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Experimental Observation of Ionic Forces in Drug-Protein Interactions as Illustrated by the Binding of Sulfaethidole by Human Serum Albumin

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SUMMARY

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When ionic forces contribute to the binding of drugs by a protein the groups involved in this electrostatic bonding may undergo pK shifts. A pH titration method is described to detect such pK changes. The procedure is illustrated by the binding of sulfaethidole to human serum albumin. For this case changes in pK of groups both of the protein and of the bound drug are observed using this method.

INTRODUCTION

Drug-protein interactions, especially those with albumin, are much studied in pharmacy and pharmacology because of their possible influence on the biological activity or pharmacokinetic behavior of the drug (1, 2). In many cases the sole aim of such studies is to determine the extent of binding by trying to measure the affinity constants and the number of binding sites by some procedure (3). The next but more fundamental question concerns the nature of the binding forces. Hydrogen bonds, Van der Waals forces and hydrophobic forces are generally considered to be important for protein-protein and protein-drug interactions (4, 5). Besides these interactions, electrostatic forces between charges of opposite sign can also be very important. Serum albumin, for example, is known to bind a large variety of drugs, especially anions (3). The fact that anionic drugs are preferentially bound suggests some form of electrostatic interaction. How may this type of interaction (also known as ion pair or salt bridge formation) be observed? It should be realized that salt bridge formation will result in a stabilization of the anionic and cationic state of the two partners involved. When the negatively charged partner is the dissociated form of an acidic drug molecule, the result of such an interaction will be a lowering of the pK of the drug, and when the positively charged partner is formed by the protonated form of an imidazole, amino or guanidine group on the protein, this group will obtain a higher pK (6). These shifts in pK should be able to be used as a probe for this type of interaction.

The question now arises whether or not such a pK shift can be measured. A direct determination of the pK of a bound drug, or of specific groups on the molecule, is very difficult and only possible in some rare instances, and so a more general approach to the problem must be based on effects which are linked to the pK shift, e.g., the release or uptake of hydrogen ions upon binding. It will be shown in this paper that the measurement of the acid-base titration curves of albumin in the presence and absence of varying amounts of drug can be used for this purpose. The sulfaethidole-

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serum albumin interaction will be used as an example.

MATERIALS AND METHODS

SETD[1] was a gift from Smith, Kline and French (lot no. 5610). HSA (fraction V) was obtained from Sigma. Sodium hydroxide and hydrochloric acid solutions used for the titration were prepared from Titrisol (Merck). All solutions were prepared with distilled and degassed water. A solution of the sodium salt of SETD was made by dissolving SETD in water containing an equivalent amount of 0.1 N NaOH. An approximately 6% solution of albumin was deionized by passing the solution repeatedly over a mixed bed ion exchange column (Amberlite IRA 400 and IR 120, mixed in a 1:1 ratio). The average pH of the resulting solution was 5.48 (standard deviation 0.01, 8 determinations) at 25° and at an ionic strength of 0.15 (KCl). Albumin concentrations were determined by drying to constant weight at 105° in air. For the calculations a molecular weight of 66,250 for HSA was used (7).

Titration of albumin and albumin-SETD solutions. Two milliliters of a deionized albumin solution and 6 ml of a solution of the sodium salt of SETD were mixed in a titration vessel. The final albumin concentration was about 2×10^{-4} M. The ratios SETD/albumin were 1, 3, 5 and 10. Due to the addition of the sodium salt of SETD the isoionic pH rose a little. The principles of the titration equipment itself have been described in detail (8); in brief this equipment is as follows: The pH of the contents of the titration vessel (thermostatted at 25°) was measured using a pH meter (Radiometer PHM 64) having a combined electrode (Radiometer GK 2401 C) and was printed out directly by a digital recorder (Digitec 6110). After reaching a constant pH, 0.01 ml of 0.1 N NaOH was added by means of a microburette (Metrohm E 457). When the pH became stable the value was recorded and a further 0.01 ml portion was added. The addition and mixing time could be varied depending upon the experimental circumstances. The whole addition and recording system was automatized in order to obtain the highest possible accuracy and reproducibility (9).

