An Exact General Analysis of Ligand Binding Displacement and Saturation Curves

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ABSTRACT: Quantitative analysis of a ligand–protein interaction relates binding to the free concentration of ligand molecules in solution. A theoretical analysis is presented herein, by which intermolecular interactions can be described as a function of the added concentrations of ligand molecules. Following this analysis, ligand binding displacement and saturation curves can be converted directly into a linear form, even when nonradioactively labeled ligands are used to detect the ligand–protein interaction. From the linearities obtained, relevant binding parameters, including the binding dissociation constant, can be calculated. On the basis of this analysis, binding parameters have been characterized for the interaction between biotin–protein A and immunoglobulins, using ELISA-type detection, and for the interaction of a fluorescently labeled fatty acid with a specific fatty acid binding protein.

Many analytical methods for identification and quantification of biologically relevant molecules rely on the ability of these compounds to interact with specific binding proteins, such as receptor molecules and antibodies. These types of molecular interaction form the basis of such important analytical techniques as radio-receptor assays (RRA),[1] enzyme-linked immunosorbent assays (ELISA), and radio immunoassays (RIA). In many cases, use is made of a labeled derivative of the compound of interest by introduction of a radioactive atom or by attachment of a fluorescent or chemical label, such as a dansyl or a biotin group (King & Catino, 1990). The amount of the compound of interest present in biological samples is then quantified by studying the extent of displacement of binding of this labeled ligand to its specific binding protein, which can be monitored from the characteristics of the introduced label.

Within the existing models of reversible interaction between a ligand and its binding protein, the extent of ligand binding can be related to the concentration of ligand molecules remaining free in solution after reaching equilibrium (Scatchard, 1949). The exact concentration of free ligand required for this type of analysis can be determined experimentally only if a radiolabeled ligand molecule is used. In the case of fluorescently or chemically labeled ligands, however, no straightforward experimental method is available for direct quantification of the concentrations of free ligand.

In the present study, a theoretical analysis of ligand binding displacement curves is presented, which relates the extent of labeled ligand binding to the added, instead of the free, concentrations of labeled and unlabeled ligands. The present analysis is therefore generally applicable, that is, it applies not only to radiolabeled ligands but also to fluorescently or chemically labeled ligands, as long as the parameter measured to detect ligand binding is proportional to the amount of labeled, bound ligand. Using this method, both ligand binding displacement and saturation curves can be expressed directly in a linear form, which is of great statistical importance for measurement of a large number of samples in routine analysis (Walker, 1977; DeLean et al., 1978). From the linearity obtained, not only the ligand binding dissociation constant but also values for the free concentrations of ligand molecules can be derived, even for nonradioactively labeled ligands. Expressions are also presented for analyzing this type of displacement curve in the additional presence of a nonspecific binding component. The examples presented here for analysis are the binding of biotin-labeled protein A to substrate-attached immunoglobulins, as detected by an avidin-coupled peroxidase reaction, and the interaction of a fluorescently labeled fatty acid to a specific fatty acid binding protein.

EXPERIMENTAL PROCEDURES

Binding of Protein A to Immobilized Immunoglobulins. Immunoglobulins (IgGs) were purified from normal rabbit serum by chromatography on protein A–Sepharose and stored in phosphate-buffered saline (PBS) at a concentration of 2 μg/mL. From this solution, 100 μL was added per well (0.33 cm^2), followed by overnight incubation at 4 °C. Plates (96 wells) were subsequently washed three times with PBS/0.05% Tween 20, and nonspecific binding was blocked by incubation for 1 h at 37 °C in a serum-free culture medium containing 1% bovine serum albumin. Subsequently biotin–protein A (Sigma) was added at a concentration of 50 ng/mL, in the presence of additional variable concentrations of unlabeled protein A, to a final volume of 100 μL. After incubation for 1 h at 37 °C, the wells were washed four times with PBS/Tween and subsequently incubated in the above albumin-containing medium now containing avidin-peroxidase (Sigma) for 1 h at a final concentration of 0.25 μg/mL. After four washes with PBS/Tween, peroxidase activity was detected by the addition of *o*-phenyldiamine as a substrate, and color development was analyzed as described previously (van den Eijnden-van Raaij et al., 1990).

