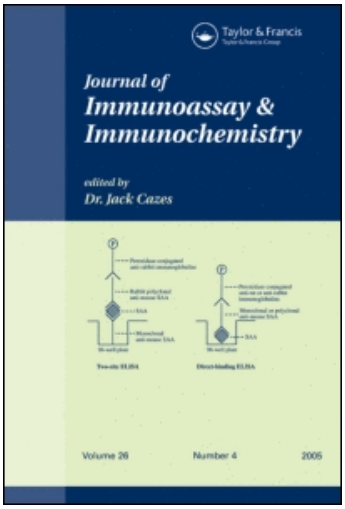


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# A Reliable Method to Estimate the Association Constant for a Monoclonal Antibody and a Protein Antigen by Enzyme Linked Immunosorbent Assay, Elisa

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# A RELIABLE METHOD TO ESTIMATE THE ASSOCIATION CONSTANT FOR A MONOCLONAL ANTIBODY AND A PROTEIN ANTIGEN BY ENZYME LINKED IMMUNOSORBENT ASSAY, ELISA.

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## ABSTRACT

The association constant $K_a$ for mouse monoclonal antibody raised against human angiotensinogen was calculated using a mathematical model, SAM I. $K_1$ represents the equilibrium constant for the binding of antibody to the solid phase with antigen previously absorbed. $K_2$ represents the interaction between antibody and antigen in solution (Ag + Ab = AgAb). $K_3$ represents binding to the antigen absorbed on the solid phase by an antigen-antibody complex. $K_4$ represents the second binding of the antigen to the antigen-antibody complex (AgAb + AgAb = (Ag)2Ab). The model unveils cooperativity for the first ($K_1$ and $K_2$) and second ($K_3$ and $K_4$) binding of antigen to antibody. The model gives the association constant in a high affinity interaction between antigen and antibody. (Keywords: Human angiotensinogen, monoclonal antibody, enzyme linked immunosorbent assay, determination of association constant, cooperativity).

## INTRODUCTION

Enzyme-linked immunosorbent assays, ELISAs, have become become widely accepted as a useful method to detect and quantify proteins and other antigens because of simplicity and economy. Simple systems as well as multilayered ELISA-

systems are widely used. These systems are used for the measurements of concentrations relative to standard dilutions with concentrations known from the use of reference methods. We have developed an ELISA-system to quantify human angiotensinogen, A, by using monoclonal and polyclonal antibodies raised against A (1). The affinities of the monoclonal and the polyclonal antibodies were determined in a direct radioimmunoassay, DRIA, of angiotensinogen. Their association constants being calculated from a Scatchard plot (2). However, the absolute determination of concentration or affinity constants using spectrophotometric absorbance methods could be investigated more precisely by getting insight into the dynamics of the system. The successful application of monoclonal antibody on the solid phase, has been the practical background for establishing a general method for calculating the affinity constant of the antigen/antibody complex. The experimental data obtained by the ELISA-system was used in a standard Broyden-Fletcher-Shanno algorithm for calculating the activity constant. The equilibrium constants have been obtained by using the protein material described earlier (Rubin et al., 1988) and optimising the experimental conditions from very low antigen concentrations up to saturated conditions. The proteins under investigation had not been modified chemically in order not to interfere with the protein structure more than the binding of antigen to the antibody molecule causes in itself. The aim of the study has been to determine the association constant of the antigen-antibody complex. For these experiments we have used a system identical to the routine assay. This system measures the reaction without modifying the participating molecules. The adsorbance to the solid phase and the binding of antigen to antibody will presumably cause changes in the protein conformation which are inherent to the reactions taking place. We have, by using this system, developed a model based on a phenomenological description using equilibrium constants. This model is used in a computer-based fitting program rendering four association constants, a determination of cooperativity and a qualita-

tive assessment of the strength of the absorbance to the solid phase. The fifth constant showed that there were no desorbtion in our system, accordingly, $K_5$, does not appear in the final model.

