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Effect of a contaminating competitive ligand on ligand-binding curves

Inverse protein concentration dependence

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A theoretical binding model is considered which provides an explanation for the inverse protein concentration dependence observed for a variety of ligands. The model describes the inhibition of binding caused by a highly bound contaminant. The complete binding equation is derived and examined in terms of form, limits, and protein dependence. Furthermore, several approximate relations are derived which are useful for obtaining initial estimates of the model parameters and for a qualitative test of the applicability of the model. It is found that the binding curve may show a characteristic plateau at a saturation equal to the uncontaminated fraction of the protein and that the free ligand concentration at half saturation depends linearly on protein concentration. The practical implications of the present findings are discussed based on an analysis of simulated as well as experimental data.

1. Introduction

It is generally assumed that a ligand-protein interaction is independent of the protein concentration. However, a dependence of the apparent association constants and of the number of binding sites upon protein concentration has been demonstrated for a variety of ligands [1–5]. Several explanations for protein concentration effects have been offered. These include the displacement of bound ligand through molecular aggregation of the protein [6,7], ligand-induced dimerization or polymerization [8,9], and the inhibition of binding by highly bound contaminants, as discussed in the present work and originally proposed by Brunckhorst and Hess [10]. Except for the inhibition of binding by a highly bound contaminant, these models have been sufficiently described in mathematical terms to allow a proper analysis.

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Contamination of proteins by other ligands is common both in vivo and in vitro. The degree of contamination strongly influences the apparent binding to the site and the interpretation of binding data may be rather arbitrary if the contaminant is not taken into account. One may argue that once the contamination has been discovered then the contaminant should be removed if at all possible. This would be sensible in some cases. However, in others, removal of the contaminant would not be desirable. For example, if one is interested in studying the binding of a ligand to a protein under in vivo conditions where another ligand competes for the same sites, then this competitor will become a contaminant in a sample taken out of the system. Depending on its regulation, the competitor may of course also have been a contaminant in vivo.

In the present study we investigate the inverse protein concentration dependence originating from a different ligand which is strongly bound to the same sites as the investigated ligand and which is

present in a fixed ratio to the protein. The exact binding equation is derived and examined in terms of form and limits. Furthermore, we derive several approximate relations which are useful for a fast initial analysis of experimental data and for distinguishing between alternative explanations of protein concentration dependence.

The derived equations are used to analyze experimental binding data of aurothiosulphate to a commercial human serum albumin [5,11], where the Cys-34 SH site is known to be contaminated to an appreciable extent [12,13].

2. Materials and methods

2.1. Materials

The albumin preparation used was purified, lyophilized human albumin (Behringwerke, Marburg, FRG). Polyacrylamide gradient gel (PAA 4/30, Pharmacia, Uppsala) electrophoresis of a 0.5% albumin solution showed only one distinct band of monomeric albumin and one very faint band due to the dimeric form. Crossed immunoelectrophoresis performed against rabbit anti-human serum (Dako, Copenhagen) showed that no peaks attributable to proteins other than albumin were detectable [14]. Sodium aurothiosulphate, Sanocrysin^{R}, was purchased from Ferrosan (Søborg, Denmark). Visking seamless cellophane tubing (8/32 inch, Union Carbide, Chicago) used for dialysis was washed and prepared as described by Pedersen [15]. All initial solutions of albumin, sodium aurothiosulphate and blanks were unbuffered solutions prepared in distilled, sterile water containing 0.15 M NaCl and with the pH adjusted to 7.50–7.55 in order to reach pH 7.2–7.4 at equilibrium.

2.2. Equilibrium dialysis

Equilibrium dialysis was performed as previously described [5,11] at 37°C, ionic strength 0.15–0.16 M, pH 7.2–7.4, at four different albumin concentrations, and over a range of free gold concentrations covering four orders of magnitude.

2.3. Measurements

For albumin determinations a quantitative electroimmunoassay technique was used according to the principles of Laurell [16] (albumin standard: standard human serum, Behringwerke). The gold concentrations were determined with a flameless atomic absorption spectrophotometer (Beckman model 485 fitted with a Masmann model 1268 cuvette) as described by Pedersen and Graabæk [17]. All pH measurements were performed at 37°C with a Radiometer PHM 72 pH meter equipped with an electrode system (BMS 2 MK 2 blood micro system).

