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Interaction of serum albumin with vinyl sulfonate azo dye

Short Communication

Jolanta Sereikaite*, Vladas-Algirdas Bumelis

Department of Chemistry and Bioengineering,
Vilnius Gediminas Technical University,
LT-10223, Vilnius-40, Lithuania

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Abstract: The interaction between bovine serum albumin and the mono azo reactive dye Orange ZT has been investigated using absorption difference spectroscopy. The influence of pH and ionic strength of the solution on the stability of the dye-protein complex has been determined. At 25°C, the complex dissociation constants were equal to 24.0, 28.0, 7.0, 11.0, 17.6 and 46.0 μM at pH 7.0, 6.5, 6.0, 5.5, 5.0 and 4.3, respectively. In the presence of 0.1, 0.2, 0.3 M KCl, at pH 6.0 and 25°C, the complex dissociation constants were 8.8, 20.0, 18.0 μM, respectively. The protein-dye complex dissociation constants show that Orange ZT could be used as an affinity ligand for protein purification.

Keywords: *Orange ZT • Serum albumin • Absorption difference spectroscopy • Affinity ligand*

1. Introduction

Albumin is one of the most abundant proteins in plasma and, together with immunoglobulin, constitutes 80% of all plasma proteins. Effective removal of high-abundance plasma proteins is of high importance in proteomics studies, which are aimed to identify biomarkers of diseases [1]. To separate the albumin, dye-affinity chromatography is commonly used, and Cibacron blue F3G-A (CB) is a ligand of high popularity [2]. In this context, CB is coupled to matrices of various forms, such as microspheres [3-4] and membranes [5-6], or used unbound for albumin extraction with a reversed micellar system [7]. The non-covalent CB-human serum albumin complex has been thoroughly investigated. The complex was isolated by applying gel-permeation chromatography on Sephadex G-100. A dye-protein molar ratio equal to 3:1 was found over the pH range 6.5-10 [8]. Albumin has three different binding sites for CB [9], and likely more than one for other dyes, too [10]. Moreover, albumin can bind not only several single molecules of dye, but also self-assembling dyes such as Congo red or Evans blue. It is likely that specific polymolecular ligands penetrate the central crevice of albumin [11].

In this report the interaction of Orange ZT (OZT) has been examined using readily available bovine serum albumin (BSA) as a model protein. We found that the affinity of OZT for bovine serum albumin is suitable for affinity chromatography. Moreover, BSA-OZT complexes are stable over a range of pH and ionic strengths. Therefore, OZT could be used as an affinity ligand for albumin separation under wide range of conditions, thereby enlarging the spectrum of suitable ligands, depending on other contaminant proteins and their ligand affinities.

2. Experimental Procedures

2.1. Materials

Bovine serum albumin was obtained from Sigma (essentially fatty acid free). Buffer salts were of the highest purity available (Reachim). OZT was a kind gift from Dr. O. Sudziuviene (Institute of Biotechnology, Lithuania).

* *E-mail: sjolanta@fm.vgtu.lt*

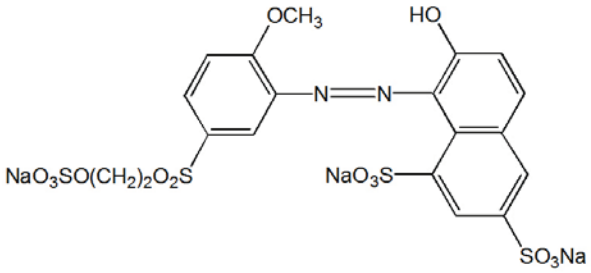

Figure 1. Chemical formula of OZT.

