

Absorption Difference Spectroscopy

Different spectra in the wavelength region 400–750 nm were recorded by means of an Ultrospec 4000 spectrophotometer (Pharmacia Biotech) provided with Swift II software; the path length was 1 cm. Difference spectral titrations were performed at 25°C. The dye concentration in the reference cuvette was the same as that used for the complex formation. Experimental curves were constructed:

1. at a constant BSA concentration of 10 μM, 5 μM or 2.5 μM in 0.025 M potassium phosphate buffer, pH 7.0, while the dye concentration was increased to 90 μM, 60 μM or 50 μM;
2. at a constant dye concentration of 25 μM in 0.025 M potassium phosphate buffer, pH 7.0, while the protein concentration was increased to 10 μM; and
3. at a constant BSA concentration of 10 μM in 0.025 M potassium phosphate buffer, pH 7.0, in the presence of 0.1 M, 0.25 M or 0.5 M KCl, while the dye concentration was increased to 80 μM.

RESULTS AND DISCUSSION

Absorption difference spectroscopy is widely used to study the binding of the well-known dye CB and other dyes to proteins [6,8,16]. This spectroscopic technique has also been used to monitor the interaction of BR with BSA.

BR is a reactive azo dye with a monochlorotriazine ring and three sulfonic acid groups. The structure of BR is presented in Fig. 1. The visible absorption spectrum of BR has a maximum at 544–545 nm and a shoulder in the wavelength region 515–530 nm (Fig. 2).

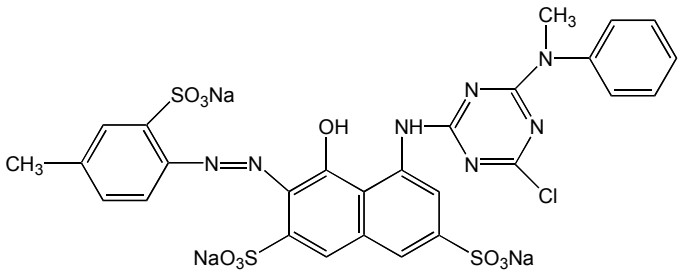

Fig. 1

Chemical formula of BR

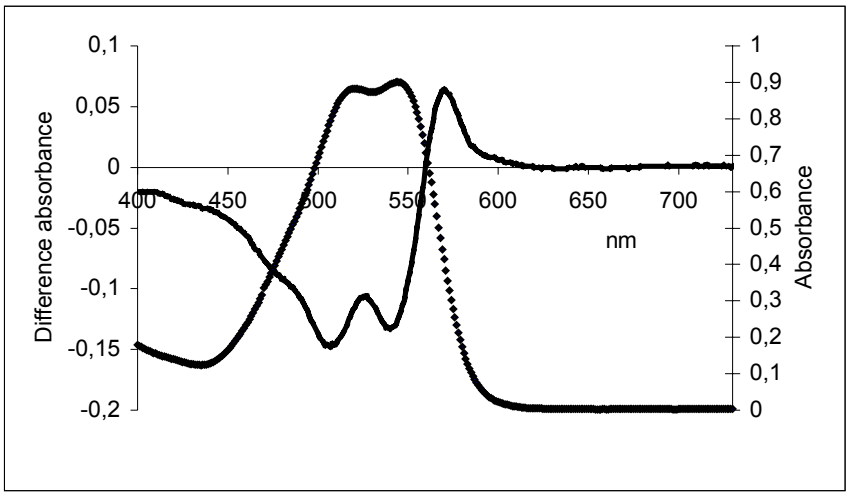

Fig. 2

Spectrum of 30 μM solution of BR in 0.025 M potassium phosphate buffer pH 7.0 (---) and the difference spectrum for BR dye binding to BSA in the same buffer (—). The protein concentration in the sample cuvette was 10 μM and the dye concentration in both the sample and reference cuvettes was 40 μM