Interaction of Fatty Acids with Fatty Acid Binding Proteins. Binding of 11-(dansylamino)undecanoic acid (DAUA) to purified porcine liver fatty acid binding protein was determined

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; RIA, radio immunoassay; RRA, radio-receptor assay; IgG, immunoglobulin G; DAUA, 11-(dansylamino)undecanoic acid; PBS, phosphate-buffered saline.

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as described before (Peeters et al., 1989). Experiments were carried out with 0.2 μM binding protein in 10 mM Tris-HCl (pH 8.0) containing a final concentration of 0.1% ethanol. Fluorescence was measured at 345 (excitation) and 505 nm (emission), a condition under which no fluorescence is observed in the absence of protein.

THEORY

The reversible binding of labeled ligand to a homogeneous class of noncooperating binding sites, in the additional presence of unlabeled ligand molecules under equilibrium, is given by (Mendel & Mendel, 1985; van Zoelen, 1989)

$$B^* = \frac{NF^*}{K_D + F^* + F^u} + K_A F^* \quad (1)$$

in which F^* is the free concentration of labeled and F^u that of unlabeled ligand molecules after reaching binding equilibrium, and B^* is the amount of labeled bound ligand under these conditions. N stands for the number of specific binding sites available and K_D stands for the binding dissociation constant, while K_A is a constant for nonspecific binding which is only of relevance in the case when additional ligand binding takes place to low-affinity binding domains in a nonspecific manner. It will be assumed that this potential nonspecific binding component is nonsaturable within the range of ligand concentrations used. When a constant concentration of labeled ligand (L_0^*) is added with varying concentrations of unlabeled ligand (L^u), eq 1 provides a theoretical description of ligand binding displacement curves.

The use of eq 1 requires that labeled and unlabeled ligands behave chemically identical. This can also be expressed as

$$F^u/L^u = F^*/L_0^* \quad (2)$$

indicating that within any given experiment the free fraction of both ligands must be similar. The concentration of free ligand (F) will differ from the added concentration (L) according to

$$F = L - \rho B \quad (3)$$

in which B is the amount of bound ligand at equilibrium, and ρ is a dimension-converting parameter. If F, L, and B are all expressed in the same units, ρ will equal unity. Otherwise $1/\rho$ will equal the specific activity expressed in radioactivity per concentration unit (e.g., cpm/nM) in the case of a radiolabeled ligand, while for a fluorescently or chemically labeled ligand, $1/\rho$ will generally be expressed in absorption units per nM. The concomitant use of eqs 1 and 3 implies that B and N are expressed in the same units.

In the absence of nonspecific binding ($K_A = 0$), the binding of labeled ligand can be fully displaced by addition of an excess of unlabeled ligand, following eq 1. B_0^* will now be defined under these conditions as the amount of labeled bound ligand in the absence of unlabeled ligand ($L^u = 0$), making $B_0^* = NF_0^*/(K_D + F_0^*)$, in which F_0^* is the free concentration of labeled ligand under conditions without displacement. The reduction in labeled bound ligand upon addition of a particular concentration of unlabeled ligand (L^u) will now be expressed as $\phi = B^*/B_0^*$.

Because of the general validity of eq 3, it follows that in the absence of unlabeled ligand $F_0^* = L_0^* - \rho B_0^*$, while upon addition of a certain concentration of unlabeled ligand, the free concentration of labeled ligand is given by $F^* = L_0^* - \rho B^*$. Under conditions that L_0^* is a constant, it therefore follows upon elimination of ρ, and by using the definition of ϕ, that

$$F^* = (1 - \phi)L_0^* + \phi F_0^* \quad (4)$$

Since ϕ and L_0^* are experimentally known, it follows that, if F_0^* can be determined, all values of F^* following the addition of unlabeled ligand can be calculated. Furthermore, it follows from the definition of ϕ, combined with eq 1 under conditions $K_A = 0$, that

