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# Binding of fluoride, bromide and iodide to bovine serum albumin, studied with ion-selective electrodes

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**Abstract**

The interactions of halide ions, $F^-$, $Br^-$ and $I^-$, with bovine serum albumin (BSA) in neutral aqueous solutions (pH = 7.1) were investigated by the use of ion selective electrodes. The number of ions bound to BSA was determined from the difference in the amount of ion added to BSA solution and the amount of ion remaining free after equilibration. These data were treated according to the Klotz equation to find the number of binding sites, their equivalency and the binding constants. It was found that BSA has one binding site each for $Br^-$ and $I^-$, and two equivalent binding sites for $F^-$ in neutral solutions. The rather small number of binding sites for the halides studied was attributed to a net charge of −18 reported in the literature for BSA. The binding constants for $Br^-$ and $I^-$ and the two stepwise binding constants for $F^-$ were also determined.


*Keywords:* Bovine serum albumin; Fluoride; Bromide; Iodide; Ion selective electrode; Binding

## 1. Introduction

It is important to study the interactions of small ions, and molecules, with proteins in order to understand the nature of transportation and distribution of these species in biological systems because such interactions play a key role in transportation and distribution processes. Bovine serum albumin (BSA) has a high affinity for various ligands. Although it is one of the most widely studied subjects (Bojesen & Bojesen, 1996; Klotz, Walker, & Pivan, 1946; Scatchard, Wu, & Shen, 1959; Shrivasta, Kanthimathi, & Nair, 1999; Tanaka, Asahi, Masuda, & Ota, 1989), the nature of binding of ions is not yet clearly understood. The structure of serum albumin and the studies on binding of various ligands to serum albumin have been reviewed by Peters (1985) and by Carter and Ho (1994). The changes in conformation of BSA upon binding of small ions or molecules are also of interest and have been the subject of various studies (Shrivasta et al. 1999; Takeda & Moriyama, 1997). BSA is a globular protein and has a compact ellipsoid structure. The stability of this structure originates mainly from hydrophobic interactions (Carter & Ho, 1994). Thermal denaturation of BSA, studied by many workers (Ayranci, 1994; Itoh, Wada & Nakanishi, 1976; Ruegg, Moor & Blanc, 1977), has been found to be affected by binding fatty acids (Bernal & Jelen, 1985) and soy soap (Ikedo, Shimoyamada, & Watanabe, 1996). Interactions of BSA with urea have been studied by Ayranci and Kaya (1990) in relation to denaturation of BSA. The amino acid composition and sequence of BSA which are important in its conformational analysis are now known (Hirayama, Akashi, Furuya, & Fukuhara, 1990).

Klotz et al. (1946) studied binding of organic anions, methyl orange and azo-sulphathiazole to BSA and derived the so-called Klotz Equation. This equation can be given as:

$$\frac{1}{r} = \frac{K}{n}\frac{1}{[L]} + \frac{1}{n} \quad (1)$$

where r is the ratio of moles of bound anion to the total moles of protein, $n$ is the maximum possible number of bound anions/protein molecule and K is a constant to be determined experimentally which is known as the

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intrinsic dissociation constant. K depends on the nature of the anion as well as on the character of the protein. [L] is the equilibrium concentration of free anion. Scatchard et al. (1959) reported binding of small anions, such as chloride, fluoride, thiocyanate and trichloroacetate, to denatured bovine serum albumin. They treated their data according to a type of non-linear form of Klotz equation which is now known as the Scatchard equation.

Various experimental methods, such as indirect spectroscopic methods (Ohyoshi, Hamada, Nakata, & Kohata, 1999; Wang, Cheng, & Sun, 2000), gel filtration (Young & Cimasoni, 1974), the use of ion-selective electrodes (Ayranci, 1995; Luehrs & Johnson, 1986) and equilibrium dialysis (Klotz et al. 1946; Liang, Tu, Zhang, Shen, Zhou, & Shen, 2000), have been used in binding studies.

The purpose of the present study was to investigate the binding of the three halide ions, $F^-$, $Br^-$ and $I^-$, to bovine serum albumin by the use of ion selective electrodes. The analysis of various ions with ion selective electrodes has been carried out successfully in various previous studies in our laboratories (Ayranci, 1995; Ayranci & Balci, 1993).

