Use of fluorescence enhancement technique to study bilirubin–albumin interaction

Humra Athar a,*, Nisar Ahmad a, Saad Tayyab b, Mohammad A. Qasim a,1

Abstract

Bilirubin–albumin solution gave an emission spectrum in the wavelength range 500–600 nm with emission maxima at 528 nm when excited at 487 nm. The magnitude of fluorescence intensity increased on increasing bilirubin/albumin molar ratio. At three different albumin concentrations, namely, 1.0, 2.5 and 10.0 µM, there was an initial linear increase in fluorescence up to a molar ratio 1.0 in all cases beyond which it sloped off or decreased. This fluorescence enhancement was used to calculate the binding parameters of bilirubin–albumin interaction and the value of binding constant was found to be $1.72 \times 10^7$ l/mol similar to the published values obtained with other methods. Different serum albumins, namely, human (HSA), goat (GSA), pig (PSA) and dog serum albumins (DSA) bound bilirubin with almost the same affinity when studied by the technique of fluorescence enhancement. Bilirubin–albumin interaction was also studied at different pH and ionic strengths. There was a decrease in bilirubin–albumin complex formation on either decreasing the pH from 9.0 to 7.0 or increasing the ionic strength from 0.15 to 1.0. These results suggest that the technique of fluorescence enhancement can be used successfully to study the bilirubin–albumin interaction.

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Keywords: Fluorescence enhancement; Serum albumin; Bilirubin binding

1. Introduction

The technique of fluorescence quenching has been extensively used for studying the bilirubin–albumin interaction [1–3] and the value of binding constant obtained by this way compares well with the one obtained with the other methods such as equilibrium dialysis, peroxidase method, etc. [4,5]. On the other hand, this technique has been found handicapped in the study of displacement of bilirubin from albumin by a variety of ligands including drugs. This is because some ligands either fluoresce or absorb in the wavelength region of albumin fluorescence, i.e. 300–400 nm. Addition of bilirubin to albumin causes quenching in its fluorescence spectrum. Besides this phenomenon, there is an appearance of a band in the wavelength region 500–600 nm if the bilirubin–albumin solution is excited near 460 nm. The fluorescence intensity increases on increasing the bilirubin/albumin molar ratio. In this region, these ligands neither fluoresce nor absorb or do so poorly. When searched, the appearance of this band corresponds to an excitation maximum near 487 nm which falls in the region of absorption of bilirubin–albumin complex. Thus, this enhancement in fluorescence has been used to study the bilirubin–albumin interaction. In this report, data is presented on the use of fluorescence enhancement to study bilirubin–albumin interaction including determination of binding parameters.
2. Experimental

Sephacryl S-200 HR, bilirubin, pig serum albumin (PSA) and fraction V of human (HSA), dog (DSA) and goat (GSA) serum albumins were purchased from Sigma, St. Louis, MO. Bovine serum albumin (BSA), fraction V was the product of Sisco Research Laboratories, India. All other chemicals were of analytical grade.

Protein concentration was determined either spectrophotometrically using a specific extinction coefficient of 6.67 [6] at 279 nm for BSA or by the method of Lowry et al. [7].

Spectral measurements in the ultraviolet and visible regions were made on a Shimadzu double beam spectrophotometer, model UV 150-02 using matched silica cuvettes of 1 cm pathlength. Fluorescence measurements were made on a Shimadzu spectrofluorophotometer, model RF-540, equipped with a data recorder, model DR-3, a water jacketed cell holder, a mini magnetic stirrer (Hellma cuv-o-stir model 333) and a constant temperature water circulator, model TB-85.

Bilirubin solution was prepared by dissolving a few crystals of bilirubin in 1 ml of 1 M sodium carbonate solution containing 1 mM EDTA and the desired volume was made up to 10 ml with water. The solution was filtered and stored in the dark. The concentration of bilirubin was determined spectrophotometrically by taking the absorbance of the bilirubin solution at 440 nm using a molar extinction coefficient of 47 500 [8] after diluting it 50 times with the buffer. The solution was prepared fresh and used within 2 h. All experiments involving bilirubin were done in dim light.

