Structural features of bromocresol purple and its binding sites on human serum albumin for a proton-exchange reaction

Shigenori Ito

Department of Physiological Chemistry, Osaka Medical College, 2-7 Daigakcho, Takatsuki 565-8686, Japan

Abstract

Human serum albumin (HSA) levels of identical samples are different when determined using the bromocresol green (BCG) and bromocresol purple (BCP) methods. The aim of this study was to determine this reason for this difference. The pH-dependent color change of the Sulton pH indicators (BCG, BCP, bromophenol blue, bromothymol blue, thymol blue, and phenol red [PR]) complexed with HSA (or poly-L-lysine) in acidic (pH 2.4–7.8) and basic (pH 7.6–11.2) solutions was determined with and without inhibition of complex formation by warfarin and ibuprofen. The structures surrounding the drug-binding sites were analyzed by determining the required characteristics of target residues predicted from the experimental data based on the crystallographic data of the drug–HSA complexes (2BXD and 2BXG). BCP specifically bound to the warfarin- and ibuprofen-binding sites in acidic pH, but BCG did not. In basic pH, all of the indicators, except for PR, bound to their sites. All of the residues for color change, proton exchange, and binding via electrostatic interaction were present in the binding sites. Given that BCP binds to the warfarin- and ibuprofen-binding sites, the influence of coexisting substances can be experimentally evaluated to facilitate precise measurement using the BCP method.

Keywords: Bromocresol purple, human serum albumin, color-change mechanism, binding site, sulton pH indicator

1. Introduction

Quantification of human serum albumin (HSA) is commonly performed in a clinical setting using the dye-binding method with two pH indicators, bromocresol green (BCG) and bromocresol purple (BCP) [1,2], based on the metachromasy phenomenon, which is a characteristic color change that occurs when dyes bind to proteins [3,4]. However, different values are obtained with these indicators [5]. BCG reacts slowly with other serum proteins in addition to albumin, such as globulins [6,7]. Therefore, the BCP method, which has greater specificity, has been commonly used instead [8]. In addition, a modified BCP method was developed based on the conversion of human mercaptalbumin (HMA) to human nonmercaptalbumin (HNA) with the oxidizing reagent 5,5’-dithiobis(2-nitrobenzoic acid) because the value of HMA was found to be lower than that of HNA using the BCP method [9].

The color change of the BCP–HSA complex is attributed to proton exchange between BCP and an acidic or basic group of HSA [10]. In this study, I evaluated the pH-dependent color change of Sulton pH indicators (BCG, BCP, bromophenol blue [BPB], bromothymol blue [BTB], thymol blue [TB], and phenol red [PR]), which have structural similarity, to determine the structural characteristics of the pH indicators required to cause a color change. In particular, I ascertained the specific features of the interaction and structure of the BCP-binding sites, i.e., the warfarin- and ibuprofen-binding sites, on HSA [11]. Therefore, I conducted a binding inhibition experiment using warfarin or ibuprofen to determine whether the tested Sulton pH indicators would also bind to the drug-binding sites. Finally, I evaluated the features of the two binding sites in HSA that contain the key residues required for binding and color change, respectively, based on comparison with the crystallographic data (PDB: 2BXD, warfarin–HSA complex: 2BXG, ibuprofen–HSA complex) [12,13]. Understanding the underlying mechanism and binding features of the interaction between HSA and pH indicators would aid in the understanding of the problems of HSA quantification.

* Correspondence Info

Dr. Shigenori Ito
Department of Physiological Chemistry
Osaka Medical College
2-7 Daigakcho, Takatsuki 565-8686, Japan
E-mail: ito@art.osaka-med.ac.jp
2. Materials and Methods

2.1 Materials

The reagents used for buffer preparation, warfarin, ibuprofen, and pH indicators (BCG, BCP, BPB, BTB, TB, and PR) were purchased from Wako Chemical Co. (Osaka, Japan). Fatty acid-free HSA and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Poly-L-lysine (PLL) hydrochloride was purchased from Peptide Institute Inc. (Osaka, Japan). The molecular weight (36.5 kDa) of PLL was determined by high-performance gel filtration chromatography using a TSKgel G2000SW column.

2.2 Preparation of buffers

Acidic buffer solutions of pH 2.4–7.8 were prepared by mixing a 0.1 mol/L citric acid solution and a 0.2 mol/L Na₂HPO₄ solution. Basic buffer solutions were prepared by adjusting the pH of a 0.1 mol/L Na₂B₄O₇ solution to 7.6–11.2 by adding 4 mol/L NaOH or a 6 mol/L HCl solution. pH indicators, warfarin, and ibuprofen were dissolved in DMSO to a concentration of 20 mmol/L. The working solution used was 10 μmol/L BCP and 5 μmol/L HSA in a 2.5x dilution of the aforementioned buffer solutions.