2.2. Absorption Difference Spectroscopy

Spectra were recorded on an Ultrospec 4000 spectrophotometer (Pharmacia Biotech), equipped with SWIFT II software, in the wavelength region 380-650 nm and using a path length of 1 cm. Difference spectral titrations were performed in 0.025 M potassium phosphate buffer, pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.3 or 3.6, and at 25°C. Spectra were additionally recorded in the presence of 0.1, 0.2 or 0.3 M KCl, at pH 6.0. The sample cuvette contained a constant BSA concentration of 10 μM, while the dye concentration in both the sample and the reference cuvette ranged from 5 to 90 μM.

2.3. Calculation of the Dissociation Constant of the Protein-Dye Complex

The dissociation constant of the complex was calculated using the equation (1):

$$\Delta A = \frac{1}{2}(E_{PL} - E_L)(L_0 + nP_0 + K_d - \sqrt{(L_0 - nP_0)^2 + K_d 2(L_0 + nP_0) + K_d^2}) \quad (1)$$

where K_d is the dissociation constant of BSA–OZT complex; L_0 and P_0 are initial concentrations of OZT and BSA, respectively; n is the number of dye binding sites per protein molecule (the value of n was assumed to be 1); E_L and E_{PL} are molar absorption coefficients of OZT and BSA – OZT complex, respectively; ΔA is the difference absorbance at 537 nm.

The experimental values were fitted to the ΔA equation (1) using the nonlinear least-squares method implemented in the Mathcad 5.0 program package (calculating the parameters of the function with the lowest coefficient of variation, CV).

The equation for ΔA (1) was obtained by rearranging the following equations (2-4) as described previously [12]:

$$K_d = \frac{(nP_0 - x)(L_0 - x)}{x} \quad (2);$$

$$A = E_L (L_0 - x) + E_{PL}\, x \quad (3);$$

$$\Delta A = A - E_L\, L_0 \quad (4),$$

where x is the concentration of BSA – OZT complex.

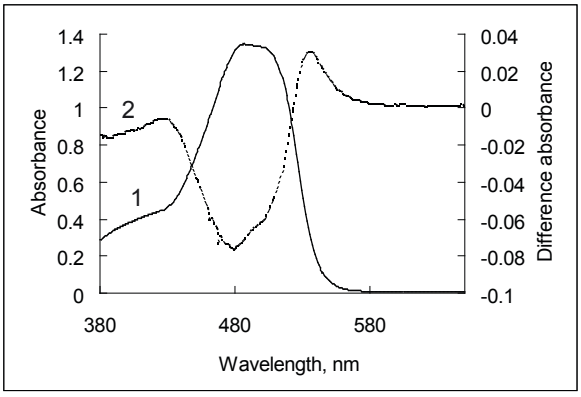

Figure 2. Spectrum of 60 μm solution of OZT in 0.025 M potassium phosphate buffer pH 6.0 (curve 1) and the difference spectrum for OZT dye binding to BSA in the same buffer (curve 2). The protein concentration in the sample cuvette was equal to 10 μM.

3. Results and Discussion

OZT is a mono azo reactive dye, containing one reactive vinyl sulfonate group and two sulfonic acid groups (Fig. 1). The visible absorption spectrum of OZT has a broad maximum at 486 - 490 nm (Fig. 2). To monitor the interaction of OZT with BSA, absorption difference spectroscopy was used. Before recording the difference spectra, the linearity of the Lambert-Beer law plot was verified under all experimental conditions used in the study, employing dye concentrations up to 100 μM. The molar absorption coefficient, calculated with the coefficient of correlation equal to 0.999, was found to be 3800 M^{-1} cm^{-1} at 537 nm. The visible difference spectrum of OZT in the presence of BSA has a maximum at 535 - 537 nm and a minimum at 479 - 480 nm. The approximate 50 nm shift of the maximum occurs to the long wavelength side (Fig. 2). These spectral changes suggest that OZT forms a complex with BSA. When a dye molecule is bound to a protein, the dye is transferred from polar environment to a less polar one, and the resulting dye absorption spectrum undergoes a shift. For example, CB binding to human growth hormone induces a shift of approximately 60 nm to the long-wave side [13]. Analysis of various active dyes binding to yeast alcohol dehydrogenase revealed that dye spectra undergo a red shift in the range of 20 - 75 nm [14]. For characterization of the dye-binding process, 10 μM BSA was titrated with an increasing amount of OZT. Typical difference spectra obtained from OZT dye binding to BSA can be seen in Fig. 3. The intensity of the 537 nm peak in each difference spectrum was plotted against the dye concentration (Fig. 4-5). Using the nonlinear least-squares method in the Mathcad 5.0 program package, K_d and E_{PL} were calculated [12]. The influence