Before recording of difference spectra the linearity of the Beer–Lambert law plot was checked for dye concentrations up to 100 μM under all the experimental conditions used. No spectral perturbations were revealed in the pH range 4.3–7.0 and KCl concentrations up to 0.5 M in 0.025 M potassium phosphate buffer at pH 7.0. The molar absorption coefficient was calculated and found to be 29000 M^{-1} cm^{-1} at 544 nm. The vi-

sible difference spectrum of BR in the presence of BSA has a maximum at 570 nm and a double minimum at 507 and 540 nm. The shift of a maximum of 56 nm occurs to the long wavelength side (Fig. 2). These spectral changes suggest that BR forms a complex with BSA. For characterization of the dye-binding process 10 μM BSA was titrated with an increasing amount of BR. The type of spectra obtained when the absorbance of mixtures of dye and BSA was measured against a dye solution of the same concentration can be seen in Fig. 3. The intensity of the 570 nm peak in each differen-

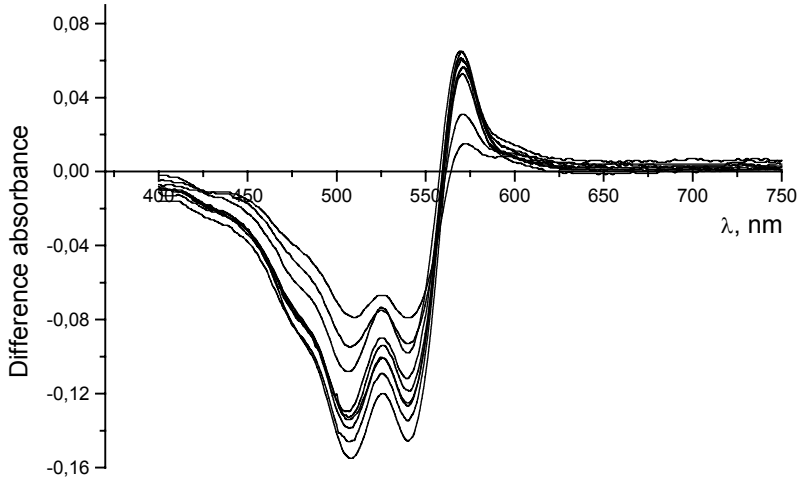

Fig. 3

Typical difference spectra obtained from BR dye binding to BSA in 0.025 M potassium phosphate buffer pH 7.0. The sample cuvette contained 10 μM BSA whereas the dye concentration in both sample and the reference cuvettes ranged from 20 to 90 μM

ce spectrum was plotted against dye concentration, as shown in Fig. 4A. The curve seemed to follow a simple saturation curve, but a shift of the saturation curve on the *x*-axis was established. No peak in the visible difference spectra was observed at dye concentrations equal to approximately twice the protein concentration and lower. The same regularity was observed for 5 μM and 2.5 μM BSA (Fig. 4). The same effect was found for bovine serum albumins from both Serva and Sigma.

The effect of pH on BR binding to BSA was also investigated in 0.025 M potassium phosphate buffer at pH 6.0 and pH 5.0 and in 0.025 M sodium acetate buffer at pH 4.3. A shift of the saturation curve on *x*-axis was observed at pH 6.0 and 5.0 in the same way as for pH 7.0. In contrast

