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EFFECT OF PROTEIN CONCENTRATION ON THE BINDING OF GOLD(I) TO HUMAN SERUM ALBUMIN

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Abstract—The binding of aurothiosulphate, gold(I), by human serum albumin has been studied by equilibrium dialysis at four different albumin concentrations, 37°, pH 7.2–7.4 and ionic strength 0.15 M. The results show that the interaction of aurothiosulphate with albumin depends on albumin concentration. This observation is linked with the previous observation that the usual independent site description cannot be used to represent the clinically important low concentration data. All the observed dependences are satisfactorily accounted for by assuming that gold(I) competes with a highly bound contaminant for the high affinity (Cys(34)-SH) site. This description is supported by the experimental observation that a fraction of this site is originally blocked both *in vivo* and in *in vitro*. The present interpretation yields a high affinity binding constant 100 times larger than found previously and provides an explanation for the lack of correlation between dose and therapeutic and toxic effects in chrysotherapy.

Gold(I) compounds in the form of thio complexes, e.g. aurothiosulphate and aurothiomalate, have been successfully used in the treatment of rheumatoid arthritis for many years. There is *in vivo* [1–3] and *in vitro* [4–6] evidence that these drugs are strongly bound to human serum albumin. Minor binding alterations may, therefore, be clinically significant due to the resulting dramatic changes in the free drug concentration. No real quantitative data are available on the effect of albumin concentration on the extent of gold binding. This aspect, however, has important therapeutic implications since hypoalbuminemia often is encountered in patients with rheumatoid arthritis.

Melethil *et al.* [7] using an ultrafiltration technique found that the binding of aurothiomalate at various concentrations of gold to 2 and 4% bovine serum albumin was independent of protein concentrations. The method of data analysis utilized is, however, not able to detect true protein concentration dependent binding.

Since it is important to understand the dependence of gold binding on albumin concentration, the binding of aurothiosulphate to human serum albumin is studied at different albumin concentrations. The resulting binding data are discussed in terms of different molecular binding mechanisms in an attempt to understand the mode of action of the compound.

Aurothiosulphate is found to be strongly bound to the dialysis membrane used. The following experiments are, therefore, performed by means of an equilibrium dialysis system with the advantage that absorption to the membrane does not influence the results.

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MATERIALS AND METHODS

Materials. The albumin preparation used was purified, lyophilised human albumin (Behringwerke AG, Marburg, F.R.G.). Polyacrylamide gradient gel (PAA 4/30, Pharmacia, Uppsala, Sweden) electrophoresis of a 0.5% albumin solution showed only one distinct band of monomer albumin and one very faint band due to dimer albumin. Crossed immunoelectrophoresis performed against rabbit anti-human serum (DAKO, Copenhagen, Denmark) showed that no peaks attributable to proteins other than albumin were detectable [8]. The sodium aurothiosulphate, Sanocrysin®, was purchased from Ferrosan (Søborg, Denmark). The visking seamless cellophane tubing (8/32 in., Union Carbide Corporation, Chicago) used for dialysis was washed and prepared as described by Pedersen [9]. All initial solutions of albumin, sodium aurothiosulphate and blanks were unbuffered solutions prepared in distilled, sterile water containing 0.15 M NaCl and with pH adjusted to 7.50–7.55 (see below).

Equilibrium dialysis. The binding of sodium aurothiosulphate, gold(I), to human serum albumin in unbuffered solutions at three different albumin concentrations was studied in an equilibrium dialysis system previously described [5]. The ionic strength was 0.15–0.16 M, and pH was 7.2–7.4. The range of concentrations of total sodium aurothiosulphate in the initial solutions was 41 μM–1633 μM at albumin concentrations 0.44 and 0.26 mM and 16 μM–612 μM at 0.07 mM. After equilibrium was reached within 48 hr the concentration of albumin was measured inside and the pH and concentrations of gold and sodium were measured on both sides of the dialysis membrane.