3. Theory

3.1. Description of the model

We wish to describe the binding of a ligand X to a protein P, which has some of its sites contaminated by a different ligand Y. A fairly general model would be to assume that the protein has two independent groups of binding sites; a single high-affinity site for which the two ligands X and Y compete and another group of lower affinity sites to which only X binds. It is not essential that the contaminated site has higher affinity than the others but the effect is more difficult to observe if this is not the case. The assumption of a single contaminated site is made mainly for ease of presentation. Any number of contaminated sites are included in the description provided the sites are independent. The competition is assumed to be mutually exclusive, i.e., only one of the two ligands can be bound to the same site at any given moment. Since the ligand Y is a contaminant, it is present in the sample at a fixed ratio to P. This ratio is denoted β and the corresponding uncontaminated fraction is represented by $\alpha = 1 - \beta$. Since the two groups of sites are assumed to be independent, they can be treated separately.

3.2. Exact treatment of the contaminated site

Binding to the contaminated high-affinity site is described by the reaction schemes

$$P + X \rightleftharpoons PX(K_x) \quad (1)$$

$$P + Y \rightleftharpoons PY(K_y) \quad (2)$$

where the association constants are given in parentheses. The usual fractional saturation of the site with X or Y, respectively, is given by

$$\nu = \frac{K_x x}{1 + K_x x + K_y y} \tag{3}$$

$$\nu_y = \frac{K_y y}{1 + K_x x + K_y y} \tag{4}$$

where the free ligand concentrations are denoted by x and y. These relations follow from the mass law equations to the above reactions and the conservation equation for the protein

$$C_p = [P] + [PX] + [PY] \tag{5}$$

As usual C indicates a total concentration and a term in square brackets represents the free concentration of the indicated species. The binding expressions, eqs. 3 and 4, are identical to those used for a standard competitor system. There is, however, one important difference between a competitor and a contaminant. A contaminant is a competitor which has a constant total concentration determined by the protein concentration

$$C_y = \beta C_p \tag{6}$$

This implies that the free concentration of the contaminant is completely determined by the free concentration of ligand X as shown below. The conservation equation for Y is

$$C_y = y + [PY] \tag{7}$$

This equation is not true for an equilibrium dialysis system since some of the contaminant can diffuse into the protein-free compartment. However, the following equations may still be used with a slight modification discussed in the following section.

An equation which expresses the free concentration of the contaminant in terms of the free concentration of ligand X is obtained by dividing eq. 7 by the total concentration of protein C_p, writing [PY] as $\nu_y C_p$ and inserting eq. 4. This yields

$$\frac{C_y}{C_p} = \frac{y}{C_p} + \frac{K_y y}{1 + K_x x + K_y y} \tag{8}$$

where the left-hand side of the equation is constant and equal to β. By multiplying eq. 8 by the denominator of the last term a quadratic equation in y is obtained which can be solved to give

$$K_y y = -0.5(\alpha b + 1 + K_x x) + 0.5\left[(\alpha b + 1 + K_x x)^2 + 4b\beta(1 + K_x x)\right]^{1/2} \tag{9}$$

The dimensionless quantity b is defined as

$$b = K_y C_p \tag{10}$$

which for a strongly bound contaminant is much greater than unity. It should be noted that the equations are valid irrespective of the strength of binding of the contaminant, i.e., for any value of b.

Finally, the desired binding curve, ν vs. x, is obtained by inserting eq. 9 into eq. 3.

Figs. 1 and 2 display a selection of theoretical binding curves calculated from the above equations (full curves) for several values of α and $b = K_y C_p$. One observes that the curves level off at $\nu \simeq \alpha$ and that a distinct plateau is obtained for $b \geqslant 1000$. Furthermore, the binding decreases with increasing protein concentration. These features and a few other characteristics that are not immediately evident from the equations are discussed more fully below.