$$K_D(F^* - \phi F_0^*) + (1 - \phi)F^*F_0^* = \phi F_0^* F^u \quad (5)$$

and in combination with eq 4 that

$$\frac{\phi}{1 - \phi}F^u = F^* + K_D\frac{L_0^*}{F_0^*} \quad (6)$$

From this equation, together with eq 2, it follows upon elimination of F^* that

$$F^u\left(\frac{\phi}{1 - \phi} - \frac{L_0^*}{L^u}\right) = K_D\frac{L_0^*}{F_0^*} \quad (7)$$

and since from combination of eqs 2 and 4 it follows $F^u = L^u(1 - \phi(1 - F_0^*/L_0^*))$, elimination of F^u results in

$$\frac{L_0^*(1 - \phi)}{L^u\phi^2 - L_0^*\phi(1 - \phi)} = \frac{1}{\phi}\frac{F_0^*}{K_D} - \frac{F_0^*}{K_D}(1 - F_0^*/L_0^*) \quad (8)$$

This final equation describes a linear relationship between the experimentally derived dimensionless parameter on the left-hand side and $1/\phi$, while on the basis of the values of the slope and the intercept of this linearity, the system parameters F_0^* and K_D can be determined. Intriguingly, eq 8 does not contain the specific activity parameter ρ, and is therefore valid for any type of binding assay in which the signal for binding obtained is proportional to the amount of labeled bound ligand.

In cases where additional ligand binding takes place to nonspecific domains ($K_A > 0$), labeled bound ligand according to eq 1 cannot be completely displaced by the addition of excess unlabeled ligand. If the fraction of bound nondisplaceable labeled ligand is defined as ϕ_∞, it follows from the considerations presented in the Appendix that

$$\frac{L_0^*(1 - \phi)}{L^u\phi(\phi - \phi_\infty) - L_0^*\phi(1 - \phi)} = \frac{1}{\phi}\left(\frac{F_0^*}{K_D(1 - \phi_\infty(1 - F_0^*/L_0^*))}\right) - \left(\frac{F_0^*(1 - F_0^*/L_0^*)}{K_D(1 - \phi_\infty(1 - F_0^*/L_0^*))}\right) \quad (9)$$

which is the equivalent expression of eq 8 for $\phi_\infty > 0$. Again, there is a linear relationship between the experimentally derived, dimensionless parameter on the left-hand side and $1/\phi$. Since ϕ_∞ can be obtained directly from the competition experiment, the parameter F_0^* is again obtained from the ratio of intercept and slope; once this value is known, K_D can be derived from the slope.

RESULTS

Data Simulation. Figure 1A shows simulated ligand binding displacement curves, both in the absence ($\phi_\infty = 0$) and presence ($\phi_\infty > 0$) of a nonspecific binding component. The parameters used for data simulation are presented in the Figure 1 legend, using the condition that the free ligand concentrations are significantly lower than the added concentrations ($F < L$). Linearization of the displacement curve