## 2. Materials and methods

### 2.1. Materials

Bovine serum albumin was obtained from Sigma (Sigma A-7906). The standard solutions of KBr, KI and $NaNO_3$ were from Elit and of NaF from Orion.

An Orion model 94-09 fluoride electrode (Boston, USA), Elit 271 bromide electrode and Elit 281 iodide electrode (Laval, Canada) were used for the analysis of $F^-$, $Br^-$ and $I^-$ ions, respectively. An Orion model 90-01 single junction electrode (Boston, USA) was used as the reference electrode for fluoride analysis. For the analysis of the other two ions, an Orion 90-02 double junction electrode (Boston, USA) was the reference electrode. A combination pH electrode, i.e. a combination of a Ag/AgCl reference electrode, and a pH glass electrode was used for pH measurements. The potentials of the $F^-$ ion were recorded with a Metrohm-654 digital pH meter (Herisau, Switzerland) in mV. The potentials of $Br^-$ and $I^-$ ions and pH values of all solutions were measured with a Jenway-3040 Ion Analyzer (Dunmow, UK).

Titrations for binding studies were carried out with a Metrohm E274 burette (Herisau, Switzerland). The temperature control of solutions was achieved with a Heto DT Hetotherm type circulation water bath (Birkerod, Denmark). It was adjusted to $25.0 \pm 0.1$ °C.

### 2.2. Methods

#### 2.2.1. Calibration

The calibration curve for the iodide-selective electrode was prepared by plotting potentials measured after adding 0.5 ml 5 M $NaNO_3$ as ionic strength adjuster (ISA) to 20 ml KI solutions of different concentrations as a function of logarithm of $I^-$ concentration. The equation of the best fitting curve with the data points was obtained by the least square analysis of the data. The same procedure was followed for the preparation of calibration lines of $F^-$ and $Br^-$ selective electrodes.

#### 2.2.2. Binding studies

For $I^-$ binding studies, 0.5 ml 5 M $NaNO_3$ was added to 25 ml $2\times10^{-4}$ M BSA solution. The concentration of BSA was determined on the basis of an average molecular weight of 66 000 g $mol^{-1}$ (Ayranci, 1995). A $7.88\times10^{-4}$ M standard $I^-$ solution was used as a titrant. The potential and pH of the solution were recorded after each addition of a small volume (about 2 ml) of titrant by allowing sufficient time for equilibration at $25.0 \pm 0.1$ °C. These potentials were converted into concentrations by use of the previously obtained calibration curve for $I^-$. The amount of bound $I^-$ was calculated as the difference between the amount of total $I^-$ added and the amount of free $I^-$ measured at equilibrium.

The same procedure was followed for the binding studies of the other two ions to BSA with slightly different volumes and concentrations of reagents, which are given in Table 1.

Table 1
Volumes and concentrations of reagents used in the binding studies of ions to BSA

| Ion | Concentration (M) of 25 ml BSA solution | Volume (ml) of ISA (5 M $NaNO_3$) | Concentration of standard titrant solution |
|---|---|---|---|
| $F^-$ | $2\times10^{-4}$ | 0.5 | $5.26\times10^{-3}$ M NaF |
| $Br^-$ | $6\times10^{-4}$ | 1.5 | $6.25\times10^{-3}$ M KBr |
| $I^-$ | $2\times10^{-4}$ | 0.5 | $7.88\times10^{-4}$ M KI |

## 3. Results and discussion

The equations of calibration lines and the corresponding linear regression coefficients for the analysis of ions studied are given in Table 2. Excellent lines were obtained, as indicated by the regression coefficients.

r Values of Eq. (1) were calculated by taking the ratio of the amount of bound halide ion to the amount of BSA after each incremental addition of halide solution to BSA solution. Then 1/r was plotted as a function of 1/[free halide ion] according to Eq. (1). These plots are shown in Fig. 1 for $F^-$, in Fig. 2 for $Br^-$ and in Fig. 3 for $I^-$.

