Influence of Hydrogen and Chloride Ions on the Interaction between Sulfaethidole and Bovine Serum Albumin Studied by Microcalorimetric and Acid-Base Titrimetric Methods

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From earlier studies it is known that bovine serum albumin has one high affinity binding site and several lower affinity sites for the sulfa drug N\textsuperscript{(5-ethyl-1,3,4-thiadiazol-2-yl)sulfanilamide} (sulfaethidole) (Kostenbauer, H. B., Jawad, M. J., Perrin, J. H., and Averhart, V. (1971) J. Pharm. Sci. 60, 1658–1660).

This binding has been further studied using equilibrium dialysis, microcalorimetry, and pH titration technique. Results of these studies show that the binding of sulfaethidole to the first (high affinity) site may be accompanied by an uptake of protons. Proton uptake is found to be zero at pH 7.4 and approximately 0.6 at pH 8.5 for each sulfaethidole molecule bound. The other binding sites for sulfaethidole are not proton linked. The first, and probably the other binding sites, are also Cl\textsuperscript{−} ion linked; for example, the binding of sulfaethidole to the first binding site is accompanied by the displacement of (on average) one Cl\textsuperscript{−} ion at pH 7.4 in 0.1 M NaCl.

This explains the observation that the heat of binding of sulfaethidole to the high affinity site is -33.0 kJ\textper mоль\textsuperscript{−1} in the absence of chloride ions, but only -22.8 kJ\textper mоль\textsuperscript{−1} in the presence of 0.1 M Cl\textsuperscript{−} (at pH 7.4).

The binding of drugs to serum albumin is a well known and much studied phenomenon. The fact that some drugs are bound to albumin to a large extent may have an influence on the biological activity of the drug (1). For many drugs the binding properties in terms of binding constants and number of binding sites are known (2). Most in vitro binding studies are carried out at a fixed pH and given ionic strength of the medium and often in the presence of (the physiologically important) Cl\textsuperscript{−} ions. The influence of variations in the concentration of H\textsuperscript{+} and Cl\textsuperscript{−} ions on the binding of the sulfa drug N\textsuperscript{(5-ethyl-1,3,4-thiadiazol-2-yl)sulfanilamide} (sulfaethidole) to bovine serum albumin is the subject of this study. Besides possible physiological implications, this type of study provides more insight into the mechanism of small molecule/protein interactions.

The involvement of protons in the binding of SETD\textsuperscript{1} by albumin has been studied by means of an acid/base titrimetric method (3). These measurements are here combined with microcalorimetric measurements under various buffering conditions. These studies enable the influence of Cl\textsuperscript{−} ions on this type of interaction to be detected and evaluated.

EXPERIMENTAL AND ANALYZING PROCEDURES

Materials—Bovine serum albumin (Fraction V) was obtained from Sigma. SETD was a gift from Smith, Kline and French Laboratories (Lot 5610). All other chemicals were analytical or reagent grade. Phosphate and Tris buffers were used at an ionic strength of 0.1 (4).

Microcalorimetric Measurements—Microcalorimetric measurements were performed using a LKB flow microcalorimeter LKB 1070-1. This calorimeter was modified so that the calorimetric head was housed within a watertight container, submerged in a Tronac 1065 waterbath, provided with a PTC 40 temperature controller (Tronac, Inc., Orem, Utah). A Keithley 150 B microvolt ammeter was used to amplify the signal which was displayed on a Kipp B08 multi-range recorder (Kipp, Delft, The Netherlands) provided with an AG 1 zero point suppressor (Kisch, Albachten, Germany). The instrument was calibrated electrically within the expected range and with the pumping speed to be used. The calibration constant was approximately 0.069 V-watt\textsuperscript{−1}.

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Solutions were pumped into the calorimeter by means of two LKB peristaltic pumps. Flow rates (usually about 3.3 ml-s\textsuperscript{−1}) were measured at frequent intervals. Base-lines were measured with the appropriate solutions and corrections applied whenever necessary. The calorimetric experiments were all done at 25°C.

Equilibrium Dialysis—Some binding experiments were performed using the Dianorm equilibrium dialyzer (Diachema AG, Rüschlikon, Switzerland) using membranes 10-14. At the ratios studied equilibrium was reached within 96 h. Free concentrations of SETD were measured according to the method of Bratton and Marshall (6).