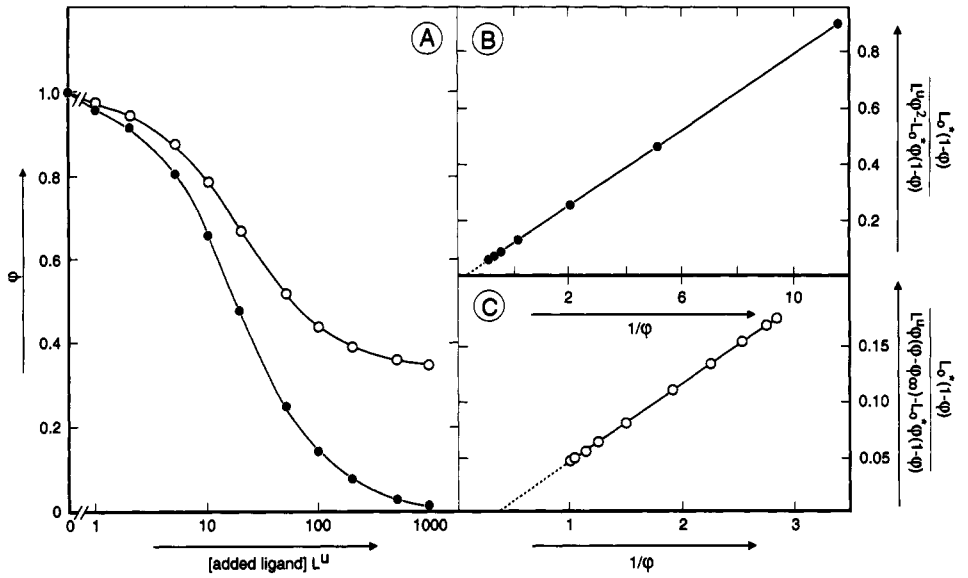

FIGURE 1: Linearization of ligand binding displacement curves in the absence (●) and presence (○) of a nonspecific binding component. (A) Simulated displacement curves for competition of labeled ligand binding upon the addition of unlabeled ligand. Binding data were simulated by iterative computer analysis based on eq 1 using the concentrations of ligand initially added (L) instead of the free concentrations (F). After calculation of B values, corresponding F values were calculated according to eq 3, which were again used in eq 1 in an iterative way. The following values were used for simulation: N = 10 000; K_D = 10; ρ = 0.0005; L_0^* = 1; and K_A = 0 (●) or 300 (○). Binding of labeled ligand represented by ϕ = 1 corresponds to non-displaced conditions (L^u = 0). (B) Linearized displacement curve in the absence of nonspecific binding according to eq 8. Data points corresponding to a value of $1/\phi > 14$ have not been included. (C) Linearized displacement curve in the presence of nonspecific binding according to eq 9. A value of ϕ_∞ = 0.3405 has been used, obtained following eq 14.

in the absence of nonspecific binding following eq 8 results in Figure 1B, while in the presence of nonspecific binding, Figure 1C is obtained following eq 9. The value for ϕ_∞ used in Figure 1C was derived from the value of K_A according to eq 14. In both cases linearity was exact, giving an intercept with its abscissa between 0 and 1. It follows from eqs 8 and 9 that both in the presence and absence of nonspecific binding the absolute ratio of intercept and slope equals $(1 - F_0^*/L_0^*)$, therefore an intercept with the abscissa equal to 0 denotes binding characteristics in which the fraction of labeled ligand bound following interaction with the binding protein is negligible ($F_0^* \approx L_0^*$). By contrast, an intercept with its abscissa equal to 1 indicates binding in which, under non-displaced conditions, nearly all added ligand molecules become bound as a result of the interaction with the binding protein ($F_0^* \approx 0$).

Statistics of Data Analysis. Analysis of the simulated binding data of Figure 1A,B (L_0^* = 1, K_D = 10, K_A = 0; see legend) according to eq 8 resulted in generated values for F_0^* and K_D as shown in Table I (0% relative random error). In order to investigate how errors in experimental data points propagate when using the present linear expressions, relative random error levels of 1, 2, and 4% were superimposed on the simulated B^* values, and the effects on the system parameters F_0^* and K_D were determined. Table I shows that, although the sample standard deviation in the parameters F_0^* and K_D gradually increases with increasing error level, the mean values of these parameters remain close to the values generated in the absence of statistical error. Only in the case of a 4% error level are the mean values of the two parameters within a 90% confidence interval (F_0^* = 0.700–0.724, K_D = 10.23–10.59) significantly different from the undisturbed values.