3.3. Modifications using equilibrium dialysis

In an equilibrium dialysis experiment, the free contaminant may diffuse into the protein-free compartment, which implies that the total concentration of contaminant in the protein-containing compartment is no longer constant. However, the total number of moles of contaminant in the two compartments is constant and equal to

$$N_y = V_1[PY] + V_1[Y]_1 + V_2[Y]_2 \tag{11}$$

where V_1 and V_2 are the volumes of the compartments with and without protein, respectively. At equilibrium the free concentrations of contaminant in the two compartments, denoted $[Y]_1$ and $[Y]_2$, are identical and equal to y in the absence of the Donnan effect. Eq. 11 can then be rewritten as

$$N_y = \nu_y C_p V_1 + (V_1 + V_2) y \tag{12}$$

where [PY] has been replaced by $\nu_y C_p$. Dividing eq. 12 by the total volume of the two compartments yields

$$N_y/(V_1 + V_2) = \nu_y C_p' + y \tag{13}$$

where C_p' is defined as

$$C_p' = C_p V_1/(V_1 + V_2) \tag{14}$$

The total number of moles of contaminant in the two compartments, N, can be evaluated as $\beta C_p^0 V_1^0$, where the superscript zero indicates the value in the original sample before dialysis. Since the protein cannot diffuse through the dialysis membrane $C_p^0 V_1^0 = C_p V_1$. Using these relations and dividing eq. 13 by C_p' yields

$$\beta = y/C_p' + \nu_y \tag{15}$$

which is formally identical to eq. 8 with the substitution C_p' for C_p. Thus, the equations can be applied to an equilibrium dialysis system simply by replacing C_p by C_p' in the equations. Note that C_p' can be calculated from the protein concentration and the volume of the compartments before dialysis.

3.4. Approximate relations

The exact binding equation given by eqs. 3 and 9 is sufficiently complicated to prevent an intuitive understanding of the effect of changing the values of the parameters, although the graphical illustrations in figs. 1 and 2 do give some indication thereof. In order to compensate for the above, we derive two approximate relations that express specific experimental values in terms of the unknown parameter values. These relations are valid for a strongly bound contaminant only and can be used as a qualitative test of the model description and for obtaining initial estimates of the parameters.

A useful approximation for the association constant K_x is obtained by considering the value of ν for which $K_x x = 1$. By expanding the square root in eq. 9 in powers of small arguments one finds $K_y y \simeq 2\beta/\alpha$ which, when inserted into eq. 3, yields

$$\nu \simeq \alpha/2 \quad \text{for } K_x x = 1 \tag{16}$$

Thus, if α is known or can be estimated separately, then eq. 16 provides an initial estimate of the association constant K_x as the reciprocal of the free concentration of ligand, for which $\nu = \alpha/2$.

Another useful approximation is obtained by considering the ligand concentration $x_{1/2}$ for which $\nu = 0.5$. Provided that $\alpha < 0.5$ a simple expression can be derived. It follows from eq. 3 that $\nu = 0.5$ implies that $K_x x_{1/2} = K_y y + 1$. Neglecting one compared with the other terms, inserting eq. 8 and squaring the resulting expression yields

$$x_{1/2} = (\beta - \alpha) K_y C_p/(2K_x) \quad \text{for } \alpha < 0.5 \tag{17}$$

which shows that $x_{1/2}$ depends linearly on the protein concentration C_p. This equation is useful for an initial examination of whether the observed protein concentration dependence is consistent with the present binding model and for obtaining a crude estimate of K_y/K_x.

The basic approximation used in deriving eq. 17, i.e., that unity is negligible compared to $K_y y$, deserves some comment. Let y_0 denote the free concentration of contaminant for $x = 0$. Since, $K_y y = 1$ implies $\nu_y = 0.5$ for $x = 0$, it follows that $\beta > 0.5$ (or equivalently $\alpha < 0.5$) implies $K_y y_0 > 1$. Since the free concentration of Y increases as ligand X is added, it follows that $K_y y \gg 1$, which justifies unity being neglected. On the other hand, if $\alpha > 0.5$ then $K_y y_0 < 1$ and we cannot predict the magnitude of $K_y y$ for $x \neq 0$.

Both ends of the binding curve, that which is far from saturated and the almost saturated part, can be approximated by expressions that are formally identical to the independent site binding equation. These expressions facilitate the understanding of the form of the curve.

Consider first the low-saturation part of the binding curve, $\nu < \alpha$. For low concentrations of ligand X, i.e., $K_x x \ll 1$, one can carry out Taylor expansion of eq. 9 about $x = 0$, yielding

$$K_y y \simeq (1 + K_x x)\beta/\alpha \tag{18}$$

Inserting this expression into eq. 3 gives

$$\nu \simeq \alpha \frac{K_x x}{1 + K_x x} \tag{19}$$

which is a standard binding curve for a system

with a binding capacity equal to the uncontaminated fraction α and an association constant equal to K_x corresponding to binding of X to the free fraction of the contaminated site.