Analysis of Calorimetric Data—For a protein P having t classes of binding sites for a ligand L, the observed mean number of ligands bound \( \bar{r} \) is composed of a contribution from several sites such that \( \bar{r} = \sum_{\nu} \bar{r}_{\nu} \). Each of the classes of sites has a heat of binding \( \Delta H_{\nu} \), which is assumed to be a constant at constant pH. In the mixing cell of the calorimeter a protein solution having an initial concentration \( [P]_{i} \) with a flow rate \( f_{P} \cdot 1 \cdot s \textsuperscript{−1} \) is continuously mixed with a ligand solution having a concentration \( [L]_{i} \) and a flow rate \( f_{L} \cdot 1 \cdot s \textsuperscript{−1} \). The resulting heat of reaction evolved per s is recorded as a microvolt signal which is converted to microwatts by means of the calibration constant. When appropriate corrections for heat of dilution are applied, the measured heat per s \( Q \) is due only to binding of the protein by the ligand, as given in Equation 1.

\[ Q = \sum_{\nu} \Delta H_{\nu} \cdot \text{(number of moles of protein reacting per s)} \]  

The number of moles of protein reacting per s follows from the product of the protein concentration in the calorimeter, which is given by \( (P)_{f_{P}}/(f_{P} + f_{L}) \), and the volume element (formed per s) in

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1 The abbreviations used are: SETD, sulfaethidole; albumin, bovine serum albumin.

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which the reaction takes place, which is approximated by \( f_1 + f_2 \). Therefore, \( Q = \sum f_i \Delta H_i^{(f)} \) or \( Q/\{f_i \} = \sum f_i \Delta H_i \) (2)

The calorimetric experiments are performed such that \( f_1, f_2, \) and \( \{f_i \} \) are held constant and only \([L]_1\) is varied. A kind of titration curve is obtained in which \( \sum f_i \Delta H_i \) is plotted against the drug to protein ratio \( r \), given by

\[
r = (c_1 \{L\})/\{AP\}.
\]

(3)

A drawback of this curve is that when experimental conditions such as flow rates and concentrations are changed, the shape of the curve will also change since \( r \) depends on these factors. Only when \( \sum f_i \Delta H_i \) is plotted against \( \tilde{r} \) will a unique curve be obtained, for a given \( \tilde{r} \) corresponds to a unique combination of \( r_1, r_2, \) etc., as determined by the relative magnitude of the corresponding binding constants.

Assuming that two \( \sum f_i \Delta H_i \) versus \( r \) curves are measured, obtained by changing, for example, \( \{P\} \), then the same value of \( \sum f_i \Delta H_i \) may now be observed at two different values for \( r \), e.g., \( r_1 \) and \( r_2 \). If the corresponding protein concentrations in the calorimeter are \( P_1 \) and \( P_2 \), then it follows that \( r_1, P_1 = c + \tilde{v} P_1 \) and \( r_2, P_2 = c + \tilde{v} P_2 \), where \( c \) denotes the free ligand concentration. Note that \( \tilde{r} \) and \( c \) must be equal in both equations. These two equations can be solved for \( P \), as \( r_1, r_2, P_1, \) and \( P_2 \) are all known. This method was used in some cases to obtain a \( \sum f_i \Delta H_i \) versus \( \tilde{r} \) curve. The advantage of this method is that \( r \) can be determined without any separation procedure.

When protons are involved in the interaction between \( P \) and \( L \), \( \Delta H \) becomes dependent on the presence and type of buffer used and, moreover, \( \Delta H \) will vary with \( pH \). Scheme 1 is a simplified view of what is imagined to happen when \( L \) becomes bound to \( P \). The required quantity is the heat of reaction associated with reaction \( a \), where reactants and products are at the same \( pH \) (\( pH_1 \) in Scheme 1).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>( \Delta H ) (kJ/mol H(^+))</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>-56.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>-46.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Tris</td>
<td>-4.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Albumin (0.5 mM)</td>
<td>-28.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 1: Heat of protonation and buffer capacities (\( \beta \)) at \( pH \) 7.4 of several solutions used

For simplicity of notation the right part minus the number of protons bound by the substances at the right part minus the number of protons bound by the substances at the left part of the equation, per mol of \( PL \).