Determination of pK of SETD. Eight milliliters of a SETD solution (about 3×10^{-4} M, ionic strength 0.15 KCl) were made basic (pH 10–11) by adding 1 ml 0.1 N NaOH. This solution was then titrated with 0.02 N HCl using the equipment described above. A blank titration was performed and subtracted from the SETD titration. Using the known concentration of SETD, the pK was calculated from the half-neutralization point. Six determinations gave a mean pK of 5.33 (standard deviation of the mean 0.01).

TREATMENT OF DATA

As stated in the INTRODUCTION pK shifts of groups on the protein are revealed by measuring acid-base titration curves of the protein in the presence and absence of the ligand which causes these pK shifts. The principle of this procedure, also termed difference counting, is well known (6). The situation becomes more complicated when the ligand can also bind protons. The analyzing procedure for this situation will be given now. Some basic equations adapted from refs. 6 and 10, should be recalled for this purpose.

The number of residues of a protein in aqueous solution which are positively charged in the protonated form determine the maximum positive protonic charge of the protein, represented by Z_{max}. Addition of base to a protein in this state will cause a decrease in charge due to the successive dissociation of groups according to their pK values. When all n_i residues having the same pK_i are combined in one class i, then

$$Z_H = Z_{max} - \sum n_i \alpha_i \qquad (1)$$

where Z_H is the charge of the protein due to the binding of protons only; α_i is the degree of dissociation of the n_i residues belonging to the same class i. Z_H is measured experimentally starting from an isoionic albumin solution. When r_{NaOH} equivalents of NaOH has been added (r_{NaOH} taken relative to the protein concentration) then

[1] The abbreviations used are: SETD, sulfaethidole; HSA, human serum albumin.

$$Z_H = - r_{NaOH} \quad (2)$$

Equation 2 allows one to calculate Z_H after each successive addition of NaOH. In our procedure equal amounts of base are added stepwise. Experimental conditions are chosen such that the corresponding change in charge is about 0.7 for each addition.

Suppose that now the titration curve in the presence of a drug has to be measured. When the drug does not bind protons in the pH region of observation, Eq. 2 can be used to evaluate the charge of the protein. A certain fraction of the added drug will be bound to the protein. Due to this binding the ionization of some groups on the protein may change. This means that (at the same pH), the charge $Z_H(PD)$ of the protein-drug complex may differ from the charge $Z_H(P)$ of the free protein. The quantity ΔZ_H is defined as $Z_H(PD) - Z_H(P)$. Using Eq. 1, ΔZ_H can be written as

$$\Delta Z_H = \sum n_i[\alpha_i(P) - \alpha_i(PD)] \quad (3)$$

and thus ΔZ_H may be used as an indication for pK changes. When one group is involved a positive ΔZ_H signifies an upward pK shift. ΔZ_H as a function of pH is mostly bell-shaped. The absolute value of the maximum and its position on the pH axis can easily be calculated assuming some model (6, 11).

The measurement of a titration curve in the presence of a drug which is able to bind protons can now be discussed. Suppose to an originally isoionic protein solution r_{NaOH} equivalents NaOH and r_D equivalents NaD are added. NaD is the sodium salt of a drug; in water NaD fully dissociates into Na^+ and D^-; D^- in turn is in equilibrium with its protonated form HD. At the new pH, a fraction $(1 - \alpha)$ of the added drug exists as HD which means that $(1 - \alpha)r_D$ extra equivalents of base have been added. Therefore, instead of Eq. 2, the following equation should be used to calculate $Z_H(PD)$

$$Z_H(PD) = - r_{NaOH} - (1 - \alpha)r_D \quad (4)$$

α being calculated from the pK and the observed pH.