2.3 Calculation of dissociated BCP at 3 pKa values

The method employed for the calculation of dissociated BCP was described in my previous study [10]. In brief, the concentration of a particular ionic species (or molecule) at a given pH is calculated by using the Henderson–Hasselbalch equation. The concentrations of a univalent ion of a pH indicator (pHi−) such as BCP− and a bivalent ion (pHi²−) such as BCP²− at a given pH were calculated using the pKa value of the pH indicator and that of the acidic and basic groups in HSA. According to the following reactions, the maximum amount of the bivalent ion and univalent ion formed is proportional to the amount of the dissociated carboxyl group and protonated amino groups in HSA, respectively.

\[
\text{pHi}^- + \text{HSA-COO}^- \rightarrow \text{pHi}^{2^-} + \text{HSA-COOH} \quad \text{(at pH lower than the pKa value)}
\]

\[
\text{pHi}^{2^-} + \text{HSA-NH}_3^+ \rightarrow \text{pHi}^- + \text{HSA-NH}_2 \quad \text{(at pH higher than the pKa value)}
\]

2.4 Experiment of pH-dependent absorbance change

The reaction mixture contained 10 μmol/L pH indicator, 5 μmol/L HSA, and 0.05% DMSO in buffer solutions of various pH (2.4–7.8). The absorbance was measured at 435 nm to detect the decrease in the undissociated indicator formed by the addition of HSA. Furthermore, the reaction mixture contained 10 μmol/L pH indicator, 5 μmol/L HSA or PLL, and 0.05% DMSO in buffer solutions of various pH (7.6–11.2). The absorbance was measured at 616, 589, 592, 616, and 597 nm to detect the decrease in dissociated BCG, BCP, BPB, BTB, and TB resulting from addition of HSA.

2.5 Experimental inhibition of warfarin or ibuprofen

The reaction mixture was prepared using 10 μmol/L pH indicator, 5 μmol/L HSA, and 0.05% DMSO and either no other drug or 0.4 mmol/L drug (warfarin or ibuprofen) was added to this mixture. The absorbance was measured at 435 nm to determine the amount of the pH-indicator-HSA complex formed following the addition of HSA in a solution with acidic pH solution (pH 3.6, 4.8, and 3.0 for BCG, BCP, and BPB, respectively). The absorbance was measured at 616, 589, 592, 616, and 597 nm in a solution with basic pH solution (pH 8.0, 8.4, 8.0, 8.4, and 9.6 for BCG, BCP, BPB, BTB, and TB, respectively). Each value shown is the mean of three experiments, and the %CV values are as follows: (A) warfarin: 1.4 (BCG), 7.4 (BCP), 1.0 (BPB); (A) ibuprofen: 2.0, 6.2, 3.7; (B) warfarin: 2.6 (BCG), 6.5 (BCP), 3.5 (BPB), 3.0 (BTB), 5.6 (TB); (B) ibuprofen: 1.3, 3.7, 5.6, 2.7, 3.7 (Figure 3).

3. Results and discussion

3.1 Proton-exchange reactions between Sulton pH indicators and HSA

A pH indicator has two main forms in aqueous solution: pH⁻ at a pH lower than the pKa value and pH²⁻ at a pH higher than the pKa value. The transfer of a proton from pH⁻ to water or vice versa causes a visible color change in the solution at a specific pH with a difference in the resonance structures [14,15]. When albumin, which can adsorb various organic compounds [16], is added to a buffer solution containing a pH indicator, the resulting color change in the indicator is equivalent to that occurring in a solution of high or low pH. This property is applied to quantify HSA levels using dye-binding methods [1,2]. In this study, I evaluated the pH-dependent color change to clarify the color-change mechanism of other Sulton pH indicators in addition to BCP, including BCG, BPB, BTB, TB, and PR. Two ionic specimens (pHi⁻ and pHi²⁻) such as BCP⁻ and BCP²⁻ bind to separate binding sites. My
previous experimental results showed that proton-exchange reactions of BCP–HSA complexes occur at separate binding sites in acidic and basic pH solutions [17].