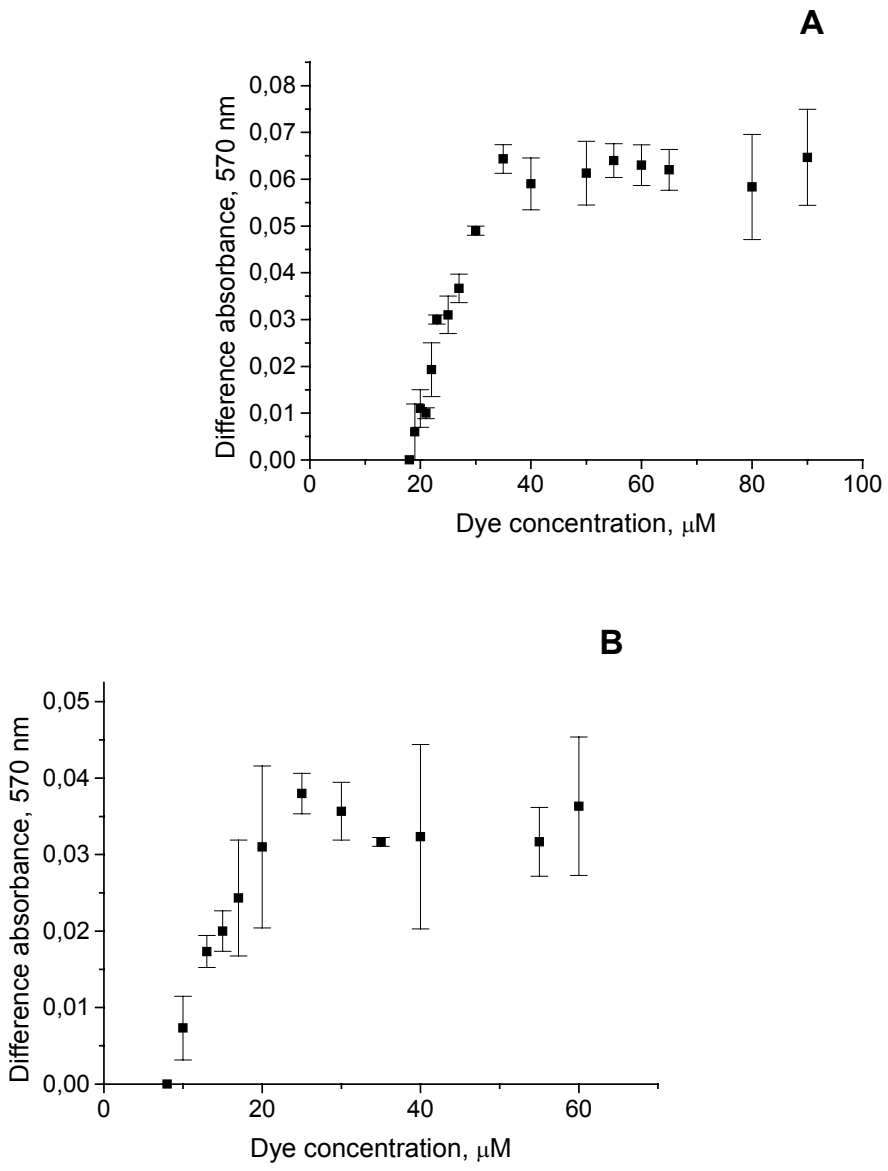

Fig. 4

Titration at λ = 570 nm of BSA with BR in 0.025 M potassium phosphate buffer, pH 7.0. The protein concentration in the sample cuvette was 10 μM (A) and 5 μM (B). Data are presented with standard deviations calculated from results from three parallel experiments

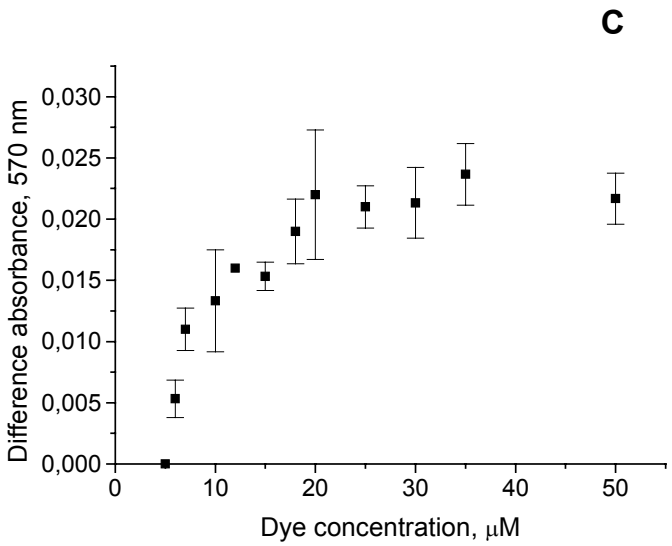

Fig. 4 (continued)

Titration at λ = 570 nm of BSA with BR in 0.025 M potassium phosphate buffer, pH 7.0. The protein concentration in the sample cuvette was 2.5 μM (C). Data are presented with standard deviations calculated from results from three parallel experiments

with to pH 7.0, at pH 6.0 and 5.0 saturation was not achieved up to a BR dye concentration of 90 μM at pH 4.3 and the saturation curve moved nearer the *x–y* intersection (data are not shown). The different interaction of BSA with BR at pH 4.3 is probably be related to acid-induced structural changes of bovine serum albumin characterized by changes in secondary and tertiary structures [17]. It is known that at pH 4.3 the N form of BSA is converted to the F form. This transition is accompanied by a slight decrease in helical content from 55% to 45% [18].