Near saturation, i.e., $\alpha/2 \ll \nu \simeq 1$, the concentration of ligand X is so large that $K_x x \gg 1$. Consequently, one can ignore all terms of unity in eqs. 3 and 9 and approximate the resulting expressions by Taylor expansions. This yields, after some algebraic manipulations,

$$\nu \simeq \alpha + \beta \frac{(K_x/b)x}{1 + (K_x/b)x} \tag{20}$$

Eq. 20 shows that the apparent binding constant in this region is equal to K_x/b, which has an inverse dependence on the protein concentration, since $b = K_y C_p$.

In order to obtain a rough indication of the overall form of the binding curve (ν vs. log x) eqs. 19 and 20 are combined into a single approximate binding equation

$$\nu \simeq \alpha \frac{K_x x}{1 + K_x x} + \beta \frac{(K_x/b)x}{1 + (K_x/b)x} \tag{21}$$

The accuracy of this equation is illustrated in fig. 1 which displays both the exact binding curves (full lines) and the corresponding approximate curves (dotted lines) for several values of the parameters α and $b = K_y C_p$. It can be seen that the approximate expression gives rise to an overemphasized plateau and that it underestimates

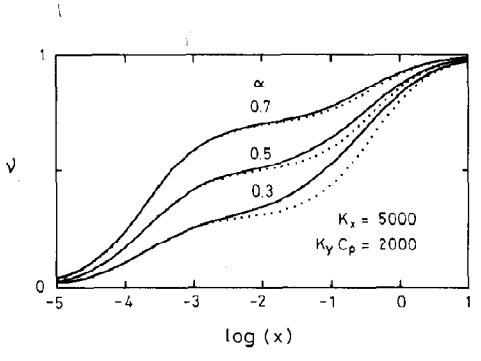

Fig. 1. Simulated binding curves illustrating the effect of contamination. The free ligand concentration x is in arbitrary units. The exact (full line) and approximate (dotted line) curves are calculated from eqs. 3, 9 and 21, respectively. The parameter values are indicated.

the binding in the important intermediate region $\alpha < \nu < 1$ which indicates that eq. 20 is not nearly as accurate as eq. 19. This deficiency in the approximate expression is due to the interaction between the contaminated and the uncontaminated fractions of the high-affinity site being neglected. In fact, eq. 21 describes exactly the situation where the contaminant is an irreversible binder and where ligand X also binds to a site already occupied by the contaminant. Fig. 1 then illustrates the observable differences between these two situations. Eq. 21 may be useful for obtaining approximate estimates of the association constants by application of linear graphical methods, such as Scatchard plots, but the value of K_x/b obtained should not be expected to be reliable, since eq. 20 is not particularly accurate. Application of eqs. 16 and 17 gives more accurate results.

3.5. *Analysis based on approximate relations*

The approximate relations were derived with the intention of permitting a qualitative test of the applicability of the model and for obtaining initial estimates of the parameters α, K_x, and $K_y C_p$. The usefulness of these approximate relations was investigated by analyzing the exact curves in figs. 1 and 2.

The exact curves in fig. 1 all show a clear plateau and by estimating α as the midpoint of the plateau the exact values of α are obtained. It is also possible to determine $\alpha/2$ as the first inflection point on the curve; this gives 0.15, 0.25 and 0.37 which is also acceptable. The log x values for $\nu = \alpha/2$ are seen to coincide, as expected from eq. 16, and have a value between -3.6 and -3.7 as judged by the accuracy that can be read from the graph. According to eq. 16, this corresponds to $K_x = 4000$–5000, which is as close to the correct value 5000 as can be expected. Since eq. 17 requires $\alpha < 0.5$ only the curve with $\alpha = 0.3$ can be further analyzed. For $\nu = 0.5$ one finds that log $x_{1/2} \simeq -1.13$ which, when inserted into eq. 17, with the above estimated values of α and K_x, yields $K_y C_p \simeq 1700$. This value is also quite close to the exact value 2000.