Binding of Biotin–Protein A to Immobilized Immunoglobulins. Binding of biotin-labeled protein A to rabbit immunoglobulins can be detected by incubating the complex with avidin peroxidase and a suitable substrate which is converted by a peroxidase reaction into a colored derivative. Figure 2A shows a displacement curve of biotin–protein A binding by unlabeled protein A, according to the protocol described in the legend. In the presence of a 200-fold excess of unlabeled over labeled ligand, a value of ϕ = 0.06 was obtained which is indicative of near-complete binding competition. For linearization of these data according to eq 9, the parameter ϕ_∞ is required. Using a trial-and-error method, various values of ϕ_∞ between 0 and 0.06 were therefore tested for the best linear fit. Statistics for linearity were carried out by minimizing studentized residuals, as described in detail elsewhere (van Zoelen, 1989). The best linear fit, as shown in Figure 2B, was obtained using a value of ϕ_∞ = 0.03 (see the dashed horizontal line in Figure 2A). From the intercept with the abscissa at $1/\phi$ = 0.87, a value of F_0^*/L_0^* = 0.13 was obtained, which in combination with the slope resulted in K_D = 0.0152 μg/mL. These data show that under non-displaced conditions only a small fraction of the added biotin–protein A remains free in solution and that as a consequence the K_D value obtained is much smaller than the concentration of unlabeled protein A required for half-maximum binding competition (approximately 0.25 μg/mL).

Table I: Effect of Random Errors on Parameter Estimation from Linearized Displacement Curves[a]

relative random error level (%)	F_0^* ± SSD	K_D ± SSD
0	0.681	10.00
1	0.684 ± 0.047	10.05 ± 0.69
2	0.678 ± 0.097	9.96 ± 1.43
4	0.712 ± 0.195	10.41 ± 2.87

[a] Binding data were simulated using the parameters given in the legend of Figure 1 (K_A = 0). Subsequently, for each data point a normal distribution was generated with mean value B^* and standard error pB^*, in which p in the relative random error level. Binding data were then generated from these normal distributions and plotted in a linear fashion according to eq 8 to obtain values for F_0^* and K_D, using unweighted linear regression analysis. For each relative random error level tested, mean values and sample standard deviations for these two parameters are indicated, on the basis of 1000 repeated calculations. Under all conditions tested, the values of F_0^* and K_D thus obtained appeared to obey a normal distribution.

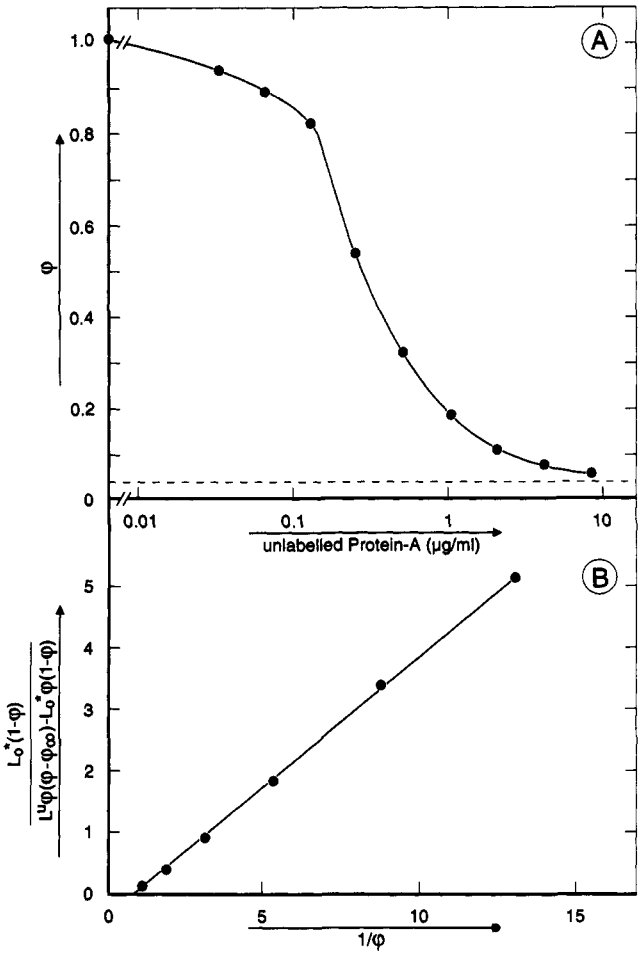

FIGURE 2: Analysis of protein A binding to immobilized immunoglobulins. (A) Displacement curve for biotin–protein A binding (50 ng/mL) to immunoglobulins (IgGs), following addition of increasing concentrations of unlabeled protein A. Binding of biotin–protein A was detected by incubation with avidin peroxidase and *o*-phenyldiamine as the peroxidase substrate. Care was taken that the reaction was stopped while the increase in color intensity was still linear with time. (B) Linear representation of displacement curve A according to eq 9, using a value of $\phi_\infty = 0.03$ (see dashed line in A) as the nonspecific contribution.