## MATERIALS AND METHODS

The angiotensinogen used for coating was partially purified from pregnant women's sera as described earlier ( Rubin et al., 1988 ). The immunoplates I (NUNC, Roskilde, Denmark) were coated with human angiotensinogen, A, 150 $\mu$l/well, in concentrations of 1.82 x $10^{-9}$ mol/l (0.1 $\mu$g/ml) diluted in PBS, 10 mmol/l sodiumphosphate, pH 7.2, 150 mmol/lNaCl, with 10 $\mu$g BSA (Bovine serum albumin, Behring Werke, Germany), for 20 h at $4^0$C.



After washing, the wells were blocked for 1 h with 175 $\mu$l 1% TWEEN 20 (SIGMA,USA) in PBS. The test solution consisted of a constant concentration of monoclonal antibody, A2F8 4.67 x $10^{-10}$ mol/l with varying concentrations of HA, 17 - 2120 ng/ml = 3.08 x $10^{-10}$ - 3.86 x $10^{-8}$ mol/l in PBS/ 0.5% BSA. The test solutions were equilibrated for 20 h at $20^0$C before applicating 150 $\mu$l test solution to the well. The plates were incubated for 1 h at $20^0$C. The antibodies bound to the solid phase were visualised using 150 $\mu$l peroxidase conjugated goat anti-mouse antibody, (TAGO, USA) diluted appropriately in PBS/0.5% BSA, 0.5% TWEEN 20. OPD was used as substrate, 150 $\mu$l of 2.2 mmol/l 1,2-phenylenediaminehydrochloride in 0.1 mol/l sodium citrate buffer, pH 5.0, with 6.5 mmol/l hydrogen peroxide. The enzyme reaction was allowed to run for 20 min. It was stopped with 50 $\mu$l 4.5 mol/l sulphuric acid, the absorbance was measured at 492 nm on an ELISA - reader (SLT EAR 400 AT, Austria). All reactions except for the coating of the wells were performed at $20^0$C shaking at 4 cycles/sec. Between each reaction the plate was washed 3 x 1 min with PBS/ 0.5% TWEEN 20. The absorbance at the different antigen levels was found as a mean of three determinations made in

neighbouring wells. Zero-calibration was performed in wells treated identically with all chemicals/solutions except for antigen and/or antibody.

Using the same antigen concentration the absorbance was measured using either three or four different concentrations of antibody in the test solution.The experiment was made on two plates each having 96 wells.Thus in each experiment we obtained 16 corresponding values of antigen concentration and absorbance. This was obtained with three or four different antibody concentrations, giving a total of 48 or 64 corresponding pairs.

## RESULTS



We have earlier calculated the affinity constant from a Scatchard plot, $K_a$, as the negative slope of the curve determined by regression analysis, by direct radioimmunoassay, DRIA. The calculated $K_a$ was $1.67 \times 10^{10}$ l/mol $\pm$ 0.02 (n=5). As a control all r-intercepts were found to be close to 1, as r approaches 1 when (Ag) gets large. The clone A2F8, which is used in our routine laboratory work, has been selected because of its high affinity, making it possible to measure antigen concentrations in all physiological samples, with a lower limit of one femtomol/well.

The model of the experiment gives rise to four equilibria with association constants $K_1$ - $K_4$, (Fig.1). These constants may be calculated numerically as described below. We found however that the emerging least square model was numerically unstable in the sense that many combinations of the K's produced least square sums which were almost identical. Such numerical instabilities are common in non-linear optimisation though. To allow for steric interference, we introduced a factor "x", thus putting $K_3 = xK_1$ and correspondingly $K_4 = xK_2$, where factor x presumably is a measure of steric interference. By this procedure we got a numerically