In the derivation of this equation it is assumed that the pK of the free and bound drug do not differ. However, the free and bound drug should be assigned a different pK. This means that Eq. 4 needs to be modified as follows. When the number of drug molecules bound is represented by ν_D, then $(r_D - \nu_D)$ equivalents of the drug are free (having a pK_F), whereas ν_D equivalents of the drug are bound (having a pK_B). Analogous to Eq. 4 $Z_H(PD)$ is now given by

$$Z_H(PD) = - r_{NaOH} - (1 - \alpha_F)(r_D - \nu_D) - (1 - \alpha_B)\nu_D \quad (5)$$

where α_F and α_B represent the degree of dissociation of free and bound drug. Note that the difference between Eq. 4 and Eq. 5 is equal to $(\alpha_F - \alpha_B)\nu_D$.

The problem now arises that only the pK of the free drug is known, so it is impossible to apply Eq. 5. Therefore in this study Eq. 2 was used to calculate $Z_H(P)$ and Eq. 4 to calculate $Z_H(PD)$. In this way a ΔZ_H versus pH curve was obtained. From the discussion above it will be clear that this experimentally observed ΔZ_H is given by

$$\Delta Z_H = \sum n_i(\alpha_i(P) - \alpha_i(PD)) + (\alpha_F - \alpha_B)\nu_D \quad (6)$$

Albeit that Z_H and ΔZ_H have been defined as measures of the protein charge only, by the calculating procedure outlined above ΔZ_H also includes the pK shifts undergone by the drug on binding. It should be noted that a downward pK shift has a negative contribution to ΔZ_H. When more than one drug molecule is bound, Eq. 5 and Eq. 6 should be modified to include the possibility that the bound drugs may have different pK values. Equation 6 will be used to simulate the observed ΔZ_H versus pH curve.

As ΔZ_H is a function of the amount of drug bound, the titrations were performed at several drug to protein ratios. The study was limited to the pH region between 5.5 and 8.5, primarily because the measurements can then be performed more accurately. This implies that pK changes far outside this region are not detected by this method.

RESULTS AND DISCUSSION

The interaction between SETD and HSA was studied by the method described above. In Fig. 1 ΔZ_H values from two independent experiments at several drug to albumin ra-

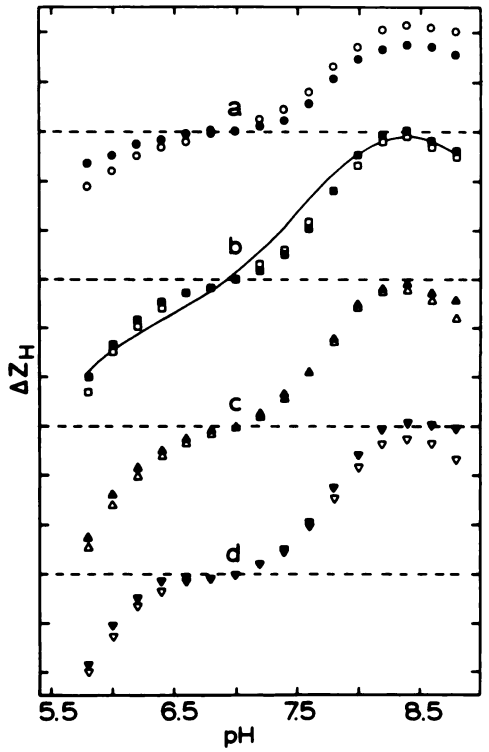

FIG. 1. *Difference in protein protonic charge of HSA in presence and absence of SETD*

Experimental conditions and determination of ΔZ_H are mentioned in the text. The zero of ΔZ_H was assumed to be located at pH 7, and is represented by the dashed lines. At pH values higher than 7 ΔZ_H is positive. Each indicated unit corresponds to 0.2. The results of two experiments are shown. Open and closed symbols relate to independent experiments. Curves a, b, c and d represent experiments at a drug to protein ratio of respectively 1, 3, 5 and 10. The drawn line in curve b has been calculated as indicated in the text.