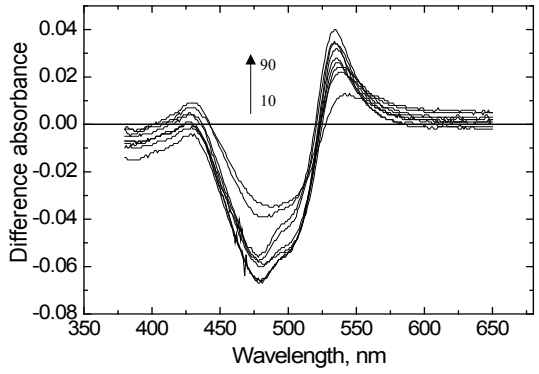

Figure 3. Typical difference spectra obtained from OZT dye binding to BSA in 0.025 M potassium phosphate buffer pH 7.0. The sample cuvette contained 10 μM BSA whereas the dye concentration in both sample and the reference cuvettes ranged from 10 to 90 μM.

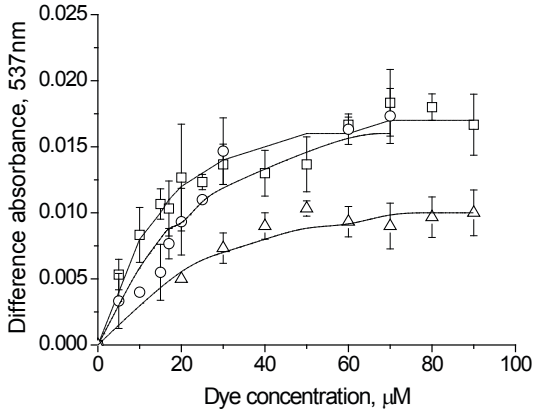

Figure 5. Titration at λ = 537 nm of BSA with OZT in 0.025 M potassium phosphate buffer, pH 6.0, in the presence of 0.1 M KCl (□), 0.2 M KCl (○) and 0.3 M KCl (Δ). The protein concentration in the sample cuvette was 10 μM. Data are presented with standard deviations calculated from the results of three parallel experiments. Symbols are the experimental data and --- is the theoretically calculated curve for the case of one binding site.

of pH on BSA-OZT complex stability was investigated, and the dissociation constants of the complex are presented in Table 1. The strongest interaction between BSA and OZT was found to be at pH 5.5 - 6.0, with K_d dramatically increasing at more acid pH values. It seems most plausible that the decrease of complex stability is related to acid-induced structural changes of bovine serum albumin, characterized by changes in secondary and tertiary structure. 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% [15,16]. Contrary to other pH values, at pH 3.6 the saturation of 10 μM BSA was not reached using the dye concentration up to 90 μM. It is known that below pH 4.0 BSA undergoes another isomerization, characterized by the appearance of a so-called expanded form (E), whose helical content decreases to 35% [16]. It seems