Effect of \( H^+ \) and \( Cl^- \) ions on Sulfathidole/Albumin Interaction

Heat of protonation was measured calorimetrically by reacting the indicated solutions with HCl solutions which varied between 0.001 M and 0.01 M. These heat effects are in good accord with literature values (7, 8). The value for albumin reported here is in accordance with the results obtained from titration curves at different temperatures (9). Values for the buffer capacity \( \beta \) (defined in the text) of Tris and phosphate are from Ref. 4.
protons bound (calculated per mol of protein) by the protein-drug complex and by the free protein, both at the same pH. When \( \Delta H^* \) is positive the protein-drug complex has more protons bound than the free protein. These measurements were performed at several drug to protein ratios, as the experiments can only be performed at a constant ratio and not at a constant fraction of occupied sites. In the pH region investigated \( \Delta H^* \) is not constant but clearly positive. This positive value might be caused by an increase in pK of a positively charged, ligand binding-linked group on the protein. When the SETD becomes involved in salt bridge formation such a situation might be expected. The curves presented here for bovine albumin are similar to the one published for human serum albumin (3).

At SETD/albumin ratios of 3 and higher \( \Delta H^* \) is fairly constant. The binding constants for SETD binding to albumin have been measured by equilibrium dialysis at pH 7.4 (phosphate buffer and at physiological Cl\(^-\) concentration); both Fraction V and crystalline albumin have one primary binding site (\( k_1 = 1.5 \times 10^5 \text{ M}^{-1} \)) and three secondary sites (\( k_2 = 1.6 \times 10^2 \text{ M}^{-1} \)) (10). This means that under the experimental conditions at pH 7.4 at the ratios 1, 3, 5, and 10 of SETD to protein the fraction of primary sites occupied amounts to 0.77, 0.97, 0.99, and 0.99; for the secondary sites the values are 0.08, 0.65, 1.10, and 1.77, respectively. The constant value of \( \Delta H^* \) at \( r \geq 3 \) in Fig. 1 signifies that the pH dependence of ligand binding is associated with the first site only, as was reported for human albumin (3). In contrast with human albumin no clear maximum was observed at alkaline pH, which makes a simulation of the experimental curve more difficult. It will be evident, however, that at least one basic group on the protein is involved.

It is seen that at the pH used for the calorimetric experiments (7.4) \( \Delta H^* \) is almost zero. By performing calorimetric binding measurements under different buffering conditions more information can be obtained.

In Fig. 2 the results of several calorimetric experiments are shown. It is seen that the curves in phosphate buffer and water coincide exactly over the whole ratio range studied.

This confirms the results represented in Fig. 1, namely that \( \Delta H^* \) is very small at pH 7.4. The results in phosphate and water at the values \( 0 \leq r \leq 0.7 \) fit the equation

\[
\Sigma_v \Delta H_i = -33.0 (\pm 1.4) r - 0.1 \ (n = 6, \ r = 0.999, \ s = 0.368) \ (5)
\]

Here \( n \) indicates the number of data points, \( r \) the correlation coefficient, and \( s \) the standard deviation as obtained from a linear least square analysis. The number in parentheses in Equation 5 gives the value of twice the standard deviation in the slope, which might be considered as a fair measure for nonsystematic errors (11). At a ratio of 0.7, up to 96% of the added ligand is bound as calculated from the known binding constants (10). This linear portion in the plot indicates that all the added SETD may be assumed to be bound, so that \( r = \nu, \) and \( \Sigma_v \Delta H_i = \nu \Delta H_i. \) Therefore, the heat of binding associated with the first binding site is about \(-33 \text{ kJ}, \) both in phosphate and water, at pH 7.4.