Obvious linearity, observed in 1/r vs. 1/[halide] plots for $F^-$, $Br^-$ and $I^-$, indicates that there are $n$ equivalent binding sites in BSA for binding these ions. It is known that deviations from linearity in 1/r vs. 1/[halide] plots indicate the presence of non-equivalent binding sites (Chang, 1981). In order to find the value of $n$ and the intrinsic dissociation constant K, the data of Figs. 1–3 were linearly regressed according to Eq. (1). The reciprocal of the $y$ intercept of the best fitted line gives the value of $n$ and then the slope of the same line times $n$ gives the K value. Correlation coefficients of the best-fitted lines, $n$ and K values for binding of $F^-$, $Br^-$ and $I^-$ to BSA are given in Table 3. It should be noted that the more precise values of $n$, calculated from the reciprocal of the intercept, were 1.86 for $F^-$, 1.02 for $Br^-$ and 0.96 for $I^-$. However, they were rounded to whole numbers in Table 3 since the number of bound ion cannot be fractional.

It is seen that the maximum number of ions, $n$, bound /BSA molecule is two for $F^-$ and one for $Br^-$ and $I^-$. This means that the binding occurs in two steps for $F^-$ and in one step for $Br^-$ and $I^-$. In general, the binding constant of the $i$th step, $K_{f,i}$, is calculated from the intrinsic dissociation constant by the following equation (Klotz et al., 1946),

$$K_{f,i} = \frac{n-(i-1)}{i} \times \frac{1}{K} \quad (2)$$

where $n$ is the maximum number of ions bound and K is the experimentally observed intrinsic dissociation constant. The $K_{f,i}$ values calculated from Eq. (2) are given in the last two columns of Table 3. They correspond to the equilibrium constants of the following reactions

$$BSA + F^- \rightleftharpoons [BSA.F]^- \quad K_{f,1}(F^-) \quad (3)$$

$$[BSA.F]^- + F^- \rightleftharpoons [BSA.F_2]^{2-} \quad K_{f,2}(F^-) \quad (4)$$

$$BSA + Br^- \rightleftharpoons [BSA.Br]^- \quad K_{f,1}(Br^-) \quad (5)$$

$$BSA + I^- \rightleftharpoons [BSA.I]^- \quad K_{f,1}(I^-) \quad (6)$$

The binding constants, $K_{f,1}$, of the three halide ions are found to decrease in the order $F^- > Br^- > I^-$ (Table 3) which can be explained by the increase in ionic size of these ions in the same order ($F^- < Br^- < I^-$). The value of $K_{f,2}$ for $F^-$ is lower than that of $K_{f,1}$ for the same ion as expected.

It is important to note that the pH of the solution was monitored throughout the binding studies and found to remain almost constant. It was measured from 7.04 to 7.05 for $F^-$, 7.16–7.18 for $Br^-$ and 7.07–7.09 for $I^-$ throughout the course of binding studies, so that there

Table 2
Equations of calibration curves and the corresponding linear regression coefficients for the analyses of ions under study

| Ion | Equation of the calibration line | Regression coefficient |
|---|---|---|
| $F^-$ | $E = -178.29 - 59.57 \log [F^-]$ | 0.9994 |
| $Br^-$ | $E = -176.60 - 58.96 \log [Br^-]$ | 0.9994 |
| $I^-$ | $E = -374.80 - 59.04 \log [I^-]$ | 0.9992 |

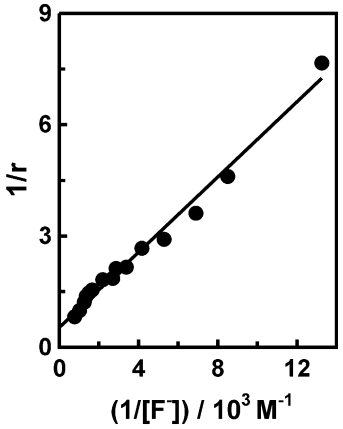


Fig. 1. Klotz plot for $F^-$–BSA system.