The process of binding of BR to BSA has been studied in solutions of different ionic strength. BSA titration with an increasing amount of BR was performed in the presence of 0.1 M; 0.25 M, and 0.5 M KCl in 0.025 M potassium phosphate buffer, pH 7.0 (Fig. 5). Titration curves moved nearer the *x–y* intersection when the KCl concentration was increased. The curve was hyperbolic in shape and passed through the origin at 0.5 M KCl (Fig. 5C).

The protein–dye interaction was also investigated using a constant concentration of dye with increasing concentrations of protein [6]. BR titration with BSA was performed with 25 μM dye. The titration curve obtained followed a simple saturation curve and it is given in Fig. 6.

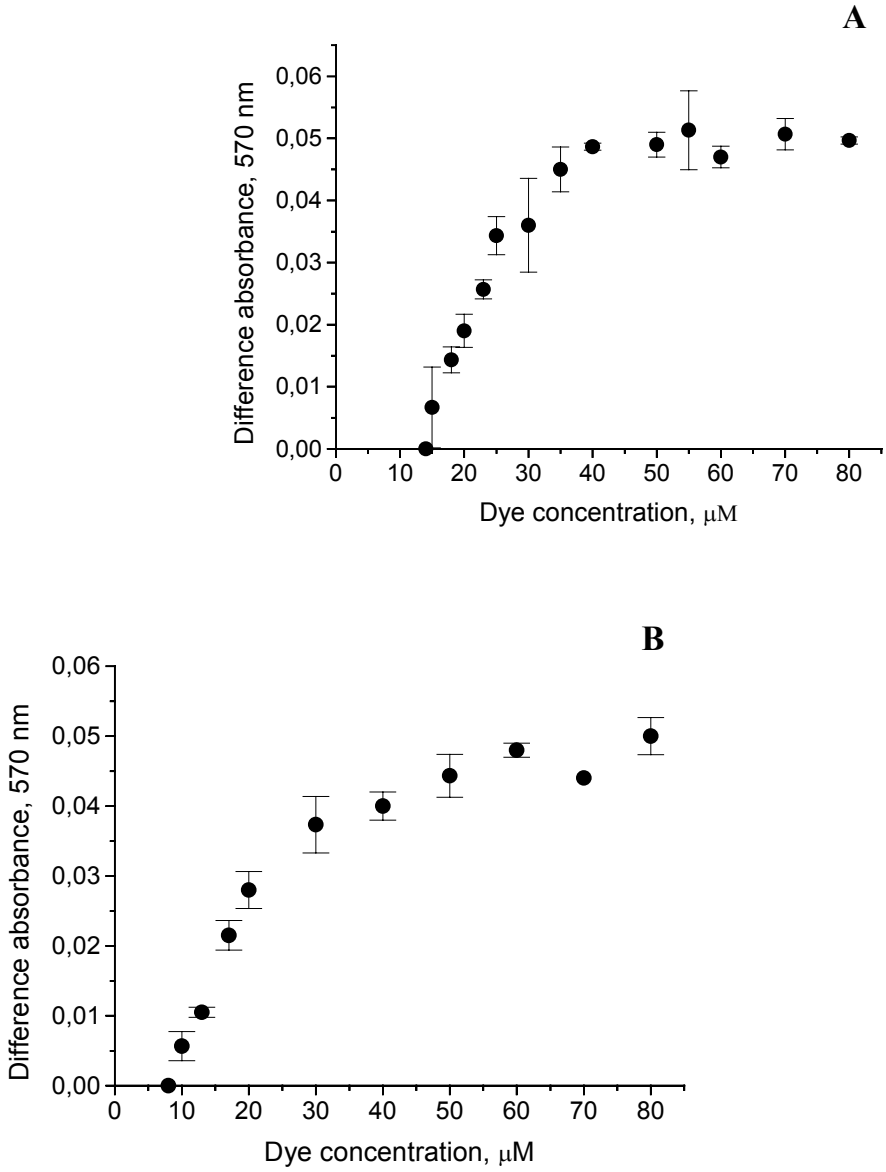

Fig. 5

Titration at λ = 570 nm of 10 μM BSA with BR in 0.025 M potassium phosphate buffer, pH 7.0, in the presence of 0.1 M KCl (A) and 0.25 M KCl (B). Data are presented with standard deviations calculated from results from three parallel experiments