Binding of Fluorescent Lipids to a Fatty Acid Binding Protein. Binding of 11-(dansylamino)undecanoic acid (DAUA) to porcine liver fatty acid binding protein results in strong fluorescence enhancement, which can be used to analyze lipid–protein interactions (Peeters et al., 1989). Figure 3A shows the enhancement of fluorescence when increasing concentrations of DAUA are incubated with a constant concentration of binding protein, as outlined in the legend. Although binding shows saturation characteristics, an additional nonsaturable component appears to be present which may result from nonspecific interaction. This type of saturation binding curve, which relates the extent of ligand binding (B) to the added concentration of labeled ligand (L), can be readily converted into a displacement-type curve by defining, for a chosen data point, the added ligand concentration as L_0^* and the corresponding binding as B_0^*. By taking this selected data point as a reference, B^* values are now defined for all other data points according to $B^* = B(L_0^*/L)$, and L^u values according to $L^u = L - L_0^*$. A plot of ϕ ($=B^*/B_0^*$) versus L^u now has all of the characteristics of a ligand binding displacement curve. This conversion strategy requires only that labeled and unlabeled ligands behave in a chemically identical manner.

Figure 3B shows the displacement curve derived from Figure 3A, using the data point at 0.15 μM DAUA as a reference value. Using the same trial-and-error strategy as that described for Figure 2, a value of $\phi_\infty = 0.025$ (see the dashed horizontal line in Figure 3B) was obtained to give the best linear fit of these data when expressed according to eq 9. From this linearity (see Figure 3C), values for $F_0^*/L_0^* = 0.727$ and $K_D = 0.19$ μM were determined. Furthermore, from the value of ϕ_∞ the parameter K_A for nonspecific binding could be determined according to eq 14, resulting in a value of 0.68 absorbance units (au) per μM lipid. Once the concentration of free lipid in the chosen reference point was known, free concentrations for other data points could be calculated according to eq 4. By subtracting the nonspecific binding component (K_AF) from the experimental data points of Figure 3A, the specific binding data thus obtained were plotted according the method described by Scatchard (1949), as shown in Figure 3D. From this linear plot, it is shown that maximum specific ligand binding results in a fluorescence enhancement of 11 au; together with the known value of K_D, a complete saturation curve for specific binding with the fatty acid binding protein can thus be obtained.

DISCUSSION

In the present study, theoretical equations have been derived to express ligand binding displacement and saturation curves in a linear form and to determine relevant system parameters from the slopes and the intercepts of the linearities obtained. Because data can be expressed directly as a function of the total ligand concentration added, the method described here can be used for any type of labeled ligand, provided that the signal measured to detect ligand binding is proportional to the amount of the ligand–protein interaction. On the basis of the results obtained, values for the free concentration of ligand molecules can be calculated, even for nonradioactively labeled ligands; the data can then be plotted conventionally in a Scatchard plot if desired. Application of the above equations is limited to situations where a single, noncooperative class of binding sites is present which binds labeled and unlabeled ligands with the same affinity. In addition, it is assumed that the free ligand molecules are not complexed by other molecules in solution and that subsequent protocols to detect ligand binding, for example by incubation with avidin or a second antibody, do not significantly interfere with the amount of labeled bound ligand.