Ionic strength. According to general practice the contribution of albumin to ionic strength was

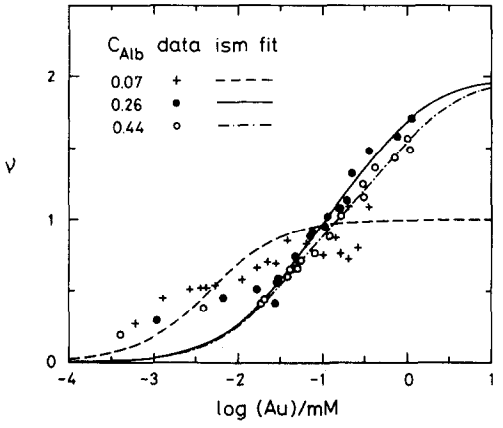

Fig. 1. Experimental data for binding of gold(I) to albumin at three different albumin concentrations as indicated on the figure in mM. The curves are the best fits of the data to the independent site model given by Eq. (1). The parameter values are displayed in Table 1.

ignored, and it was found that in the concentration range of sodium aurothiosulphate used the contribution of sodium aurothiosulphate to the ionic strength in each albumin solution could be neglected.

pH. During dialysis it was found that pH decreased by 0.15–0.30 in the albumin containing solution. In order to obtain pH equal to 7.2–7.4 at equilibrium the pH was adjusted before dialysis to 7.50–7.55 in each initial solution.

Measurements. For albumin determinations a quantitative electroimmunoassay technique was used according to the principles of Laurell [10] (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 [11]. All pH measurements were performed at 37° with a Radiometer pH meter PHM 72 supplied with the electrode system BMS 2 MK 2 blood Micro System. The sodium concentrations were measured with a IL 343 Digital Flame Photometer.

RESULTS

Equilibrium dialysis experiments

Figure 1 shows the experimental data for the binding of aurothiosulphate to human serum albumin at three different albumin concentrations in the range 0.07–0.44 mM. The binding data are plotted as ν versus log(Au), where ν is the average number of gold atoms bound per albumin molecule and (Au) is the concentration of unbound gold at equilibrium. In the calculations the molecular weight of albumin is assumed to be 67,000 throughout.

No correction for the Donnan effect is made as the concentration of added salt (0.15–0.16 M NaCl) is sufficiently high to suppress this effect.

Evidence for protein concentration effect

It is immediately clear from the experimental data of Fig. 1 that varying the albumin concentration has an effect on the binding of gold. At least for small values of ν (i.e. ≤ 0.7) the binding decreases with increasing albumin concentration. Furthermore it appears as though the number of binding sites decreases with decreasing albumin concentrations.

Another but less direct method for differentiating apparent and true protein concentration dependence in the interaction of a ligand with a macromolecule [12] is based on an analysis of the data according to the equation

$$\nu = \sum_{i=1}^{n} \frac{K_i\,(\mathrm{Au})}{1 + K_i\,(\mathrm{Au})} \qquad (1)$$

This equation describes the binding of a ligand by n pre-existing, non-interacting binding sites on the protein. The summation is over all n sites, K_i is the apparent association constant for site i, and (Au) is the concentration of the unbound gold ligand. An effect of protein concentration will show up as a dependence of K_i and/or n on the protein concentration, provided all other assumptions of equation (1) are valid for the particular interaction. This, however, is not the case for the present data as shown below.

The best fits to equation (1) using a non-linear least square curve-fitting procedure are shown as the displayed curves in Fig. 1. The values obtained by fitting the experimental data of Fig. 1 to equation (1) are presented in Table 1. The high affinity binding

Table 1. The apparent independent association constants for binding of aurothiosulphate to human serum albumin at different albumin concentrations*

Albumin† (mM)	K_1 $\times 10^{-3}$ M	K_2 $\times 10^{-3}$ M	K_3 $\times 10^{-3}$ M	K_4 $\times 10^{-3}$ M	$\Sigma_{i=1}^{n} K_i$ $\times 10^{-3}$ M	rms‡
0.066 ± 0.004	215.1	0	0	0	215.1	0.144
0.26 ± 0.02	36.4	2.2	0	0	38.6	0.097
0.44 ± 0.02	34.3	1.2	0	0	35.5	0.087
0.53 ± 0.02§	38.8	0.44	0.44	0.44	40.2	0.108

* For each concentration of albumin, assuming 4 independent binding sites, the association constants are obtained by analysis of the experimental data in Fig. 1. I = 0.15–0.16 M, temperature 37°, and pH 7.2–7.4.