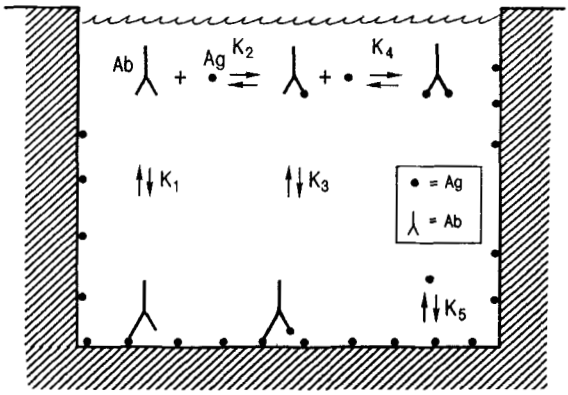


Fig.1. The reactions in a well depicted using the association constants under investigation.



stable procedure. We assumed that the association constant between antibody Ab and antigen Ag, $K_2$, was larger than the association constant between AgAb and Ag, described by $K_4$ and that the same goes for $K_1$ and $K_3$.

Here we shall briefly describe our model in mathematical terms. The antibody binds to Ag to form AgAb and $(Ag)_2Ab$. Ab also binds to the ELISA-plate to form $Ab_a$ and correspondingly AgAb binds to yield $AbAg_a$. Thus we have the following equilibria:

(1) $[Ab_a] / [Ab] = K_1$

(2) $[AbAg] / [Ab][Ag] = K_2$

(3) $[AbAg_a] / [AbAg] = K_3$

(4) $[Ab(Ag)_2] / [AbAg][Ag] = K_4$

The initial or total values of antibody and antigen, $Ab_t$ and $Ag_t$ are:

(5) $[Ab_t] = [Ab] + [AbAg] + [Ab(Ag)_2] + [Ab_a] + [AbAg_a]$

(6) $[Ag_t] = [Ag] + [AbAg] + 2[Ab(Ag)_2] + [AbAg_a]$ which may be expressed as

(7) $[Ab_t] = [Ab] + K_2[Ab][Ag] + K_2K_4[Ab][Ag]^2 + K_1[Ab] + K_2K_3[Ab][Ag]$

(8) $[Ag_t] = [Ag] + (K_2 + K_2K_3)[Ag][Ab] + 2K_2K_4[Ab][Ag]^2[Ab]$ from equation (7) and substitute in equation (8) from which we find

(9) $K_2K_4[Ag]^3 + (2K_2K_4[Ab_t] + K_2 + K_2K_3 - K_2K_4[Ag_t])[Ag]^2 + (1 + K_1 + (K_2 + K_2K_3)([Ab_t] - [Ag_t]))[Ag] - (1 + K_1)[Ag_t] = 0$

Thus [Ag] may be found as a solution to a third degree equation, and subsequently [Ab] is obtained from equation (3). In the computer program we solve equation (5) numerically (bisection and Newton iteration). Once [Ag] and [Ab] are found we calculate the total bound antibody, $[Ab_{at}]$, as:

(10) $[Ab_{at}] = [Ab_a] + [AbAg_a] = K_1[Ab] + K_2K_3[Ab][Ag]$

In the experiments $[Ab_{at}]$ is recorded as a function of total antigen $[Ag_t]$. We optimise the values $K_1$ to $K_4$ in a standard non-linear least square optimisation of these four variables minimising the function.

(11) $F(K_1,K_2,K_3,K_4) = \Sigma([Ab_{at}(i)] - [Ab_{at}(i)]^*)^2$ where $[Ab_{at}]^*$ are measured values and $[Ab_{at}]$ are calculated values, as functions of current values of the K's as described above. The minimisation was programmed based on a standard Boyden-Fletcher-Shanno algorithm.

To be specific the algorithm for estimating the K's are then:

(I) Guess a set of values of the K's.

(II) Select a value for $[Ab_t]$.

(III) Select a value for $[Ag_t]$.

(IV) Solve equation (9) for [Ag], find [Ab] from equation (7) and [$Ab_{at}$] from equation (V) Add the squared deviation to the least square sum, equation (11).

(VI) Repeat with a new value of [$Ag_t$], i.e. go to (III) above, until list of measured values of [$Ag_t$] is exhausted.