tios are plotted against pH. The zero of ΔZ_H was assumed to be located at pH 7 and is represented by the dashed lines. At pH values higher than 7, ΔZ_H is positive. No labels are given for the ΔZ_H axis. Each indicated unit corresponds to 0.2 (protonic charge units); in curve b for example, ΔZ_H amounts to + 0.6 at pH 8.4. The shape of the ΔZ_H versus pH curves indicates pK shifts of more than one group. Before examining the meaning of these shapes in more detail, the accuracy and reproducibility of the curves will be discussed.

It is evident from Fig. 1 that the shape of the curves is well reproducible, particularly when the small number of protons involved is taken into account. This conclusion followed from more experiments than is indicated in the figure. Although the shape proved to be reproducible, the absolute values of ΔZ_H appeared to be subject to some variation in the order of a few tenth units. These shifts are expected and arise mainly from the pH calibration procedure. Measuring the absolute pH value better than 0.01 to 0.02 pH unit appears to be a very difficult task. For example consider the observed standard deviation of 0.01, as mentioned above, for the isoionic pH of a number of albumin solutions.

The effect of this on the measurement of ΔZ_H can be illustrated as follows. Suppose that two titration curves are identical except for a constant shift in pH. Subtracting two such curves results in an (apparent) ΔZ_H versus pH curve. However, it should be realized that when the pH shift is constant the resultant (apparent) ΔZ_H is not constant but will depend on the slope of the pH versus Z_H curve. It is readily shown that when the slope is given by $dZ/d\text{pH}$, then the absolute value of this (apparent) ΔZ_H is given by the product of the pH shift and the slope value. In Fig. 2 the experimentally measured slope $-\Delta Z_H/\Delta\text{pH}$ of HSA has been plotted as a function of pH. This value of $\Delta Z_H/\Delta\text{pH}$ was obtained directly from the normal titration curves. A pH shift as discussed above of say 0.01 will result in a contribution to the observed difference curve ΔZ_H which is essentially constant: it

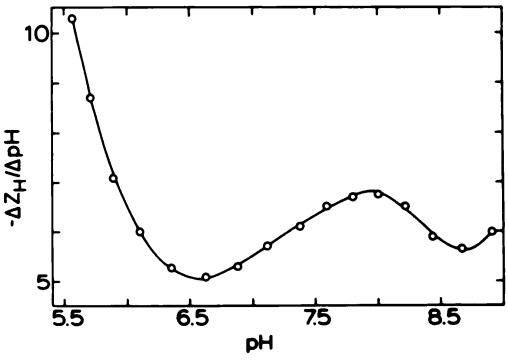

FIG. 2. *Slope of the titration curve of human serum albumin versus pH*

Experimental conditions are mentioned in the text. Note that the meaning of ΔZ_H in this figure and in Fig. 1 are quite different.

lies between 0.1 and 0.07 in the pH region 5.6 to 5.9 and is 0.06 ± 0.01 in the other pH region investigated. This effect will be the main cause of the nearly constant shifts sometimes observed during the experiments, since $-\Delta Z_H/\Delta$pH for the titration in the presence of SETD is very similar to the one shown for HSA alone.

In general the observed ΔZ_H appeared to be near zero around pH 7. This effect combined with the observation of a small plateau in this pH region was used as an argument to set the zero point at pH 7 for all curves represented in Fig. 1.

The details of the difference titration curve can now be discussed. From Fig. 1 two facts are immediately evident. First, ΔZ_H is certainly not zero and second, the curves for the ratios r = 3, 5 and 10 are indistinguishable from each other. This last effect leads to the following conclusion: under the experimental conditions at r = 1, the fraction of the high affinity sites occupied is 0.8; at the secondary sites this fraction amounts to 0.1 as calculated from the bovine serum albumin binding constants (12). At the higher ratios the first site is nearly fully occupied such that at r = 10 the mean number of molecules at the secondary binding sites is near 2. From the constancy of the ΔZ_H curves at a ratio of 3 and higher it is concluded that the observed pH effect is due to binding at the first (high affinity) site only.