The exact curves in fig. 2 resemble the experimental data at three different protein concentra-

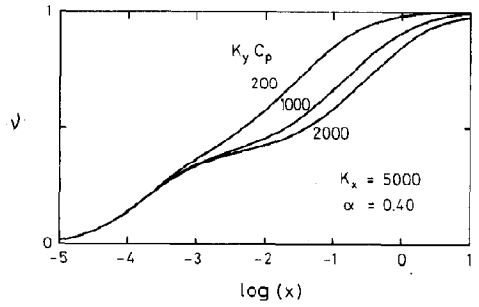

Fig. 2. Simulated binding curves illustrating the inverse protein concentration effect on the binding of a ligand X to a contaminated site. The binding curves are calculated from eqs. 3 and 9 with the parameter values indicated.

tions. The curves coincide for low values of x, indicating that α has the same value for all three curves. The uncontaminated fraction of the site can be determined quite accurately to 0.40 via $\alpha/2$ at the first inflection point and for the two high protein concentration curves also as the midpoint of the plateau. The association constant K_x determined from eq. 11 is approx. 4000 according to the accuracy by which the log x value for $\nu = \alpha/2$ can be read from the graph. Since $\alpha < 0.5$, eq. 17 can be used to check that the protein concentration dependence is consistent with the model. The $x_{1/2}$ values are found to be 5.0×10^{-3}, 2.0×10^{-2} and 4.0×10^{-2}, i.e., the relative magnitudes are 1 : 4 : 8 which are very close to the exact relative magnitudes of the albumin concentration 1 : 5 : 10. Thus, within the accuracy of analysis of the graph, $x_{1/2}$ depends linearly on protein concentration in accordance with the model. The product $b = K_y C_p$ can be determined from eq. 17 with the same accuracy.

We conclude that the approximate expressions are useful for obtaining approximate estimates of the parameters and for a rough check of model inconsistencies provided the data are sufficiently accurate and cover a sufficiently large interval.

3.6. *Inclusion of uncontaminated sites*

So far only binding to a contaminated site has been discussed. Since, however, the contaminated and uncontaminated sites are assumed to be independent of each other, they can be treated separately and the average number of X ligands bound to the protein is thus simply given by the sum

$$\nu = \nu(\text{cont. site}) + \nu(\text{uncont. sites}) \tag{22}$$

The first term represents the average number of ligands bound to the contaminated site and is given by eqs. 3 and 9. The last term represents the average number of ligands bound to the other sites. If the sites are independent, then

$$\nu(\text{uncont. sites}) = \sum_i \frac{k_i x}{1 + k_i x} \tag{23}$$

where k_i is the binding constant for site i. More generally, this contribution may be written as the Adair equation

$$\nu(\text{uncont. sites}) = \frac{\sum i\beta_i x^i}{\sum \beta_i x^i} \tag{24}$$

where the summations are from zero to the number of uncontaminated sites. Interpretation of the Adair constants β_i in terms of site binding constants and interaction between the sites is discussed in ref. 18.

4. Analysis of experimental data

The experimental data used in the analysis are displayed in fig. 3 and represent the binding of aurothiosulphate to human serum albumin under

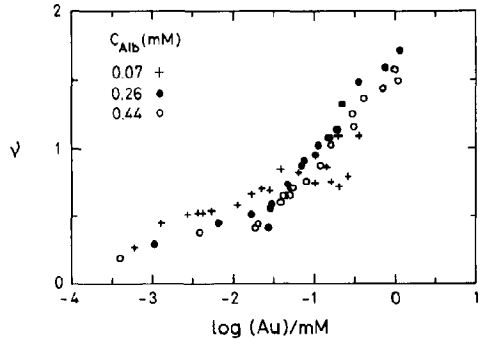

Fig. 3. Experimental data for aurothiosulphate binding to human serum albumin at three indicated albumin concentrations; from ref. 5.

physiological conditions at three different albumin concentrations [5]. It has been observed previously [5,11] that the low concentration data show a plateau which can be described by neither the independent site model nor the Adair treatment. It has also been shown [11] that the binding sites consist of a single high-affinity site, most likely the sulphydryl group in Cys-34 on albumin, and a small number of lower affinity sites. Furthermore, it was found that in the albumin preparation a fraction (α) of approx. 0.3 of the Cys-34 SH site was free, the rest being contaminated [13]. Given these observations the present model appears attractive for analyzing the data. The experimental data were analyzed both by the approximate relations, eqs. 16 and 17, and by a computerized method based on the exact binding equation given by eqs. 3 and 9.