(VII) Select a new value for [$Ab_t$], i.e. go to (II) above, until list of measured [$Ab_t$] is exhausted.

(VIII) Now the sum of squares, equation (11), has been calculated for the specific guess of the K's made in (I). Use previous values of the K's and the Broyden-Fletcher-Shanno algorithm to find a new guess for the K's and continue after (I), until no further reduction in the least squares sum is possible.

The resulting set of K's is the best fit of equilibrium constants to the experimental data of the current model.

Moreover we found, that changing the value of "x" had a significant effect on the quality of the fitting, judged by the values obtained and by the graphic display of the curves. (Fig.2, A - D). By testing changing values of "x" we observed a distinct difference in the fitting quality when "x" is changed from 0.5 through 0.05 to 0.005 with an optimum at x= 0.05. This gives an approach to the determination of cooperativity in binding of the two antigen molecules to the antibody.

Furthermore we found the incorporation of the phase-shift of antigen to solid phase as compared with the fluid phase to be without influence on the calculations. As a consequence of this we considered it irrelevant to incorporate $K_5$ from the model, leaving only four association constants, $K_1$ - $K_4$.The determination of the association constants was made in a number of experiments (n=7) using identical antigen and antibody, table II.

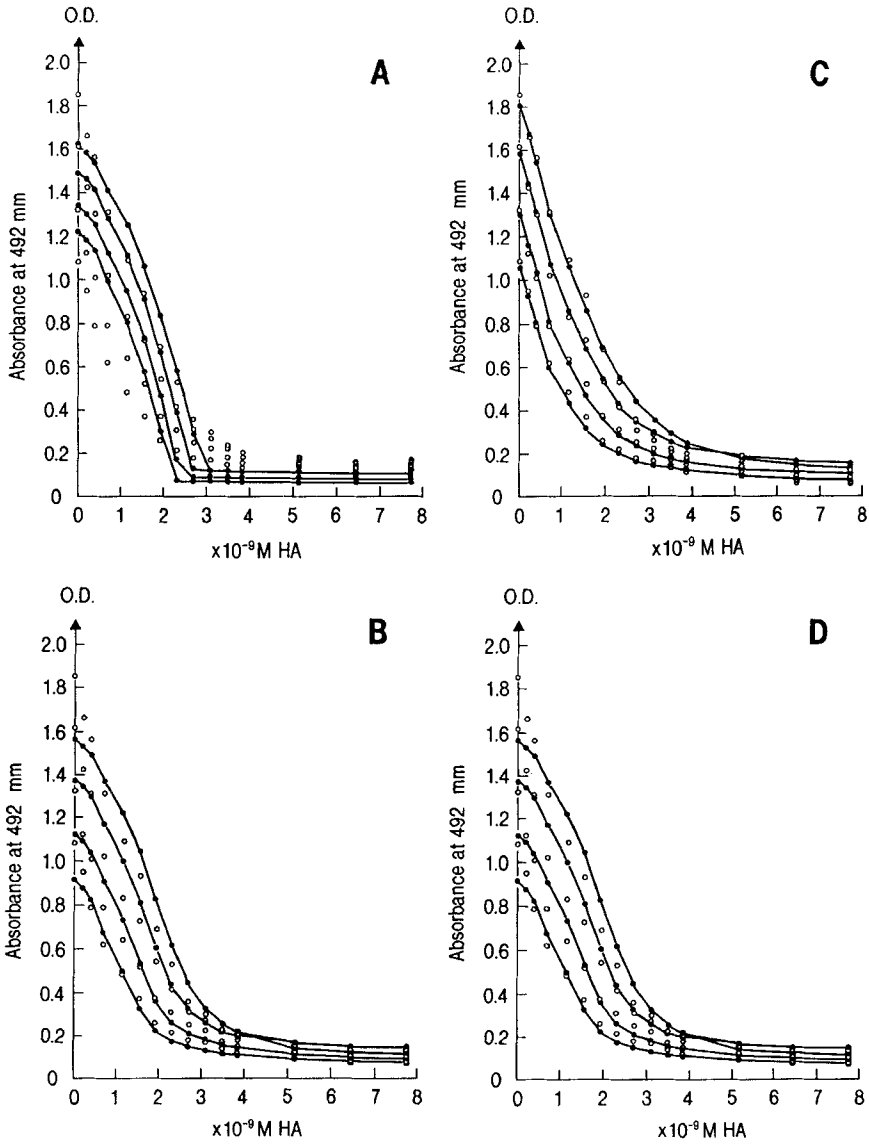


Fig.2. A - D depict four calculations of K - values. A: $K_1 = K_2 = K_3 = K_4$. B: $K_3 = 0.5 \times K_1$;, $K_4 = 0.5 \times K_2$. C: $K_3 = 0.05 \times K_1$; $K_4 = 0.05 \times K_2$. D: $K_3 = 0.005 \times K_1$; $K_4 = 0.005 \times K_2$. Open circles are the calculated values, closed circles are the measured values. Example C gives the closest fit between measured and calculated values.

Table I.