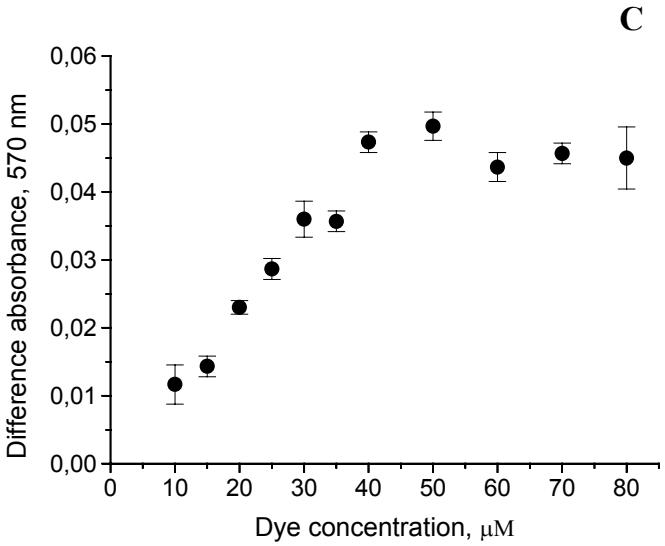

Fig. 5 (contineud)

Titration at λ = 570 nm of 10 μM BSA with BR in 0.025 M potassium phosphate buffer, pH 7.0, in the presence of 0.5 M KCl (C). Data are presented with standard deviations calculated from results from three parallel experiments

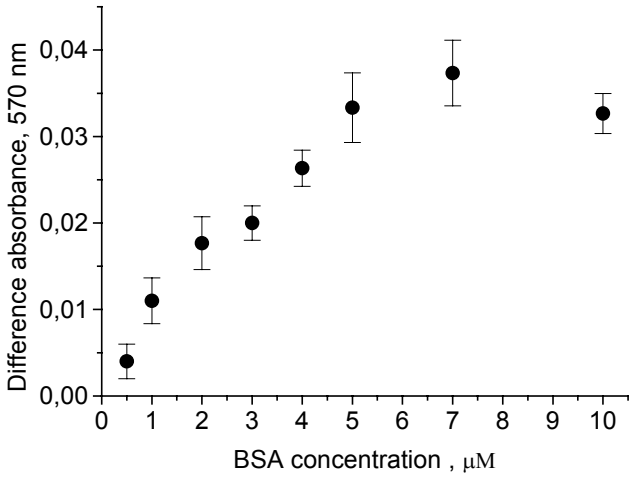

Fig. 6

Titration at λ = 570 nm of 25 μM BR with BSA in 0.025 M potassium phosphate buffer, pH 7.0. Data are presented with a standard deviations calculated from results from three parallel experiments

Our results suggest that at least two sites on the protein molecule can be occupied by BR. These sites are different in their affinity to the BR molecule and in the polarity of the environment of dye–protein complex formation. It is likely that one of the binding sites has higher affinity than the second. It seems, however, that the binding environment of the first site is much more polar than that of the second. The effect on the BR spectrum of the first dye-binding site is slight. Changes of the spectrum are not revealed by difference visible spectroscopy. Dye spectrum changes were observed and recorded only for the second dye-binding region of the protein. If a saturation curve is constructed for constant dye concentration and increasing protein concentration it is likely that differences between the affinities of the binding sites on a protein molecule could not be ascertained. Because saturation curves moved nearer the *x–y* intersection with increasing KCl concentration, one can assume KCl has different effects on the first and the second binding sites and on their affinity for the dye.

It should be mentioned that CB binding to BSA follows a typical saturation curve [10]. Although both CB and BR have sulfonic acid groups the mode of binding to the protein molecule is different. Structural studies are needed to explain BR binding to BSA.

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