4.1. Approximate data analysis

From fig. 3 it appears as if the experimental data for the different albumin concentrations would coincide for $\log[\mathrm{Au}]/(\mathrm{mM}) \leqslant -3.5$, corresponding to $\nu \simeq 0.17$, but the experimental data are sparse in this region. It is clearly impossible to ascertain the first inflection point and a precise determination of the plateau is difficult although it is in the range 0.2–0.5. The best estimate of α is probably to note from fig. 2 that the point $\nu = \alpha/2$ is approximately the highest ν value for which the curves corresponding to different protein concentrations coincide. This gives an estimated value of $\alpha \simeq 0.34$ which is very close to that measured. Estimations based on the plateau give higher values of α and seem to depend on albumin concentration. The value of K_x estimated from eq. 16 is 3.2×10^6 M^{-1}. The values of $\log x_{1/2}$ can be determined fairly precisely from fig. 3 to be (-2.6, -1.8 and -1.55) yielding $x_{1/2} = (4.0 \times 10^{-6}$, 1.6×10^{-5} and 2.8×10^{-5} M) corresponding to the albumin concentrations (0.066, 0.26 and 0.44 mM). The values of $x_{1/2}/C_{alb}$ obtained (6.1×10^{-2}, 6.2×10^{-2} and 6.4×10^{-2}) are indeed independent of albumin concentration, indicating that $x_{1/2}$ depends linearly on albumin concentration in agreement with the model. Finally, using eq. 17 and the estimated values of α, K_x and $x_{1/2}/C_{alb}$, the value of the association constant for the contaminant is estimated as $K'_y \simeq 1.2 \times 10^6$ M^{-1}.

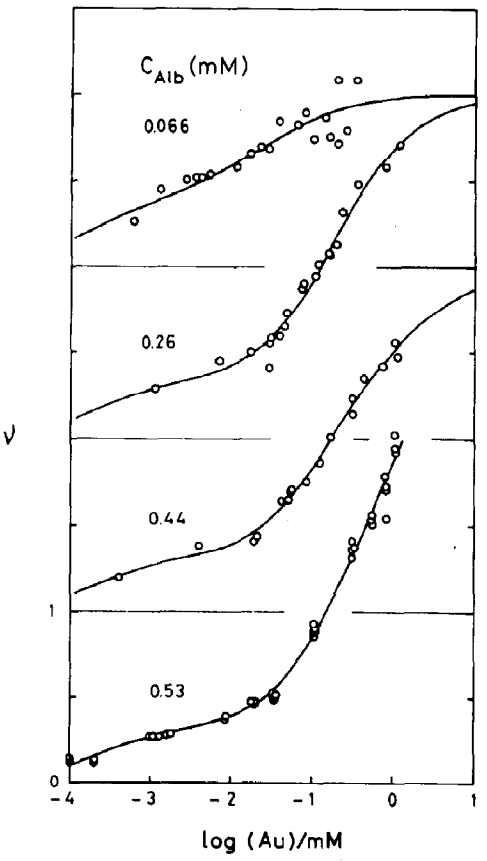

Fig. 4. Experimental data for aurothiosulphate binding to human serum albumin at four different albumin concentrations and the best fits to the exact binding equation (eqs. 3 and 9). The free fraction of the contaminated site is 0.32. The parameter values obtained by fitting are given in the text. The experimental data are from refs. 5 and 11.

4.2. Exact data analysis

Fig. 4 displays the three-data set from fig. 3 and an extra data set with albumin concentrations equal to 0.53 mM from refs. 5 and 11. The best fits to the exact binding equation, eqs. 3 and 9, obtained by a non-linear least-square regression method, are also shown. In the calculations α was considered to be constant and equal to 0.32, the previously determined value of the uncontami-

nated fraction of the sulphydryl groups on the albumin molecule.

The fits are remarkably good and represent the low-concentration data ($\nu < 0.5$) extremely well. The model equations are clearly consistent with the experimental data which are scattered randomly around the curve with no obvious trends. This is in strong contrast to a fit based on the independent site model where the low-concentration data lie far above the independent site binding curve.