† The mean value ±1 SD.

‡ The SD of ν on C from the best least-square fit to equation (1).

§ Experimental data from Fig. 1 in ref. 5.

constants show no dependence on albumin concentration in the clinically important range 0.26–0.53 mM, but a marked effect is observed at the low albumin concentration 0.07 mM. The apparent number of binding sites available on the albumin molecule is seen to decrease with decreasing albumin concentrations.

It is evident that the concentration dependence displayed by the experimental data is not reflected in the obtained values for the independent site binding constants. This is a consequence of the inability of equation (1) to account for the observed data, in particular for $\nu < 0.5$. Equation (1) is also unable to explain the observed levelling off of the data around $\cong 0.4$. For these reasons and in accordance with what is immediately clear from Fig. 1 the independent site model is rejected.

Several explanations for concentration effects have been offered, e.g. inhibition of binding by highly bound contaminants of the albumin preparation [13], the displacement of bound ligand through molecular aggregation of albumin at high protein concentration [14, 15], and ligand induced dimerization of the protein [16]. Of these the former and the latter are most likely to be of importance for the present system and are discussed below.

In order to distinguish between the various possible explanations the data were fitted to the appropriate theoretical expression for the binding curve and the obtained parameters were tested for independence of albumin concentration. In particular it was required that the low gold concentration data should be accounted for by the model description since the behaviour of these data points is the most significant and unusual feature of the data. The independent site binding model underestimates the binding in this region by two orders of magnitude. The low concentration data are reproducible and accurate and are in fact for clinically purposes the most relevant data.

Competition caused by a contaminant

Previous studies indicate that aurothiosulphate and aurothiomalate bind to human serum albumin as Au^+ to a single high affinity site [6, 17, 18] and as monomeric anion, $Au(S_2O_3)_2^{3-}$ or $AuSH_3C_4O_4^{2-}$, to three or more lower affinity binding sites [6, 18–21]. The single high affinity site is most likely the free sulphydryl (SH) group in cysteine (34) and the lower affinity sites might be the protonated basic side chain groups, i.e. ε-amino groups. The free SH content of the albumin preparation used was determined to 0.3 [6] by the method of Janatova *et al.* [22]. The remaining SH content is blocked by cysteine, glutathione or another albumin molecule [22, 23]. The high affinity binding site could thus have a very strong interaction of gold with the free Cys(34)–SH and a much weaker interaction of gold with the remaining blocked Cys(34)–SS– via the break down of the disulphide linkage to the contaminant.

Based on the above observations the following picture is applied in an attempt to explain the observed effects. Albumin, Alb, is assumed to have two *independent* groups of binding sites. A single high affinity binding site for which the two ligands gold(I), Au, and competitor, Cys, compete and a group of lower affinity binding sites to which only Au binds. The competition to site one is described by the reactions

$$\mathrm{Alb} + \mathrm{Au} \rightleftharpoons \mathrm{AlbAu} \quad (K_0) \tag{2}$$

$$\mathrm{Alb} + \mathrm{Cys} \rightleftharpoons \mathrm{AlbCys} \quad (K_{\mathrm{Cys}}) \tag{3}$$

with the association constants given in parentheses. The usual fractional saturation of site one with Au is given by, see e.g. [24],

$$\nu = (\mathrm{AlbAu})/C_{\mathrm{Alb}} = \frac{k_0(\mathrm{Au})}{1 + k_0(\mathrm{Au}) + K_{\mathrm{Cys}}(\mathrm{Cys})} \tag{4}$$

where (Au) and (Cys) denote the free concentrations of Au and Cys. In the present experiments the concentration of Cys is not measured. This is a usual situation when a competitor is not expected. Fortunately, in this situation the total concentration of the competitor is constant which implies that (Cys) can be expressed by (Au) as