The color change of the Sulton pH indicators, which occurs via proton exchange when complexed with HSA, can be estimated by comparing experimental and theoretical pH-dependent data calculated using the Henderson-Hasselbalch equation (Figures 1 and 2). The pKa values of acidic or basic groups on HSA that best fit the equation are summarized in Table 1. However, no color change of PR was observed in either of the pH solutions, and no color change of BTB and TB was observed in the acidic pH solution. The pKa value (1.4 or 1.6) of the acidic group obtained from experiments with BCG, BCP, or BPB was lower than the values of α, β, and γ carboxyl groups (Table 1). This decrease in pKa is considered to be caused by an electrostatic interaction between the acidic group and its neighboring basic group. The same result was obtained in an experiment with methyl orange–HSA complex, and the conformational arrangement of the two residues on HSA has been determined through protein-structure analysis [18]. pKa values are known to increase or decrease in the presence of favorable interactions (e.g., hydrogen bonding, electrostatic interactions, and hydrophobic interactions) between amino acid residues of proteins [19]. Furthermore, the pKa values of the indicators with basic groups were similar (Table 1). This suggests that the ε-amino groups participate in proton-exchange reactions.

**Figure 1: Comparison of pH-dependent plots in an acidic pH range generated from experimental (■) and calculated (□) absorbance change data of (A) BCG, (B) BCP, and (C) BPB**

<table>
<thead>
<tr>
<th>pH indicator</th>
<th>BCG</th>
<th>BCP</th>
<th>BPB</th>
<th>BTB</th>
<th>TB</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic group</td>
<td>1.6</td>
<td>1.6</td>
<td>1.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pH indicator</td>
<td>4.7</td>
<td>6.0</td>
<td>3.7</td>
<td>7.1</td>
<td>8.5</td>
<td>–</td>
</tr>
<tr>
<td>Basic group</td>
<td>10.6</td>
<td>10.4</td>
<td>10.6</td>
<td>10.1</td>
<td>10.5</td>
<td>–</td>
</tr>
<tr>
<td>Amino group of PLL</td>
<td>(9.7)</td>
<td>(8.1)</td>
<td>(9.4)</td>
<td>(9.8)</td>
<td>(10.5)</td>
<td>–</td>
</tr>
</tbody>
</table>

Moreover, pH-dependent experiments were performed using PLL rather than HSA to confirm the participation of the amino group in the binding reaction with the pH indicators (Figure 2). A pH indicator binds to PLL via electrostatic interactions between the sulfo group and a protonated basic group, followed by proton exchange between the phenoxy group and a protonated ε-amino group. The pH-dependent absorbance change values were similar to those of the HSA complexes, but slightly variable (Figure 2). These results demonstrate that: (1) the color change of Sulton pH indicators, except for PR, occurs via proton exchange between the phenoxy and protonated ε-amino group of HSA in a basic pH solution; and (2) the tertiary structure is important for stabilization in forming the interaction at the binding site of HSA.
Figure 2: Comparison of pH-dependent plots in a basic pH range generated from experimental (human serum albumin [■] or poly-L-lysine [▲]) and calculated (○) data for (A) BCG, (B) BCP, (C) BPB, (D) BTB, and (E) TB

3.2 Binding of Sulton pH indicators to two drug-binding sites on the HSA molecule

I investigated whether a molecule of the other pH indicators would also bind to the same drug (warfarin and ibuprofen)-binding sites as BCP [11] by conducting an inhibition experiment. In a low-pH solution, warfarin inhibited the absorbance change of the BCP–HSA complex by 23% compared to the control, whereas ibuprofen enhanced the absorbance change by 60% (Figure 3A). These results indicate that BCP interacts with the binding sites for these drugs on HSA. However, the drugs had no effect on the binding of BCG or BPB to HSA (Figure 3A). The effect for ibuprofen can be easily explained as follows: after binding of ibuprofen to the binding site, the carboxyl group of ibuprofen participates in a proton-exchange reaction to induce the color change. The binding ratio of BCP to a single HSA molecule was 2 at pH 4.8 through surface plasmon resonance (SPR) analysis (data not shown). BCP only binds to warfarin- and ibuprofen-binding sites and does not bind to the other sites. The color of BCG- and BPB-HSA complexes changes through proton exchange at other sites.

In a high-pH solution, absorbance changes of all of the indicators except for PR were inhibited by 23–66% with warfarin or ibuprofen (Figure 3B). Inhibition was observed in the solution with basic pH regardless of the pH indicator tested. Furthermore, the indicators changed color through binding to PLL, which has low solid-binding specificity. Therefore, the color change occurred in the presence of two basic groups for binding and proton exchange at a binding site. These results indicate that the binding specificity of pH molecules to drug-binding sites in a high-pH solution is lower than that in a low pH solution. The different binding features in low- and high-pH solution suggest that there are separate binding sites for the two ionic forms (pHi− and pHi2−) of a pH indicator on an HSA molecule, or that there are different configurations corresponding to each form at a binding site. The two BCP-binding sites were previously identified using a photometric method [17].
3.3 Structural features of Sulton pH indicators required to achieve a color change