The estimated values of K_x and K'_y, i.e., the association constants for aurothiosulphate and the contaminant to a Cys-34 SH site on albumin, are (25, 8, 4 and 6) $\times 10^6$ M^{-1} and (44, 58, 56 and 48) $\times 10^6$ M^{-1}, respectively, for the four albumin concentrations in increasing order. For the three highest albumin concentrations a lower affinity site was found with an association constant equal to 7×10^3 M^{-1}. Within the experimental accuracy the estimated values of the association constants are independent of albumin concentration. The apparent increase in K_x at the lowest albumin concentration (0.07 mM) might be explained by an increase in the free sulphydryl content of the albumin preparation. If the free sulphydryl content α was increased from 0.32 to 0.40 a better fit was obtained and K_x decreased to 5.9×10^6 M^{-1} which is similar to the values obtained for the other albumin concentrations.

The estimated value of K_x, 4×10^6 M^{-1}, is 100-times larger than that of the apparent association constant K_1 determined in earlier works [5,13,19] where similar data were analyzed using the independent site description. The association constant K_y for the contaminant is of the order of 10^7 M^{-1}, indicating a highly bound competitor, in agreement with the fact that the free SH group in commercial albumin preparations is highly contaminated [12].

The successful analysis of the binding data by the present model with a value of α equal to the separately measured value of the uncontaminated fraction of the sulphydryl groups on the albumin molecule provides conclusive evidence that the high-affinity site on albumin for gold(I) compounds is Cys-34 SH. This demonstrates that the presence of a contaminant may give rise to valuable extra information concerning the binding mechanism.

5. Discussion

Several explanations for protein concentration effects have been offered, e.g., the displacement of bound ligand through molecular aggregation [6,7], ligand-induced dimerization or polymerization [8,9], and inhibition of binding by highly bound contaminants as discussed in ref. 10 and above. Clearly, all possibilities must be considered. A computerized non-linear regression analysis of the experimental data (or other computerized methods) based on the exact binding equations is the most reliable method but it is often helpful and faster to use some of the general features of the binding equations first. For example, the binding equation corresponding to cross-linking of acceptor by a bivalent ligand implies that the binding curves at different protein concentrations all intersect at a point with $\nu = 0.5$ [9]. This is obviously not the case for the experimental data in fig. 3. On the other hand, the present theoretical description predicts that the ligand concentration corresponding to $\nu = 0.5$ varies linearly with protein concentration (cf. eq. 17) and this was observed for the experimental data in fig. 3. Such simple examinations of the data are extremely useful for an initial distinction between several possible mechanisms. Crude estimates of the parameters may also be obtained by application of the approximate relations, eqs. 16 and 17.

The exact analysis of the experimental data of binding of aurothiosulphate to human serum albumin showed that the approximate estimates were quite close to those obtained from the computerized analysis. However, only the computerized method can be used to prove that the binding model is in agreement with the data. This requires that the r.m.s. value of the fit is of the order of the experimental uncertainty in determining ν and that the experimental points are randomly scattered around the fit with no obvious trends. Furthermore, the estimated parameter values K_x and K_y must be independent of the protein concentration within the associated accuracy

to which these values can be determined. There exist statistical procedures for estimating this accuracy. However, they are not directly applicable, since the parameters K_x and K_y are strongly correlated. There are also statistical tests for deciding whether a given model is inconsistent with a data set, but a direct visual test of the resulting fit is just as good.

Graphical methods such as Scatchard plots may be useful for a qualitative interpretation of binding data but are not sufficiently accurate to allow quantitative conclusions. In general, only the largest parameter value, obtained as an intercept with an axis, has any significance. For example, by using Scatchard plots, Danpure [20] found that aurothiomalate was bound to albumin at a total of one site of which 0.7 had an association constant $K_1 = 1.5 \times 10^6\ M^{-1}$, the remaining 0.3 sites having an association constant of $K_2 = 1.1 \times 10^5\ M^{-1}$. These results can be rationalized in terms of the approximate binding expression, eq. 21, i.e., a fraction equal to 0.3 of the high-affinity binding site is contaminated and the association constant to the site is equal to K_1. The value of K_2 is not significant for two reasons. Firstly, the points used for the extrapolation practically coincide with the coordinate axis. Secondly, in order to give some meaning to K_2, one must use eq. 21 which was shown above to be inaccurate in the intermediate region.

In conclusion, we note that the complete mathematical expression for the binding curve derived above permits computer-generated optimized fitting and consequently quantitative analysis of binding data. Such quantitative analysis is necessary for a conclusive distinction between alternative binding mechanisms; the approximate relations are useful in the initial part of this process.

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