$$\begin{aligned} K_{\mathrm{Cys}}(\mathrm{Cys}) = {} & -\tfrac{1}{2}(\alpha b + 1 + K_0(\mathrm{Au})) \\ & + \tfrac{1}{2}[\alpha b + 1 + K_0(\mathrm{Au}))^2 \\ & + 4b\beta(1 + K_0(\mathrm{Au}))]^{\frac{1}{2}} \end{aligned} \tag{5}$$

where

$$b = K_{\mathrm{Cys}} C_{\mathrm{Alb}}, \tag{6}$$

α is the fraction of free sites when (Au) = 0, and $\beta = 1 - \alpha$ is the corresponding fraction of contaminated sites. When equation (5) is inserted in equation (4) the fractional saturation of the site with Au is obtained.

When equilibrium dialysis is applied it is not the concentration but the total amount of cysteine in the two compartments that is constant since cysteine diffuses from the albumin containing compartment into the protein-free compartment. The above equations can still be used, however, provided that the albumin concentration is multiplied by the "dilution factor", i.e. $V_1/(V_1 + V_2)$ where V_1 and V_2 is the volume of the albumin containing compartment and the protein-free compartment, respectively.

Since the lower affinity sites are assumed to be independent of the high affinity site the average number of gold atoms bound to the two groups of sites can be calculated separately and their sum is equal to the total number of gold atoms bound per albumin molecule. The binding to the lower affinity sites is assumed to be given by equation (1).

Several works have dealt with the theoretical description of a system with more ligands (competitors) but the precise specification to the present situation where the total concentration of competitor is constant has apparently not appeared and is discussed in more details in a separate paper [25].

Results of data analysis

Figure 2 shows the four sets of experimental data with albumin concentrations equal to 0.07, 0.26, 0.44 and 0.53 mM (the latter from ref. 5) and the best fits (full lines) to the competitor binding equations (5) and (6) obtained by a non-linear regression method.

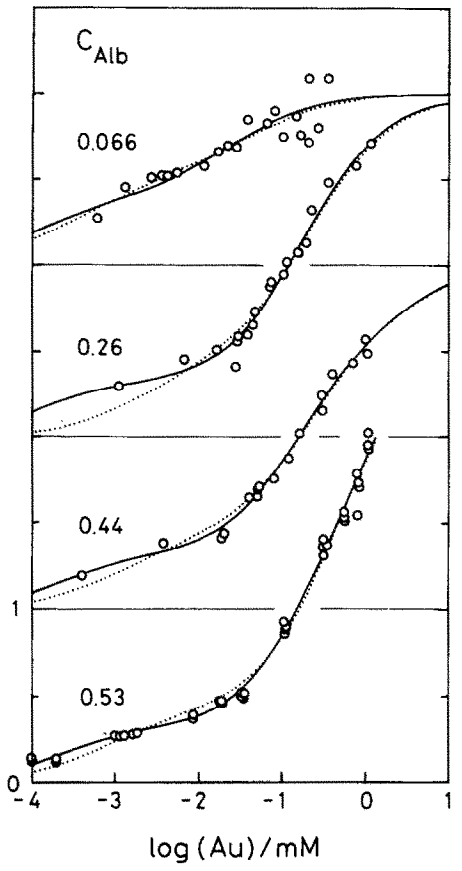

Fig. 2. Experimental data for the binding of gold(I) to albumin at four different albumin concentrations as indicated on the figure in mM. The full curves are the best fits to the competition binding isotherm given by Eqs. (5) and (6); the corresponding values of the model parameters are displayed in Table 2. The dotted curves are the best fits to the gold induced dimerization model; the parameter values are given in the text. The curves and corresponding data are displaced one unit in the ordinate direction.