In previous studies, exact mathematical descriptions have been presented for ligand binding displacement curves, both in the case of a single (Horovitz & Levitzki, 1987) and in the case of multiple (Almagor & Levitzki, 1990) receptor classes. The linearities obtained by those methods can also be used in the case where labeled and unlabeled ligands have different receptor affinities. The present method has the advantage that data from displacement curves can be directly converted into linear form, without a separate determination of the available concentration of receptor molecules. Moreover, the present equations take into account the possibility that additional ligand binding takes place to nonspecific domains in a nonsaturable manner. After the analysis of displacement curves by a homologous unlabeled ligand according to the present method, the binding affinity for nonhomologous ligand molecules can be subsequently determined on the basis of an equivalent competition principle, as described in detail elsewhere (van Zoelen, 1992).

On the basis of the general eq 9, the system parameters for receptor–ligand interaction can be obtained directly from

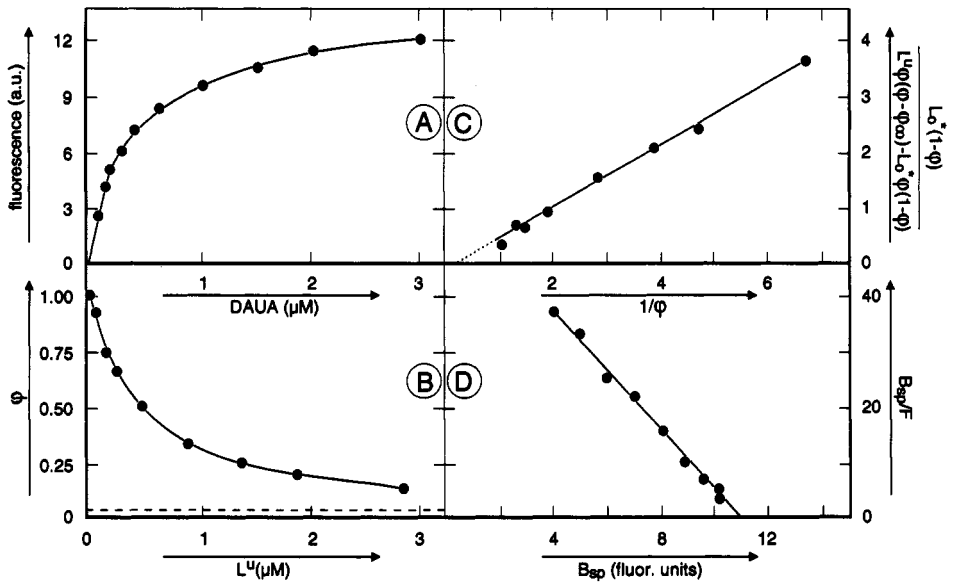

FIGURE 3: Binding characteristics of 11-(dansylamino)undecanoic acid (DAUA) to purified porcine liver fatty acid binding protein. (A) Saturation binding curve obtained by incubation of increasing concentrations of DAUA with 0.2 μM binding protein. Fluorescence increase is indicated in arbitrary units (au). (B) Transformation of saturation binding curve (A) into a displacement-type curve, using the method outlined in the text. The data point corresponding to 0.15 μM DAUA was chosen as a reference value. (C) Linear representation of displacement curve B according to eq 9, using a value of $\phi_\infty = 0.025$ (see dashed line in B) as the nonspecific contribution. (D) Scatchard plot for specific interaction of DAUA with fatty acid binding protein. As the values of F_0^* and ϕ_∞ were known from the linearity under C, data from A were corrected for their nonspecific component, and specific binding (B_{sp}) was expressed as a function of the free ligand concentration.

displacement curves either by computer-assisted nonlinear regression analysis or by expression of the data in a linear form as presented in this study. The first approach is generally more accurate, while a linear expression is particularly sensitive for detection of significant deviations from a proposed theoretical model. As for other equations describing ligand binding (Zivin & Waud, 1982), alternative linear expressions can be derived for eqs 8 and 9, for example, by multiplying both sides of the equations by ϕ or by placing $1/\phi$ on the left-hand side of the equation. The present format has been chosen because it can be used for any range of F_0^* between 0 and L_0^*, without the possibility that the slope may become independent of ϕ or that the slope and intercept may reach infinite values. As a result, the present equations generally result in a high correlation coefficient, in spite of possible data scattering, which is of great advantage when applying this method for the linearization of binding calibration curves to be used for routine analysis on a large number of samples.[2] We realize, however, that this does not imply that, following the statistical considerations given in Table I, the present format of eqs 8 and 9 necessarily gives rise to the highest accuracy in determining binding parameters.