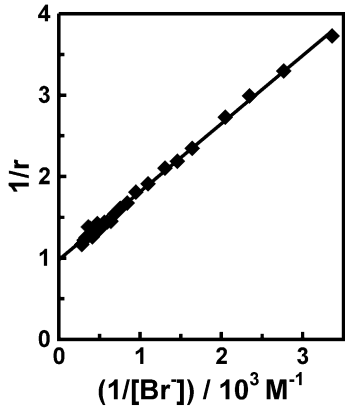


Fig. 2. Klotz plot for $Br^-$–BSA system.

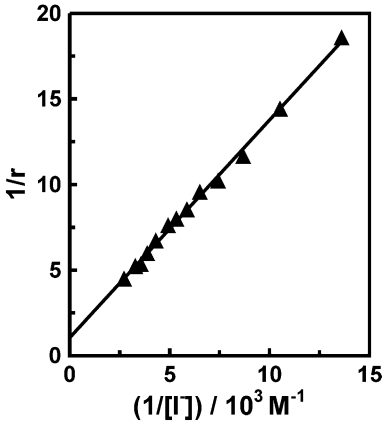


Fig. 3. Klotz plot for $I^-$–BSA system.

Table 3
Correlation coefficients, $n$, K and $K_f$ values for binding of $F^-$, $Br^-$ and $I^-$ ions to BSA

| Ion | Correlation coefficient | n | K (M) | Stepwise binding constant ($M^{-1}$) | |
|---|---|---|---|---|---|
| | | | | $K_{f,1}$ | $K_{f,2}$ |
| $F^-$ | 0.994 (Fig. 1) | 2.0 | $9.4\times10^{-4}$ | $2.0\times10^{3}$ | $9.9\times10^{2}$ |
| $Br^-$ | 0.996 (Fig. 2) | 1.0 | $8.6\times10^{-4}$ | $1.2\times10^{3}$ | – |
| $I^-$ | 0.997 (Fig. 3) | 1.0 | $1.3\times10^{-3}$ | $7.7\times10^{2}$ | – |

was no need to buffer the solutions. The use of buffer solution would introduce some more undesired charged species into the medium.

Two equivalent sites found for binding of $F^-$, and a single site each for $Br^-$ and $I^-$, seems to be unexpectedly small in number considering the huge size of BSA. However, Peters (1985) reported that BSA has a net charge of −18 at pH 7.0. Repulsive forces may have prevented the binding of anions in larger number to BSA. In Peters' review (1985), it was reported that, at a neutral pH (of about 7), BSA is in a so-called N-form, meaning normal form. This form is globular and the shape of BSA in this form is ellipsoidal with axes 41×141 Å. It is also reported that the helical content is 55% in the N-form. The net charge of −18 was distributed as −10 in the N-terminal domain (domain I), −8 in domain II and 0 in the C-terminal domain (domain III) of ellipsoidal BSA. So, there seems to be no domain in BSA, under neutral conditions, to attract halide ions electrostatically. On the other hand, in a denatured form, obtained by heat, denaturing agent or pH effect, the compact structure is deformed and BSA adopts an elongated form (F or E form, as defined by Peters, 1985), in which more sites may become available for binding of ligands that could be anionic, cationic or neutral. Ligands of a hydrophobic character may also find binding sites in BSA. For example, in the work of Scatchard et al. (1959), three classes of binding sites were found in bovine serum mercaptalbumin, which is BSA denatured by $Hg_2Cl_2$. They reported the presence of one binding site in the first class, eight sites in the second and eighteen in the third class for $Cl^-$, $F^-$, $SCN^-$ and $Cl_3CCOO^-$ ions. Small numbers of bindings (1–3) to serum albumin at neutral pH values, have also been reported for anions of a few fatty acids and for some biologically important compounds, as noted by Peters (1985). No binding at all was observed for $I^-$ to protamine (Ayranci, 1995).

BSA was found to contain 20 amino acids, ranging from 0.34% to 10.46% (Hirayama et al., 1990). Therefore, it is hard to specify the exact binding site in the amino acid chain of BSA for $F^-$, $Br^-$ and $I^-$. However, in the light of the above discussion, the bindings of the three halides are expected to be in the C-terminal domain of BSA, since it has been reported by Peters (1985) that the least net negative charge is in the C-terminal domain.

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