Table 2. Parameter values obtained by curve fitting

Albumin conc. mM	0.066	0.26	0.44	0.53
α	Binding constant for gold to site one: $K_0 \times 10^{-6}$ M			
0.32	25	8.3	4.1	4.6
0.34	14	4.7	3.4	3.7
0.36	10	3.2	2.8	3.0
N*	1	2	2	3
	Binding constant $\times 10^{-3}$ M for gold to site two (and three)			
0.32		7.4	8.1	6.4 (0.6)
0.34		6.9	7.5	5.8 (0.6)
0.36		6.5	7.0	5.3 (0.6)
	Binding constant for competitor to site one: $K_{Cys} \times 10^{-6}$ M			
0.32	44	58	56	48
0.34	28	32	46	36
0.36	20	22	40	27
rms	0.092	0.057	0.036	0.053

* The total number of gold (I) binding sites used in the calculations.

In the calculations the free fraction α of the high affinity site was considered to be a known constant equal to the previously determined [6] value ($\simeq$0·3) of the free fraction of the sulphydryl group.

The fits are remarkably good and represent the low concentration data points ($\nu < 0.5$) extremely well. The model equations (4)–(6) are clearly consistent with the experimental data which are scattered randomly around the curve with no obvious trends. This is in strong contrast to the fit based on the independent site model, cf. Fig. 1, where there is both large deviations and a very strong trend; all the low concentration data lie far above the curve.

The values of K_0 and K_{Cys}, i.e. the association constants for gold(I) and competitor to a free site one (Cys(34)–SH) on the albumin molecule are displayed in Table 2 for different values of the fraction of free sites α and albumin concentration. It is seen that K_0 and K_{Cys} depend on the value of α but the goodness of the fit is rather insensitive to the value of α within the experimentally acceptable range 0.30–0.36; the typical variation of rms is $\pm$0.001. The obtained values of K_0 and K_{Cys} are reasonably independent of the albumin concentration, which in addition to the goodness of the fits are necessary requirements for adopting the applied picture and the associated binding mechanism. It is concluded that the experimental data are properly represented by the proposed competition binding mechanism, and that there is no experimental evidence for rejecting the model.

The association constants K_0 for gold(I) to a free site one on the albumin is determined to $4 \times 10^6\ M^{-1}$. The association constant K_{Cys} for the competitor is of the order of $10^7\ M^{-1}$, indicating a highly bound competitor, in agreement with the fact that the SH group in commercial albumin preparations is highly contaminated [23].

The fit for the lowest albumin concentration (0.07 mM) is not quite as good as the others although the small concentration region is represented well. (cf. Fig. 2 and Table 2). This could be due to the inherent larger experimental errors associated with the small albumin concentration. But it also could indicate that another mechanism is active at very low albumin concentrations.

Ligand induced dimerization

Another possibility which might give some explanation of the albumin concentration dependence and the dramatic deviation of the experimental data from the independent sites binding curve at small values of ν (<0.5) is that gold at low ratio of gold to albumin binds to the high affinity binding site by a gold-bridged dimer of albumin, i.e. AlbSAuSAlb. This for example is well known for mercuric ions which perform dimers of mercaptoalbumin only when Hg^{2+}/–SH is 0.5 or less [16].

A ligand induced dimerization model [26] was extended to more than one site by assuming the other sites were independent, i.e. the fractional saturation to the site which induces dimerization is given by Nichol and Winzor [26] and the average number of gold ligands bound to the other sites are assumed given by equation (1), cf. the identical discussion above for the competition model.

Applying this model to the experimental data of Fig. 1 resulted in fits, shown as dotted curves in Fig. 2, that were markedly improved with respect to the fits based on the independent sites in the small concentration region. However, the agreement between the experimental data and the theoretical binding curve is not as satisfactory as that presented above based on competitive binding. It is seen that the dimerization model does not account satisfactorily for the levelling off of the experimental data for small values of ν and that a too small binding is predicted for small concentrations, although the discrepancy is not as pronounced as with the independent site model. The rms values for the fits (0.063, 0.050, 0.071 and 0.089) are significantly higher than the corresponding values for the competition model (0.053, 0.036, 0.057 and 0.092) except for the smallest albumin concentration where both values are rather large due to the larger experimental error at this concentration. Note also that the experimental data are not randomly scattered around the dimerization fits but show obvious trends. In large groups the data points alternate from lying above to lying below the dimerization fit. Such a behaviour indicates an inconsistent model. The ligand induced dimerization model is therefore rejected.