2.1. Binding of bilirubin to serum albumin

Interaction of bilirubin with albumin was studied by the fluorescence enhancement titration method. The titration was carried out at 25°C in Tris-HCl buffer, pH 8.0, I = 0.06 by adding aliquots of bilirubin (0.05–4.0 ml of 11.62 μM concentration) to a fixed amount of albumin (1 ml of 2.5 μM concentration) taken in various test tubes to obtain a molar ratio between 0.05 and 3.75. At other albumin concentrations, different stocks of bilirubin were used to obtain the same molar ratios. The final volume was made up to 5 ml with Tris–HCl buffer, pH 8.0, I = 0.06. Fluorescence emission spectra were recorded in the wavelength range 500–600 nm by exciting the protein solution at 487 nm using a slit width of 5 nm. The fluorescence data were analyzed according to the following method.

2.2. Analysis of binding data

The fluorescence enhancement data were analyzed according to the method of Levine [9] modified in the laboratory. For the first binding site (R = 1.0), the maximal enhancement, eᵱ was calculated by the following equation:

\[ e_1 = F_1 - F_0 \]  

where \( F_1 \) is the fluorescence intensity at the bilirubin/albumin ratio 1.0 and \( F_0 \) is the fluorescence intensity at zero bilirubin concentration. For each fluorescence value, \( F \), the fractional enhancement, \( E \) was calculated by the following equation:

\[ E = \frac{F - F_0}{e_1} = \frac{F - F_0}{F_1 - F_0} \]  

Since enhancement is linearly related to the extent of binding, therefore,

\[ E = \frac{[\text{Bil} - \text{Alb}]}{[\text{Alb}]_T} \]  

where \([\text{Bil} - \text{Alb}]\) is the concentration of bilirubin bound albumin and \([\text{Alb}]_T\) is the total albumin concentration.

Finally, the unbound and bound bilirubin and albumin concentrations were found by using equations:

\[ [\text{Bil}] = [\text{Bil}]_T - [\text{Bil} - \text{Alb}] \]  

\[ [\text{Bil}] = [\text{Bil}]_T - E[\text{Alb}]_T \]  

and

\[ [\text{Alb}] = [\text{Alb}]_T - [\text{Bil} - \text{Alb}] \]  

or

\[ [\text{Alb}] = [\text{Alb}]_T - E[\text{Alb}]_T \]  

Using the association constant, \( K_a \)

\[ K_a = \frac{[\text{Bil} - \text{Alb}]}{[\text{Alb}][\text{Bil}]} \]  

Substituting with Eqs. (3)–(5) and (8)

\[ K_a = \frac{E[\text{Alb}]_T}{(1 - E)[\text{Alb}]_T ([\text{Bil}]_T - E[\text{Alb}]_T)} \]  

or,

\[ K_a = \frac{E}{(1 - E)([\text{Bil}]_T ([\text{Alb}]_T - E[\text{Alb}]_T))} \]  

Since ratio (R) is given by

\[ R = \frac{[\text{Bil}]_T}{[\text{Alb}]_T} \]  

Therefore,

\[ K_a = \frac{E}{(1 - E)(R - E)[\text{Alb}]_T} \]  

The transformation of the above equation into Scatchard equation gave the following relationship
\[ nK_a - K_aE = \frac{E}{[\text{Bil}]} = \frac{E}{(R - E)[\text{Alb}]_T} \]  

(14)

The value of association constant, \( K_a \) was determined from a straight line plot between \( E/[\text{Bil}] \) and \( E \). The slope of the plot gave the association constant \( (-K_a) \) and the intercept on \( X \)-axis gave the binding capacity, \( n \).

Binding was also studied using different serum albumins by fluorescence enhancement. Effect of pH and ionic strength was also seen by taking the fluorescence intensity at 528 nm.

3. Results and discussion

When a mixture of bilirubin and albumin was excited at 487 nm, it resulted in the appearance of a fluorescence band in the wavelength region 500–600 nm with an emission maximum at 528 nm. This seems to arise from the fluorescence properties of the bilirubin–albumin complex. The magnitude of fluorescence intensity at 528 nm increased on increasing bilirubin concentration as shown in Fig. 1.