The angiotensinogen concentration, ($Ag_t$), is shown in the first column. The optical density measured at 490 nm for four antibody concentrations is shown in column 2 - 5. As mentioned in "Materials and Methods" section, the wells were coated with 0.1 $\mu$g angiotensinogen/ml.

| $Ag_t$ (x $10^{-9}$M) | OD. Ab x 2.37 x $10^{-9}$ (mol/l) | OD. Ab x 2.13 x $10^{-9}$ (mol/l) | OD. Ab x 1.94 x $10^{-9}$ (mol/l) | OD. Ab x 1.78 x $10^{-9}$ (mol/l) |
|---|---|---|---|---|
| 0.0 | 1.85 | 1.61 | 1.32 | 1.08 |
| 0.193 | 1.66 | 1.42 | 1.12 | 0.949 |
| 0.386 | 1.56 | 1.30 | 1.01 | 0.790 |
| 0.772 | 1.31 | 1.02 | 0.788 | 0.620 |
| 1.16 | 1.09 | 0.832 | 0.640 | 0.482 |
| 1.54 | 0.927 | 0.725 | 0.517 | 0.370 |
| 1.93 | 0.688 | 0.542 | 0.373 | 0.258 |
| 2.31 | 0.529 | 0.413 | 0.307 | 0.212 |
| 2.70 | 0.358 | 0.311 | 0.251 | 0.176 |
| 3.09 | 0.294 | 0.270 | 0.222 | 0.168 |
| 3.47 | 0.230 | 0.224 | 0.176 | 0.142 |
| 3.86 | 0.197 | 0.178 | 0.148 | 0.124 |
| 5.14 | 0.141 | 0.146 | 0.138 | 0.117 |
| 6.43 | 0.099 | 0.124 | 0.108 | 0.085 |
| 7.72 | 0.081 | 0.113 | 0.085 | 0.068 |

Table II.

The calculated values for all association constants, is seven consecutive experiments. $K_3 = 0.05 \times K_1$; $K_4 = 0.05 \times K_2$.

| | $K_1 \times 10^9 M^{-1}$ | $K_2 \times 10^9 M^{-1}$ | $K_3 \times 10^9 M^{-1}$ | $K_4 \times 10^9 M^{-1}$ |
|---|---|---|---|---|
| 1 | 2.79 | 28.9 | 0.139 | 1.45 |
| 2 | 1.44 | 78.7 | 0.719 | 3.93 |
| 3 | 5.94 | 68.2 | 0.297 | 3.41 |
| 4 | 9.97 | 63.8 | 0.498 | 3.19 |
| 5 | 4.80 | 34.0 | 0.240 | 1.70 |
| 6 | 7.94 | 139.0 | 0.397 | 6.93 |
| 7 | 6.87 | 90.1 | 0.343 | 4.50 |
| SD | ± 2.95 | ± 37.1 | ± 0.189 | ± 1.85 |
| Mean | 5.68 | 71.8 | 0.373 | 3.59 |



## DISCUSSION

In order to approach a true quantitative determination of antigen concentration it is desirable to be able to make a determination of affinity constants in a similar experimental system. The present mathematical model gives us an estimation of the association constants and a possibility to estimate the cooperativity in the antigen binding to the first and second binding site on the antibody molecule.