In order of decreasing albumin concentration the values obtained for the association constant for binding to the high affinity site K_0 and the dimerization constant K_d are respectively (1.01*, 18.9, 29.7 and 298) and (1524*, 62.4, 37.6 and 126) in units of $10^3\,M^{-1}$. These values show a larger dependence on albumin concentration than do the values obtained by the competition model. The values indicated by asterisks are unstable and arbitrary; all values of K_0 and K_d which satisfy $K_d < 2$ and $K_d \times K_0 = 1539$ yield identical fit. These findings also support the rejection of the dimer model as an explanation of the experimental data.

DISCUSSION

In the present investigation it is found that the binding of aurothiosulphate by human serum albumin increases when the concentration of albumin is decreased, cf. Fig. 1. Analysis of the data showed that in the clinical important range of gold concentration ($\nu < 0.5$) the independent site model of data analysis is not applicable. Two explanations for this large deviation appeared feasible; gold induced dimerization and inhibition of gold binding by a highly bound contaminant. The former possibility assumes that the gold compound upon binding to albumin induces a dimerization of albumin. The latter assumes that gold(I) and an already present contaminant, most likely a thiol ligand, compete for the free sulphydryl group on the albumin molecule. Only the competitive binding could satisfactorily explain the low concentration behaviour of the binding curves. The excellent agreement between the experimental data points and the theoretical binding curve, illustrated in Fig. 2, provides conclusive evidence for this binding mechanism which implies that the high affinity side on the albumin molecule for gold (I) compounds is the (Cys(34)-SH) group.

Table 2 shows that the "true" association constant K_0 for binding of gold(I) to the free sulphydryl group on albumin is independent of the albumin concentration. The apparent increase in K_0 at very low albumin concentration (0.07 mM) might be explained by a decreased formation of albumin dimers or polymers and a subsequent increase in the free sulphydryl content of the albumin preparation. In fact if the free sulphydryl content α was increased from 0.32 to 0.40 a better fit was obtained and K_0 decreased to $5.9 \times 10^6\,M^{-1}$ which is similar to the values obtained for the other albumin concentrations. The estimated value of K_0 is $4 \times 10^6\,M^{-1}$ which is 100 times larger than the value of the apparent association constant K_1 found above and in the earlier works [5, 6, 20] where the data were analyzed using the independent site description. The present value of K_0 corresponds to 99.9% binding of the gold compound under normal physiological conditions while the earlier value corresponds to 94–96% binding. The sum of the lower affinity binding constants was determined to $6 \times 10^3\,M^{-1}$ which is of the same order of magnitude as found previously by neglecting competition. This supports the assumption that significant competition occurs to the high affinity site only.

Using a linear graphical method known as the Scatchard plot Danpure [4] found that aurothiomalate was bound to albumin at a total of one interaction site of which 0.7 sites had an association constant $K_1 = 1.5 \times 10^6\,M^{-1}$ and 0.3 sites had $K_2 = 1.1 \times 10^5\,M^{-1}$. It is well known that this method of data analysis is not reliable when the curve is not a perfect straight line. Nevertheless, it is interesting to note that the obtained value of K_1 is only a factor of three smaller than the present association constant for the binding of gold to the free sulphydryl group. Danpure's value of K_2 is extremely uncertain.

The main sulphydryl containing species in serum is albumin. Patients with rheumatoid arthritis has between 0.7 and 0.3 free sulphydryl groups per albumin molecule depending on disease activity [27]. The remaining sulphydryl groups are either blocked by cysteine or other thiol ligands or are missing altogether. Due to the large value of the high affinity binding constant K_0 the interaction of gold with the free sulphydryl group on the albumin molecule would expect to predominate at therapeutic plasma gold concentrations. The proposed competitive binding mechanism shows that the free gold concentration might vary considerably with various values of albumin concentration and free sulphydryl groups per albumin molecule, and thereby explain the lack of correlation between total plasma gold concentration and therapeutic and toxic effects described in the literature.

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