![Fig. 1. Fluorescence emission spectra of bilirubin–albumin complex in the presence of different concentrations of bilirubin obtained in Tris–HCl buffer, pH 8.0, \( I = 0.06 \) after exciting it at 487 nm. Albumin concentration was 2.5 \( \mu \)M. Concentration of bilirubin was (from bottom to top) 0.0, 0.12, 0.25, 0.37, 0.49, 0.61, 0.73, 0.85, 1.0, 1.1, 1.34, 1.46, 1.60, 1.70, 1.83, 2.10, 2.20, 2.30, 2.45, 5.80, 6.96 and 9.28.](image1)

At 1.0 and 2.5 \( \mu \)M albumin concentration, addition of bilirubin caused a linear increase in fluorescence intensity at 528 nm up to a molar ratio of 2.0 and 1.0, respectively, beyond which it sloped off. This has been depicted in Fig. 2. On the other hand, when 10 \( \mu \)M albumin concentration was used, fluorescence intensity at 528 nm increased continuously reaching the maximum value at a molar ratio of 1.3 and then it decreased significantly up to a molar ratio 5.0 (see Fig. 2). Thus, up to a molar ratio 1.0, the enhancement in fluorescence was dependent on albumin concentration as the enhancement was maximum with 10 \( \mu \)M albumin concentration and smaller with 1 \( \mu \)M albumin concentration. In all cases, up to a molar ratio of 1:1, there was an initial linear increase in fluorescence at 528 nm that can be used for the calculation of binding constant and binding capacity as described in Section 2. The Scatchard plot obtained from the data at 2.5 \( \mu \)M albumin concentration is shown in Fig. 3. The value of binding constant as obtained from the slope of Scatchard plot was found to be \( 1.72 \times 10^7 \) l/mol with the binding capacity as 0.89. The value of binding constant compared well with the value of \( 1.35 \times 10^7 \) l/mol obtained from fluorescence quench titration method as done in the laboratory (data not shown) and with other reported value [3]. Thus, the method can be used successfully for studying the bilirubin–albumin interaction.

Using this technique, the binding parameters of four different serum albumins namely, PSA, DSA, HSA and GSA were determined. The Scatchard plots for these data are shown in Fig. 4 and the values of binding parameters are given in Table 1. A comparison of the
values of binding constants obtained with these albumins suggests that PSA and DSA bind bilirubin with greater affinity while GSA has a lower affinity. The values of binding constants of HSA and GSA agreed with the values reported earlier [4,10].

This technique was also used to study the effect of pH and ionic strength on the bilirubin–albumin interaction. As can be seen from Fig. 5, the fluorescence of the bilirubin–albumin complex decreased on decreasing the pH from 10.0 to 2.3. The decrease in binding was more significant in the pH range 9.0–7.0 where albumin molecule undergoes the N-B transition. Increase in ionic strength to 0.15 led to an increase in the complex formation and then caused a decrease in this interaction (see Fig. 6) which suggested the role of electrostatic interactions in bilirubin binding to albumin involving negatively charged carboxyl groups of bilirubin and positively charged amino acid residues of albumin. Thus, the technique of fluorescence enhancement worked well in the study of the bilirubin–albumin interaction and can be used to study the effect of various drugs including those which absorb in the wavelength region of albumin fluorescence, on the bilirubin–albumin interaction.

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**Table 1**

<table>
<thead>
<tr>
<th>Albumins</th>
<th>Binding constant (l/mol)</th>
<th>Capacity (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig serum albumin (PSA)</td>
<td>$4.41 \times 10^7$</td>
<td>0.83</td>
</tr>
<tr>
<td>Dog serum albumin (DSA)</td>
<td>$4.30 \times 10^7$</td>
<td>0.93</td>
</tr>
<tr>
<td>Human serum albumin (HSA)</td>
<td>$1.31 \times 10^7$</td>
<td>0.94</td>
</tr>
<tr>
<td>Goat serum albumin (GSA)</td>
<td>$1.20 \times 10^7$</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Fig. 5. (A) Fluorescence emission spectra of the bilirubin–albumin complex obtained at different pH values, viz. (from top to bottom) 10.0, 9.3, 8.2, 8.0, 7.6, 7.3, 7.0, 6.5, 6.0, 5.6, 5.0, 4.6, 4.0, 3.6, 2.7 and 2.3. Various buffers of different pH and same ionic strength (0.10) were used. (B) Effect of pH on the binding of bilirubin to bovine serum albumin (BSA). Relative fluorescence values at 528 nm as calculated from the data of (A) were plotted against pH.

Fig. 6. (A) Fluorescence emission spectra of the bilirubin–albumin complex obtained at different ionic strengths viz. (from top to bottom) 0.15, 0.05, 0.01, 0.5 and 1.0. In these experiments 0.005 M sodium phosphate buffer, pH 8.0 was used and desired ionic strength was adjusted by adding a requisite amount of sodium chloride. (B) Effect of ionic strength on the binding of bilirubin to bovine serum albumin (BSA). Relative fluorescence values at 528 nm as calculated from the data of (A) were plotted against ionic strength.
References