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THE EFFECT OF pH ON THE BINDING OF SODIUM AUROTHIOSULPHATE TO HUMAN SERUM ALBUMIN

A POSSIBLE BINDING MECHANISM

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Abstract—The effect of pH on the binding of aurothiosulphate to human serum albumin was studied in unbuffered solutions at 37° and ionic strength 0.15–0.16 M. In the investigated pH range, 6.3–8.4, the effect of pH on the high affinity association constant K_1 was very different from that on the lower affinity constants K_2–K_4. K_1 was virtually constant except for a two-fold decrease in the narrow pH range 7.5–7.9, which was explained as a H^+ induced local conformation change in the environment of site 1. Contrary to this, K_2–K_4 decreased monotonically with increasing pH, which could be entirely accounted for by a change in electrostatic interaction. A conceivable binding mechanism consistent with the results might be: that gold binds as Au^+ to the high affinity binding site by exchanging a H^+ and that this site might be the free sulphydryl group in cysteine or the terminal α-amino group; and that gold binds as $Au(S_2O_3)_2^{3-}$ to the lower affinity binding sites which might be the protonated basic side chain group, i.e. ε-amino groups.

Although gold compounds in the form of thio-complexes have been successfully used in the treatment of rheumatoid arthritis for many years only little quantitative information concerning the binding of gold to plasma protein is available [1, 2]. In recent work [2], it was shown that sodium aurothiosulphate *in vitro* was bound reversibly, to human albumin at physiological conditions, with regard to albumin concentration, pH, temperature, and ionic strength at a single site, with an apparent association constant of $3.0 \times 10^4\ M^{-1}$ and three or more sites with association constants of the order of $10^3\ M^{-1}$.

To obtain an optimal and safe treatment of patients in chrysotherapy, it is important to know whether changes of pH have any effect on the binding of the gold complex to human serum albumin. A pH induced variation in the concentration of the non-protein bound gold complex can produce either toxic effects or symptoms due to insufficient treatment. Furthermore, there is no information concerning the binding mechanism and the nature of the binding sites which is of fundamental pharmacological interest. Such information might be obtained by studying the effect of pH on the binding.

The purpose of the present work, therefore, has been to investigate the influence of pH on the sodium aurothiosulphate–albumin interaction under conditions relevant for clinical interpretations and, in addition, to attempt to characterize the mechanism of the binding and the nature of the binding sites.

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MATERIALS AND METHODS

Materials. The albumin preparation was purified, lyophilized human albumin (Behringswerke AG, Marburg, West Germany). Crossed-immunoelectrophoresis [3], performed against rabbit antihuman serum (DAKO, Copenhagen, Denmark), showed that no other proteins than albumin were detectable. The sodium aurothiosulphate [$Na_3Au(S_2O_3)_2$, Sanocrysin®], was purchased from Ferrosan, Søborg, Denmark. The visking seamless cellophane tubing (8/32 in., Union Carbide, Chicago) used for dialysis was washed and prepared as described by Pedersen [4]. All initial solutions of albumin, sodium aurothiosulphate (and blanks) were unbuffered solutions prepared in distilled, sterile water containing 0.15 M NaCl. Necessary amounts of 1 M NaOH and 1 M HCl were then added to adjust pH to the desired range.

Equilibrium dialysis. The binding of sodium aurothiosulphate to human albumin at 37°, ionic strength 0.15–0.16 M, and pH in the range 6.3–8.4, was studied in an equilibrium dialysis system containing, initially, 1 ml albumin solution inside and 3 ml sodium aurothiosulphate solution outside the membrane. The range of the total concentrations of sodium aurothiosulphate was 80–1630 μM. At each pH value, control tubes with sodium aurothiosulphate solution on the outside and 0.15 M NaCl solution on the inside of the dialysis membrane were set up at two different concentrations in duplicate to establish that equilibrium was reached at the end of the experiment.

After equilibrium was reached, with 2 days, the pH and the concentration of albumin were measured

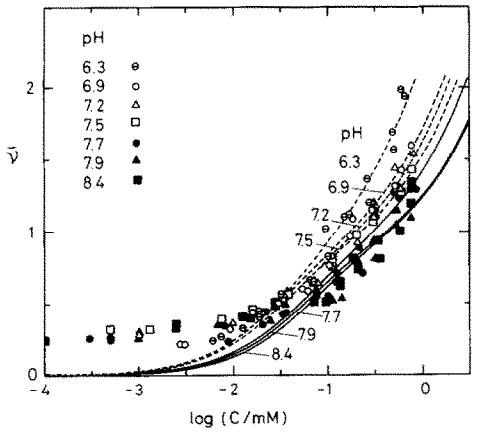

Fig. 1. The effect of pH on the binding isotherms of sodium aurothiosulphate to human albumin at 37° and ionic strength 0.15 M. The curves represent the best-fit to equation (1) using $n = 4$. Note that the curves are separated into two groups corresponding to pH ≤ 7.5 and pH ≥ 7.7. The experimental data with $C \leq 10\ \mu M$ are not included in the analysis.

inside the dialysis membrane. The concentration of sodium and gold were measured on each side of the membrane.