The present method describes an exact and direct analysis for binding of chemically and fluorescently labeled ligands. This approach can also be useful for radiolabeled ligands, particularly when, as a result of high-affinity binding, very low values for the free ligand concentrations are obtained ($F_0^* \ll L_0^*$). Other methods described in the literature for the binding analysis of fluorescently labeled ligands generally use iterative procedures based on estimated values of the various system parameters (Cogan et al., 1976; MacKay et al., 1991). In the case of ELISA reactions based on enzymatic binding detection, approximation methods have also been described for the mathematical analysis of displacement curves, particularly based on a so-called logit–log transformation (DeLean et al., 1978; Ritchie et al., 1981; Dudley et al., 1985). In contrast to these other strategies, however, there is no need to assume values for the free concentrations of ligand molecules using the present method, where experimental binding data can be directly expressed as a function of the ligand concentrations added.

[2] H. A. Ross and E. J. J. van Zoelen, unpublished results.

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APPENDIX

In the case $K_A > 0$, eq 1 will describe the amount of total labeled bound ligand and will be composed of a specific binding component B_{sp}^* given by the first right-hand term and a nonspecific binding component given by $K_A F^*$. If for a constant L_0^*, ϕ is now defined under these conditions as the ratio of total binding following displacement ($\phi = B^*/B_0^*$), while θ is defined as the ratio of only the specific binding components ($\theta = B_{sp}^*/B_{0,sp}^*$), it follows that

$$\phi = \frac{\theta B_{0,sp}^* + K_A F^*}{B_{0,sp}^* + K_A F_0^*} \tag{10}$$

Equations 2–4 will still be valid under these conditions, in which B now stands for total ligand binding, and therefore it follows from the combination of eqs 4 and 10 that

$$\theta = \phi - Z(1-\phi)L_0^* \tag{11}$$

in which Z is defined as $K_A/B_{0,sp}^*$. Equation 5 will now hold for θ instead of ϕ, and from elimination of θ by combination with eq 11,

$$\left(\frac{\phi}{1-\phi} - ZL_0^*\right)F^u = (1 + ZL_0^*)F^* + K_D\left(\frac{L_0^*}{F_0^*} + ZL_0^*\right) \tag{12}$$

which is the equivalent expression of eq 6 in the case $K_A > 0$.

The interpretation of the term ZL_0^* can be seen as follows. At an infinite concentration of unlabeled ligand ($F^u = \infty$), ϕ will reach an asymptotic value $\phi_\infty > 0$. It follows from eq 1 that $B_\infty^* = K_A F_\infty^*$, and in combination with eq 4 that $F_\infty^* = (1 - \phi_\infty)L_0^* + \phi_\infty F_0^*$. Since by definition $\phi_\infty = B_\infty^*/B_0^*$, it follows that $\phi_\infty(B_0^* - K_A F_0^*) = K_A L_0^*(1 - \phi_\infty)$. By definition, $ZL_0^* = K_A L_0^*/B_{0,sp}^*$, in which $B_{0,sp}^* = B_0^* - K_A F_0^*$, and thus

$$ZL_0^* = \frac{\phi_\infty}{1 - \phi_\infty} \tag{13}$$

When introduced into eq 12, and using the same strategy as above for elimination of F^u, eq 9 is obtained. The relationship between the value of ϕ_∞ experimentally obtained and the system parameter for nonspecific binding K_A is given by $K_A = B_\infty^*/F_\infty^*$, and thus

$$K_A = \phi_\infty B_0^*/((1 - \phi_\infty)L_0^* + \phi_\infty F_0^*) \tag{14}$$

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