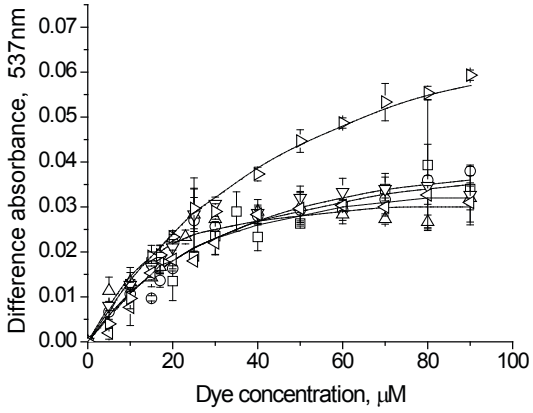

Figure 4. Titration at λ = 537 nm of BSA with OZT in 0.025 M potassium phosphate buffer, pH 7.0 (□), 6.5 (○), 6.0 (Δ), 5.5 (∇), 5.0 (◁) and 4.3 (▷). The protein concentration in the sample cuvette was 10 μM. Data are presented with standard deviations calculated from the results of three parallel experiments. Symbols are the experimental data and --- is the theoretically calculated curve for the case of one binding site.

Table 1. Influence of pH on BSA–OZT complex stability.

pH	K_d (μM)	E_{PL}, (M^{-1} cm^{-1}) at λ_{537} nm	CV (%)
7.0	24.0	8200	11.7
6.5	28.0	8800	11.7
6.0	7.0	7600	11.4
5.5	11.0	7900	7.4
5.0	17.6	7700	9.4
4.3	46.0	12400	6.2

Table 2. Influence of ionic strength on BSA–OZT complex stability at pH 6.0.

[KCl], M	K_d (μM)	E_{PL}, (M^{-1} cm^{-1}) at λ_{537} nm	CV (%)
0.0	7.0	7600	11.4
0.1	8.8	5800	8.2
0.2	20.0	5700	14.1
0.3	18.0	5000	9.2

that the expanded form E has other dye binding sites compared with N or F forms, and they are not saturated up to the dye concentration tested.

The influence of ionic strength on the dye-protein binding process was investigated. The stability of the complex decreased as ionic strength was increased. In the presence of 0.2 M KCl the dissociation constant of the complex increased about three-fold (Table 2). This trend suggests the theory that electrostatic interaction between BSA and OZT molecules is very important in the process of complex formation. The complex is most stable at pH 6.0. While pI of BSA is reported to be around 4.6 - 4.9 [17], even at pH 6.0 a protein molecule has many positively charged amino acid residues,

which can interact with deprotonated SO_3 groups of the dye molecule. Moreover, our recent investigations on the interaction of another sulfonate dye, Cibacron blue F3G-A, with human growth hormone and interferon-α2b, show that electrostatic forces make a significant contribution to the binding process. Using the method of time-resolved limited proteolysis, coupled with MALDI-TOF mass spectrometry, it was found that peptides of human growth hormone or interferon-α2b molecules, which exhibit affinity to that dye, have His or Arg and His residues, respectively, in theirs sequence. These have been shown to be important for the formation of complexes [18].

The stability of BSA-OZT complex is comparable to that of complexes of the well known dye Cibacron blue F3G-A with various proteins such as interferon-α2b, human growth hormone, nucleotide-binding enzymes and phospholipase A_2 [13 and references cited therein]. A ligand is considered to be suitable for affinity chromatography if the dissociation constant is between 10^{-4} and 10^{-8} M. With higher affinities, the problems of ligand-protein dissociation and protein elution arise [19]. Therefore, the affinity of OZT (K_d equal 10^{-5}-10^{-6} M at various pH and ionic strength) is sufficient to use this dye for serum albumin purification. Further, chromatographic matrices with immobilized OZT could be tested for protein purification. Until the conditions of protein elution are chosen it is not possible to determine if the dye is suitable to be used as an affinity ligand.

4. Conclusions

The formation of bovine serum albumin-Orange ZT complex was investigated using absorption difference spectroscopy. The influence of pH and ionic strength of the solution on the stability of the dye-protein complex has been determined. The complex is most stable at pH 5.5 - 6.0 and its stability decreases as the ionic strength of solution increases. The affinity of OZT to bovine serum albumin is on the level suitable for affinity chromatography. Orange ZT could be used for protein purification, thereby enhancing the range of suitable ligands under varying conditions.

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