At the temperature (37°) of these experiments, spectrophotometric and electroimmunoassay determination of albumin showed that the albumin solution was stable during the time required for attaining equilibrium. No bacterial growth was observed after dialysis in any of the solutions.

Measurements. All pH measurements were performed at 37° with a Radiometer pH meter (BMS2 MK2 blood Micro System). A quantitative electroimmunoassay technique was used for albumin determinations. (Albumin standard: Standard-Human-Serum, Behringwerke AG). The gold concentrations were determined with a flameless atomic absorption spectrophotometer (Beckman model 485 fitted with a Masmann Cuvette model 1268) as described by Pedersen and Graabæk [5].

RESULTS

Treatment and presentation of experimental data

Figure 1 shows the experimental data for the binding of sodium aurothiosulphate (gold) to human serum albumin at seven different pH values in the range 6.3–8.4; the temperature was 37° and the ionic strength 0.15 M in all solutions. The binding data are plotted as $\bar{\nu}$ vs log C, where $\bar{\nu}$ is the average number of gold atoms bound (in one form or another) per albumin molecule and C is the equilibrium concentration of unbound gold. The molecular weight of albumin was assumed to be 67,000 throughout.

The lines in Fig. 1 have been calculated from the equation

$$\bar{\nu} = \sum_{i=1}^{n} \frac{K_i C}{1 + K_i C} \qquad (1)$$

by a non-linear least-square curve-fitting procedure. The summation is over all n sites of the albumin molecule and K_i is the association constant for site i. Equation (1) implies that there is no interaction between the sites. The maximum number of binding sites, n, is not known and was, therefore, varied in the calculations. The fit was significantly improved by increasing n from 3 to 4 but only minor changes of the rms-value results by increasing n beyond 4. Thus, $n = 4$ was assumed in the calculations for all pH values. It should be noted that the binding isotherms are separated into two groups corresponding to pH ≤ 7.5 and pH ≥ 7.7. The association constants calculated from the experimental data of Fig. 1 are shown in Table 1. As in the previous publication [2], the binding data for values of $C \leq 10\ \mu M$ could not be fitted to equation (1) and, although highly reproducible, they were not included in the analysis. The reason for this interesting discrepancy is at present unknown. It should be noted, however, that exclusion of these binding data does not significantly change the values of the association constants. In fact only K_1 is affected; it is increased by approximately 10%.

No correction for the Donnan effect was made as the concentration of added salt (0.15 M NaCl) was sufficiently high to suppress the Donnan effect.

Table 1. The effect of pH on the association constants for binding of sodium aurothiosulphate to human albumin at $T = 37°$, and ionic strength 0.15 M*

pH†	Albumin (mM^{-1})‡	$K_1 \times 10^{-3}\ M^{-1}$	$K_{2\text{-}4} \times 10^{-3}\ M^{-1}$	rms§
6.31	0.54	28.2	0.67	0.09
6.95	0.52	30.4	0.32	0.06
7.24	0.51	27.3	0.28	0.08
7.51	0.51	27.2	0.23	0.05
7.72	0.54	18.0	0.18	0.11
7.85	0.49	16.1	0.11	0.12
8.37	0.51	14.2	0.11	0.11
		28.3‖	1.62‖	

* For each value of pH, assuming the number of binding sites, $n = 4$, the association constants were obtained by analysis of a complete binding isotherm consisting of 14–20 experimental points.

†, ‡ The indicated value is the mean value; one S.D. is typically 0.03.

§ The S.D. of $\bar{\nu}$ on C from the best least-square fit to equation (1).

‖ The intrinsic association constant, i.e. the extrapolated value of K to $Z_p = 0$, using the curves of Fig. 2.

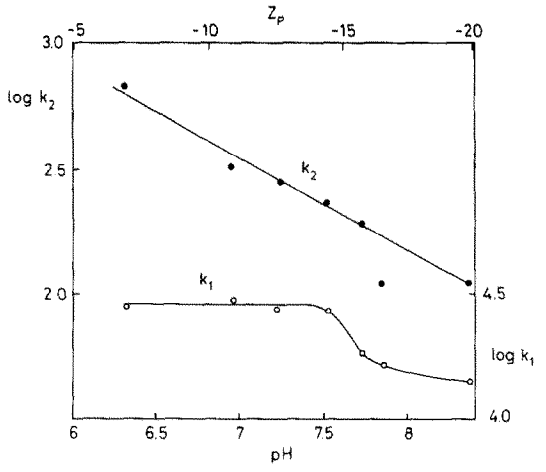

Fig. 2. The pH dependence of the association constants K_1 and K_2. The displayed linear least-square fit to the K_2 points has $r^2 = 0.99$ and a slope of 0.060 when Z_p is used as abcissa and the point at pH = 7.85 is excluded. The curve through the K_1 points is not calculated.