At alkline pH ΔZ_H is positive. According to the definition of ΔZ_H this indicates an upward pK change of one or more groups on the protein. In view of the pH region where the effect is observed, an imidazole group or the terminal amino group are the most likely candidates for being involved in this binding. At acid pH ΔZ_H is negative. According to previous discussion the most likely explanation for this effect is a fall in pK of the bound drug. The sign of both the alkaline and the acid effect are in accordance with expectations based on a contribution of electrostatic forces to the binding.

The solid line in Fig. 1 is a calculated one based on Eq. 6. To perform this calculation values for the parameters in this equation have to be found. In view of the effect of the increasing drug to protein ratios on the ΔZ_H curve, ν_D was assumed to be 1. It also is reasonable to start the calculations under the assumption that one bound drug causes a perturbation of one group on the protein (n_i = 1). To calculate the several degrees of dissociation the corresponding pK values are needed. The observed maximum ΔZ_H of about 0.6 near pH 8.4 (Fig. 1) was used as the starting value in the calculation procedure (6, 11). The solid line has been calculated assuming a pK shift from 7.6 to 9.2 for one group on the protein. A shift of the same magnitude was assumed for the SETD (from 5.33 to 3.73). The agreement between the measured and calculated curves is very satisfactory. It should be noted that the calculated curve was allowed to shift in the ΔZ_H direction in view of the uncertainty in the ΔZ_H scale discussed above. Using the above parameters the true zero in the ΔZ_H curve is observed at pH 6.4; at pH 7 the calculated ΔZ_H is 0.17. The given pK values should not be taken too rigidly as only a part of the curves could be measured, especially in the acid region. In addition it should be noted that α_i was calculated from pK, neglecting the minor influence of the protein charge on this dissociation as expressed for example in the Linderstrøm-Lang equation (6, 10).

The agreement between the measured and calculated ΔZ_H curve does not prove that the model presented here is the only explanation. Other effects, for example from pH dependent conformational changes induced by the ligand, may contribute to ΔZ_H.

The results obtained above clearly demonstrate the involvement of protons in the binding. This implies that the binding constant as measured usually must be pH dependent. It will be evident that, compared with the binding at pH 7, the binding at, say, pH 4 and pH 10 will be lower due to the unfavorable state of ionization of either the drug or the group on the protein. This pH dependence of the apparent binding constant K is given by

$$d\log K/d\text{pH} = -\Delta H^+ \qquad (7)$$

where ΔH^+ is the difference in protons bound, associated with the binding process to which log K is related (3). For this pres-

ent study when log K in Eq. 7 refers to the binding of the first binding site, ΔH^+ is (nearly) equal to the value of ΔZ_H in Fig. 1, obtained at the higher ratios. By measuring log K as a function of pH, ΔH^+ may be found. This offers a quite different experimental approach to the problem than that discussed here. However the accuracy by which ΔH^+ may be found following this method is probably no better than the titration method. The accuracy of this approach depends on the accuracy by which log K can be determined; assuming that log K is measured with an interval width of 0.2 pH, the resulting ΔH^+ has an uncertainty of 0.07 when the uncertainty in log K is 0.01, which becomes 0.7 when the uncertainty in log $K = 0.1$, a not unrealistic value. In other words a very high accuracy is necessary in the determination of log K in order to obtain a reliable value of ΔH^+. As far as we are aware this method has (therefore) never been applied in the drug-albumin binding field. One other objection to its use might be the extremely time-consuming process needed for the determination of so many binding constants.

In summary it seems that the method described here is well suited to detect changes in pK and moreover it seems to be the only feasible one presently available. It is planned to use the method for series of congeneric drugs in order to observe regularities in the effect and to estimate the contribution of ionic forces to the total binding energy.

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