The structures of PR in crystal, a lower-pH solution, and a higher-pH solution (>7.5: the pKa value of PR) are shown in Figure 4. The different groups at the positions of the phenol ring in the other Sulton pH indicators (BCG, BCP, BPB, BTB, and TB) are indicated in Figure 4 and Table 2. The influence of the substituents was examined with respect to the results of inhibition experiments (Figure 3). PR, which has no substituents on the phenol ring, did not show a color change in the reaction. This result indicates that the groups on a phenol ring play an essential role in the binding and color change. BCG, BCP, BPB, and BTB have a bromo group at position 3. The color change of BCP, which has a methyl-group at position 5, was inhibited by warfarin in a solution with acidic pH, whereas the color changes of BCG and BPB, which have a bromo group at the same position, were not inhibited. This result suggests that the bromo group at position 3 and the methyl group at position 5 are likely responsible for the binding and consequent color change. In a solution with basic pH, BCP, BTB, and TB, which possess alkyl groups at position 5, were inhibited to a greater extent by warfarin or ibuprofen than were BCG and BPB, which possess a bromo group at position 5. By contrast, the methyl group at position 2 and the bromo group at position 3 did not affect binding. These structural data indicate that the substituents of pH indicators play an important role in color changes.
3.4 Speculated key residues of HSA for binding and color change of pH indicators

HSA is a 66-kDa monomeric protein that contains three homologous helical domains, I–III, each of which is divided into two subdomains, A and B. Two drug-binding sites located in subdomains IIA and IIIA (drug-binding sites 1 and 2) have been identified on the HSA molecule [20]. BCP is considered to bind to HSA through these two drug-binding sites, which are bound by warfarin (site 1) and ibuprofen (site 2) [11]. I aimed to identify the residues in the two drug-binding sites on the HSA molecule that are responsible for the binding and proton exchange based on the PDB data of the warfarin- and ibuprofen-HSA complexes (2BXD and 2BXG) [12,13]. Based on the experimental results obtained for the low pH range, the candidate amino acid residues in the binding sites need to satisfy the following conditions: (1) a basic residue to enable binding of a Sulton pH indicator via electrostatic interactions, (2) an acidic residue that is adjacent to a basic group to enable proton exchange, and (3) a distance between the groups of two residues equal to that between the sulfo and phenoxy groups (atomic distance between O(S=O) and O(C–O): 6–7 Å) of the Sulton pH indicators. The same conditions apply in the case of the higher pH range, except that a basic residue is required for proton exchange rather than the acidic residue in condition (2) above. Based on the distance between atoms, the residues needed to satisfy the requirements mentioned above in the warfarin- and ibuprofen-binding sites are summarized in Table 3.

### Table 3: Distances between stabilizing, proton-exchange, and neighboring basic residues

<table>
<thead>
<tr>
<th>Binding site</th>
<th>PDB name</th>
<th>Binding residue</th>
<th>H⁺ exchange residue</th>
<th>Neighboring residue</th>
<th>Distance (Å)</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>warfarin</td>
<td>2BXD</td>
<td>Arg222</td>
<td>Glu292</td>
<td>Lys195</td>
<td>5.96</td>
<td>lower pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glu292</td>
<td>Lys195</td>
<td>3.87</td>
<td>lower pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arg222</td>
<td>Glu292</td>
<td>Lys195</td>
<td>6.86</td>
<td>higher pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lys351</td>
<td>Glu354</td>
<td>Arg209</td>
<td>2.88</td>
<td>lower pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glu354</td>
<td>Arg209</td>
<td>6.49</td>
<td>lower pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arg410</td>
<td>Lys414</td>
<td></td>
<td>6.64</td>
<td>higher pH</td>
</tr>
</tbody>
</table>

4. Conclusion

This study reveals the following facts regarding the binding sites of BCP and BCG on HSA: (1) the non-dissociated and dissociated forms of BCP/BCG bind to separate binding sites; (2) the color changes occur by proton-exchange reactions between the phenol group and the dissociated carboxyl group, and between the phenoxy group and the protonated ε-amino group; (3) BCP binds to warfarin- and ibuprofen-binding sites in solutions with a pH lower than the pKa value, but BCG does not; (4) in a higher-pH solution, BCP and BCG bind to the two drug-binding sites, which have an ε-amino group for proton exchange and a basic group for binding, and these binding sites have low structural specificity; and (5) the amino acid residues present in the binding sites meet these conditions required for interaction. Dye-binding methods using BCG or BCP have been used widely because of the convenience of operation and speed of measurement. However, I believe that the BCP method is more accurate than the BCG method. BCP⁺ and BCP⁺⁻² bind to the same drug-binding sites with different orientations in lower-pH and higher-pH solutions.
higher-pH solutions; thus, there is little influence of solution pH. In contrast, BCG\(^{-}\) and BCG\(^{2-}\) bind to separate binding sites with different features in both pH solutions.

References