Within experimental error, the analysis demonstrates that the association constant, K_1, was almost independent of pH, except for a two-fold decrease of K_1 in the narrow pH range 7.5–7.9. Contrary to this, the other association constants K_2–K_4 decreased monotonically with increasing pH. These pH dependences are displayed in Fig. 2. It is interesting that these important pH dependences can also be directly demonstrated, i.e. without using theoretical calculations, by plotting the experimental $\bar{\nu}$ values vs pH for constant values of the total concentration of gold (sum of protein bound and non-protein bound gold inside the dialysis membrane at equilibrium), see Fig. 3.

DISCUSSION

In the present investigation it was found that, in the pH region 6.3–8.4, the effect of pH on the binding of sodium aurothiosulphate to human serum albumin

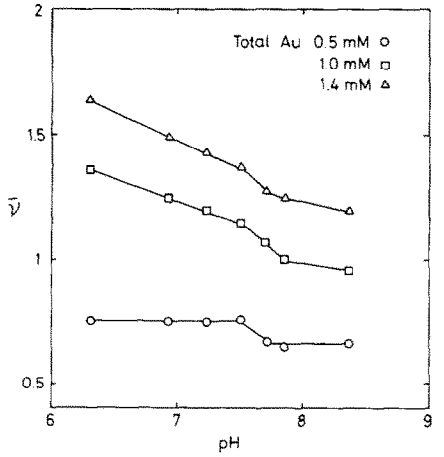

Fig. 3. The pH dependence of $\bar{\nu}$ for three constant values of the total concentration of gold, i.e. sum of protein bound and non-protein bound gold inside the dialysis membrane at equilibrium. The solid lines are not calculated.

depended strongly on the binding affinity of the sites, cf. Table 1 and Figs. 2 and 3.

The binding to the site with the highest binding affinity (site 1) was found to be almost independent of pH, except for a two-fold decrease of the binding affinity in the narrow pH range 7.5–7.9. This step function, like pH dependence, could conceivably be due to a H^+ induced conformational transition of albumin. Such a transition has been observed at pH ~7.5 [6–10]. The different values of K_1 for the two isomeric forms of albumin, thus, reflect the different surroundings of the binding site in the two forms. The possibility of characterizing the transition as global or local is discussed below in connection with the implications of the pH dependences of K_1 outside the transition region.

A common effect of pH on binding of charged ligands to protein molecules arises from the electrostatic interaction between the charge on the ligand and the net charge on the protein, Z_p, where Z_p changes with pH. This effect on the association constants is approximately described by the Linderstrøm–Lang equation

$$K_{app} = K_{int}\, e^{-2wZ_pZ_m} \qquad (2)$$

where K_{app} is the observed, apparent association constant, K_{int} is the intrinsic association constant (i.e. the association constant when $Z_p = 0$), Z_m is the charge on the ligand that binds to the site, and w is the electrostatic interaction factor which is characteristic of the conformation of the albumin molecule.

According to equation (2), a plot of log K_{app} vs Z_p should yield a straight line with a slope of $-0.869\,wZ_m$. If w is known or can be estimated, Z_m can be determined. Since Z_p for human serum albumin is not known, it is estimated as $Z_H - \bar{\nu}_{Cl}$ from the bovine serum albumin values of Z_H and $\bar{\nu}_{Cl}$ presuming that, with respect to proton and small anion binding there is little or no difference between bovine and human serum albumin. The charge Z_H on the albumin molecule, due to bound or dissociated protons in the absence of the gold complex, has been determined by Tanford *et al.* [11], and the average number of chloride ions bound per albumin molecule $\bar{\nu}_{Cl}$ has been determined by Scatchard and Yap [12] in the pH range 5.52–7.15. In the pH range 6.3–8.4, covered in the present experiments, both Z_H and $\bar{\nu}_{Cl}$ are linear in pH to a very good approximation. The $\bar{\nu}_{Cl}$ contribution to the slope of this line is only 7% and might be neglected. It should be noted that any systematic deviation between Z_p and Z_H does not affect the value of the slope.