As discussed above if \( \Delta H^* \) for the reaction at pH 7.4 was 1, \( \Delta H_i \) in phosphate buffer would be 20.4 kJ lower than \( \Delta H_i \) in water. Assuming that the detection limit is 1.4 kJ, the results suggest that \( \Delta H^* \) under the conditions considered is about 0.0 \( \pm 0.07. \)

Similar experiments have been performed in Tris buffer. Surprisingly, \( \Sigma_v \Delta H_i \) is quite different from the measurements determined in phosphate, as shown by Fig. 2. The possibility that this difference is caused by a proton effect appears to be excluded by the results discussed above. Moreover, the difference persists even at higher ratios, which are not proton linked. The most likely explanation seems to be a specific interaction of buffer substances with the protein. As shown in Fig. 2 measurements using 0.1 M KCl gave results identical with those in Tris, which demonstrates an interaction of Cl\(^-\) with albumin. The curves once again prove that \( \Delta H^* \) is zero at pH 7.4. The measurements of \( \Sigma_v \Delta H_i \) versus \( r \) at ratios 0 \( \leq r \leq 0.75 \) in Tris and KCl fit the equation

\[
\Sigma_v \Delta H_i = 22.8 (\pm 0.6) r - 0.1 \ (n = 7, \ r = 0.999, \ s = 0.213) \ (6)
\]

Therefore, in the presence of 0.1 M KCl, \( \Delta H_i \) is near \(-22.8 \text{ kJ}. \)

To investigate further the influence of Cl\(^-\) ions the SETD/albumin binding constants have been measured at several Cl\(^-\) concentrations. The results are reported in Table II. The decrease in \( \log k_1 \) with increasing Cl\(^-\) concentration corresponds with a mechanism in which Cl\(^-\) is displaced when SETD is bound. This displacement can be analyzed quantitatively by applying the relationship \( d \log k_1/d \log \text{[Cl}] = \Delta C_l \) (12) to the data of Table II (at a chloride concentration of 0.05 and 0.10 M) using the assumption that the total Cl\(^-\) concentration rather than the free concentration can be used. This leads to \( \Delta C_l = -1.0, \) which means that for the experimental conditions used, on average one Cl\(^-\) is displaced by each SETD molecule. Whether or not this is a direct displacement cannot be answered by these experiments. The differ-

![Fig. 2. \( \Sigma_v \Delta H_i \) for the binding between SETD and albumin, against the SETD to protein ratio \( r. \) Flow rates about 3.3 \mu l s\(^{-1}\), protein concentration (initial concentration) about 1 mM. Other experimental details are mentioned above; ○, phosphate buffer; ●, water; □, Tris buffer; ■, 0.1 M KCl, all at pH 7.4.](image-url)
TABLE III

<table>
<thead>
<tr>
<th>$\bar{v}$</th>
<th>$\sum v\Delta H_i$ (kJ)</th>
<th>Phosphate</th>
<th>Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-30.6</td>
<td>-21.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-51.9</td>
<td>-37.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-67.4</td>
<td>-49.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-77.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-85.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ence in $\Delta H_i$ between phosphate and Tris buffer is, therefore, attributable to the displacement of one Cl$^-$ ion having a heat of binding of $-14.3$ kJ. Lovrien and Sturtevant (13) reported a value of $-14.2$ kJ for the heat of binding of the secondary set of chloride-binding sites on bovine albumin, and it is possible that the chloride site(s) involved in SETD binding may belong to this class of sites.

By measuring two curves at two different protein concentrations a $\sum v\Delta H_i$ versus $\bar{v}$ curve can be obtained from which it is possible to hypothesize about the lower affinity sites. Table III gives some results at selected values. Firstly it should be remarked that the total number of binding sites is larger than reported in the literature, at least for phosphate and probably also for Tris. The value of $\sum v\Delta H_i$ at $\bar{v} = 1$ is almost totally due to binding at the first site (cf. Equations 5 and 6). At higher values of $\bar{v}$, $\sum v\Delta H_i$ is consistently smaller in Tris than in phosphate which might indicate that these sites are also influenced by Cl$^-$ ions. A different value of $\Delta H_i$, for $i > 1$ implies that the affinity constants for the secondary sites also depend on the presence of Cl$^-$. This in turn means that at equivalent values, $\bar{v}$ may not necessarily be composed in the same way of contributions from secondary binding sites. This effect makes a quantitative interpretation of Table III more difficult.

It would be interesting to investigate the behavior of other sulfonamides and, in general, acidic drugs to see whether the displacement of Cl$^-$ described here is a general phenomenon.

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