This approach has previously been used by several groups of investigators. They have in the majority of cases used modifications of the law of mass action as described by Scatchard and used an ELISA-system where antigen-coated wells was

incubated with a solution of antigen and antibody. Friguet et al.(4) developed an ELISA-based method which, by using the Scatchard-equation was able to determine dissociation constants in crude antigen preparations. Schots et al. (3) and van Erb et al. (5) used the original derivation either directly or by using the computer programme LIGAND (Munson and Rodbard 1980) (6).

In our opinion one should be aware of, that the derivation of the law of mass action introduced by Scatchard originally was applied to the interaction between a protein molecule and a small molecule or ion. A deviation from linearity in a Scatchard plot is due to different intrinsic constants on the two antigen-binding sites of the antibody or deviations from independent probabilities of interaction (steric hindrance). When the derivation is used to describe protein-protein interactions, it is questionable what the achieved dissociation constant reflects. Because of this problem, one must be modest in the demands upon the conclusions when the interactions are described.



To solve this problem, the method described by Friguet et al., (4) was modified by Stevens (6), who introduced a statistical modification to correct for the bivalency of the native antibodies. He found that the affinity determinations made, using the derivation of the Scatchard-equation, as described by Friguet et al., are in risk of a systematical underestimation. This is of relevance when two antibodies are compared.

Other ways to make determinations of the affinity constants have been introduced by Li (8), who used a Laplace transformation of the diffusion equation, and Beatty et al. (9), who used a derivation of the law of mass action.

The present method is based on the law of mass action. The initial purpose of the study was to find the minimal number of parameters, which in a fitting procedure could mimic the experimental data.

As presented above we found the association constants $K_3$ and $K_4$ to be related to $K_1$ and $K_2$ respectively through a factor x. These parameters together with the total amount of antigen and the relative concentrations of the antibodies was sufficient to make a stable fitting procedure. We suggest that the factor x is primarily a measure of steric interference between the binding sites, secondly, that the factor additionally reflects different intrinsic constants.

It should be noticed, that in our hands the difference in association constants at the first and second binding site reflects a negative cooperativity. This is in opposition to the positive cooperativity found by Schots et al. (3) and van Erp et al. (5) using the Scatchard equation. Additionally we do not find that the exclusion of $K_2$ - $K_4$ gives a stable result of the fitting procedure. This is supported by the finding that $K_3$ is not of a negligible value, when using our model. The determination of $K_1$ using other methods is possible, but those methods do not give an estimation of $K_2$ - $K_4$. The opportunity to get a broader insight, into the nature of our antibody, we find appealing, although we are primarily interested in one of the determined parameters. The method is based on the relationship between the relative absorbance and the relative amount of well-bound antibody. The monoclonal antibody used, A2F8, is highly potent, and this enables us to analyse very low antigen concentration. On the other hand, the antibody gets easily saturated. As a consequence of this, the antigen concentration in the coating solution has to be very low to reach usable results.

These conditions means that the experimental technique has to be very carefully selected, which impose big demands on the person, who actually perform the analysis. It is necessary to use a very pure preparation of commercial BSA as carrier protein (more than 98% pure). All glassware should be siliconized in order not to loose material.

It is established that very low protein concentrations stick to the surface because of the slight anionic character of glass. The glassware has to be handled in a special careful washer. Secondly, washing of the wells during the experiment must be done with great accuracy and timing.

Taking these precautions into consideration, the ELISA-method has shown to be a time saving alternative to the DRIA-method. Its main advantages are the use of native antigen and antibody, and the use of the same basic materials, as we use for our angiotensinogen measuring system, and in addition we can determine the cooperativity in the binding of the first and second antigen molecule to antibody.



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