Figure 2 displays the observed values of the association constants K_1 and K_{2-4} as a function of pH or Z_p. It is obvious that log K_2 vs Z_p is linear; the slope of the line is equal to 0.060. According to equation (2), the intrinsic association constant K_{2int} is obtained by extrapolating K_2 to $Z_p = 0$. The values for K_{2int} to K_{4int} so obtained are shown in Table 1. Assuming that the Debye–Hückel theory is applicable and that the albumin molecule is a compact sphere with its net charge evenly distributed over its surface, the upper limit of w is 0.032 at 37°. This value of w implies that $Z_m < -2.16$. However, a better estimate is obtained by using the experimentally observed value of $w = 0.022$ at 37°, determined by Tanford

[13], which yields $Z_m = -3.14$. Consequently, it may be concluded that the charge on the gold complex that binds to the lower affinity sites has a charge of -3. It has long been an unanswered question whether aurothiosulphate releases its thiosulphate ligands upon binding to albumin [14]. The present results and analysis could indicate that it is $Au(S_2O_3)_2^{3-}$ which binds to the lower affinity sites of albumin.

The effect of pH on the association constants K_2–K_4 could be entirely accounted for by the change of the electrostatic interaction. This would seem to imply that sites 2–4 remain unchanged in the investigated pH range. On the basis of the assumption that only protonated basic side chains participate in the binding, the sites might conceivably consist of ε-amino groups.

There is no observable effect of the transition at pH ~ 7.5 on K_2–K_4. A global unfolding would lead to an increase of the radius and, thus, to a sudden decrease of w at pH ~ 7.5. From the present data it may be concluded that the conformation change is a local change in the neighbourhood of site 1. This is in accord both with other experimental support for the existence of the transition [6–10, 15, 16] and with the observation of no change in compactness of the albumin molecule at this pH range [17].

The association constant, K_1, for binding to the site with the highest affinity is independent of Z_p in the pH range 6.3–7.5, cf. Fig. 2. In terms of the Linderstrøm–Lang equation (2) this implies that the gold complex that binds to site 1 is uncharged at this pH range. Note that the charge on the gold complex Z_m means the change of charge on the albumin molecule introduced by binding of one gold complex. The sudden decrease of K_1 when Z_p changes from -14 to -16 has already been assigned to a pH induced conformation change probably also combined with a gold complex influence on the pH region of the transition. For pH in the range 7.9–8.4 there appears to be a linear relation between log K_1 and Z_p which, when analysed as above, implies that $Z_m = -1$ in this range. However, the data are too few to exclude the possibility that K_1 is independent of pH also in that region, i.e. $Z_m = 0$. Thus, it can only be concluded that Z_m is either 0 or -1 in the pH range 7.9–8.4.

The present results and analysis permit some speculations about the nature of the high affinity binding site and the form of the gold complex which binds to that site. One possible explanation of obtaining a zero change of charge on the albumin molecule upon binding of the gold complex to the high affinity site is that Au^+ binds by replacing a H^+ on the albumin molecule. This implies that the thiosulphate ligands are released upon binding to site 1, contrary to the binding to the lower affinity sites where the aurothiosulphate is bound. Note that the binding constant, K_1, includes the effect of dissociation of gold from aurothiosulphate. Another possible explanation could be that $Au(S_2O_3)_2^{3-}$ binds by replacing three anions (i.e. chloride ions) on the albumin molecule. However, this is very unlikely as $\bar{\nu}_{Cl} < 2$ in the investigated pH range [12]. The finding of a pH independent association constant, K_1, in the pH range 6.3–7.5 indicates that site 1 remains virtually unchanged in this range. Since only a single high affinity site was found it might be suggested that the free sulphydryl group in cysteine$_{34}$ ($pK_{int} \simeq 9.5$ at 25°) or the terminal α-amino group ($pK_{int} \simeq 7.5$ at 25°) [13] constitute site 1. The observed sudden change of K_1 at pH ~ 7.5, which could be explained by a local conformation change in the neighbourhood of site 1, provides more evidence for the first assignment when compared with other experimental observations [6, 15, 16] where the transition at a similar pH is believed to involve the single thiol at cysteine$_{34}$.

One might speculate whether the reduction in K_1 in the pH interval 7.5–7.9 could also be explained by deprotonation of an amino acid residue in, or close to, site 1 [9, 10]. This implies that the binding occurs to a protonated group. However, in order to explain the pH independence of K_1 for pH < 7.5, the binding gold (or gold complex) must then be a neutral entity, which seems highly unlikely.

In the present study it was demonstrated that the binding of aurothiosulphate to human albumin was influenced by pH but, with the pH and concentrations of aurothiosulphate and albumin under relevant clinical conditions, the variation of the unbound fraction of aurothiosulphate was rather small. However, in conditions of acute alkalosis the increase in the unbound fraction of aurothiosulphate might give rise to toxic effects, as the difference between therapeutic and toxic concentrations of gold in the individual patient is small. Although the observed pH dependence was small, it did give several interesting indications of the prevailing binding mechanism.

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