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Allowance for antibody bivalence in the determination of association rate constants by kinetic exclusion assay

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ABSTRACT

This investigation completes the amendment of theoretical expressions for the characterization of antigen–antibody interactions by kinetic exclusion assay—an endeavor that has been marred by inadequate allowance for the consequences of antibody bivalence in its uptake by the affinity matrix (immobilized antigen) that is used to ascertain the fraction of free antibody sites in a solution with defined total concentrations of antigen and antibody. A simple illustration of reacted site probability considerations in action confirms that the square root of the fluorescence response ratio, R_{Ag}/R_o, needs to be taken in order to determine the fraction of unoccupied antibody sites, which is the parameter employed to describe the kinetics of antigen uptake in the mixture of antigen and antibody with defined initial composition. The approximately 2-fold underestimation of the association rate constant (k_a) that emanates from the usual practice of omitting the square root factor gives rise to a corresponding overestimate of the equilibrium dissociation constant (K_d)—a situation that is also encountered in the thermodynamic characterization of antigen–antibody interactions by kinetic exclusion assay.

Of the chromatographic methods available for the thermodynamic characterization of immunochemical reactions, the kinetic exclusion assay (KinExA)[1] affords a direct and hence rapid approach for quantifying the composition of a solution comprising an equilibrium mixture of a univalent antigen, bivalent antibody, and complexes thereof [1–7]. Basically, the procedure entails assessment of the extent of complex formation in such mixtures from the decrease in antibody uptake by an affinity matrix bearing a high concentration of immobilized antigen. However, whereas the difference between the consequent fluorescence response (R_{Ag}) and that for the same total concentration of antibody in the absence of antigen (R_o) has been taken to reflect the concentration of antibody sites occupied by antigen [1–7], it actually reflects the concentration of antibody sites present as the fully saturated complex $AbAg_2$ [8]. This leads to a situation where the KinExA results for equilibrium mixtures need to be analyzed according to an expression in which $[1-(R_{Ag}/R_o)]^{1/2}$ describes the rectangular hyperbolic dependence of response on free antigen concentration [8,9].

A similar problem pervades use of the KinExA approach to determine the association rate constant for an antigen–antibody interaction from time dependence of the decrease in R_{Ag}/R_o [7,10]. This investigation explores the changes required to convert the current invalid kinetic expression based on antibody univalence into one that takes into account the bivalence of antibody (immunoglobulin G, IgG) in its interaction with immobilized antigen on the affinity matrix.

Theoretical considerations

In the determination of an association rate constant by KinExA, there are two time courses to consider. First, there is the time course of antibody site occupancy by antigen in the reaction mixture with defined total antigen and antibody concentrations ($[Ag]_{tot}$ and $[Ab]_{tot}$, respectively). Second, there is the time course of KinExA response, which monitors the decrease in antibody uptake from the mixture by immobilized antigen (Ag^*) on the affinity matrix. Previous determinations of rate constants by KinExA have entailed the assumption that the ratio of fluorometric responses reflecting matrix-bound antibody for the same total antibody concentration in the presence and absence of antigen reflects the fraction of unoccupied antibody sites in the reaction mixture [7,10]. However, this assumption is untenable because the two 1:1 Ab–Ag complexes still retain affinity for immobilized antigen; only the Ag–Ab–Ag complex exhibits no affinity for the affinity matrix [8]. The two events are considered in turn.

Time course of complex formation in reaction mixture

The time course of antigen uptake by antibody in the reacting solution can be described by a 1:1 interaction between antigen

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[1] *Abbreviation used:* KinExA, kinetic exclusion assay.

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(presumed univalent) and antibody sites, with the relevant differential rate equation being

$$d[Ab'Ag]/dt = k_a[Ag]_t([Ab']_{tot} - [Ab'Ag]_t) - k_d[Ab'Ag]_t, \quad (1)$$

where $[Ab']_{tot}$ denotes the total antibody site concentration (= $2[Ab]_{tot}$), $[Ab'Ag]_t$ denotes the concentration of complexed antibody sites ([–Ab–Ag] plus [Ag–Ab–]) at time t, and k_a and k_d are the respective rate constants for complex formation and dissociation. For illustrative purposes, it suffices to consider a situation in which the concentration of antigen in the mixture greatly exceeds that of antibody sites ($[Ag]_{tot} >> [Ab']_{tot}$)—a simplification that allows the replacement of $[Ag]_t$ in Eq. (1) by $[Ag]_{tot}$ for all t. Eq. (1) then becomes a pseudo-first-order kinetic expression,

$$d[Ab'Ag]/dt = k_a[Ag]_{tot}([Ab']_{tot} - [Ab'Ag]_t) - k_d[Ab'Ag]_t, \quad (2)$$

which is readily integrated. In the current context, it is convenient to use the following integrated form of the expression for fractional saturation of antibody sites, $F_s = [Ab'Ag]_t/[Ab']_{tot}$,

$$F_s = (k_a[Ag]_{tot}/k_{obs})[1 - \exp(-k_{obs})t] \quad (3a)$$

$$k_{obs} = k_a[Ag]_{tot} + k_d, \quad (3b)$$

which is the counterpart of that introduced by O'Shannessy and coworkers [11] for analysis of the adsorption stage of Biacore sensorgrams. The corresponding expression for the fraction of unoccupied antibody sites, therefore, becomes

$$(1 - F_s) = 1 - (k_a[Ag]_{tot}/k_{obs})[1 - \exp(-k_{obs}t)] \quad (4)$$

Time course of corresponding KinExA response ratio

As noted above, the current assumption [7,10] that $(R_{Ag})_t/R_o$ can be substituted for $(1 - F_s)$ in Eq. (4) is clearly incorrect because the experimental parameter being monitored is the time dependence of $[Ag–Ab–Ag]_t$ instead of $[Ab'Ag]_t$. From reacted site probability theory [12–14], the concentration of $AbAg_2$ is P_{Ab}^2 $[Ab]_{tot}$, where P_{Ab} is the probability that an antibody site is occupied by an antigen molecule. On the basis that the fluorometric response ratio is thus monitoring $(1 - F_s^2)$, the KinExA equivalent of Eq. (4) becomes

$$[(R_{Ag})_t/R_o] = 1 - \{(k_a[Ag]_{tot}/k_{obs})[1 - \exp(-k_{obs}t)]\}^2 \quad (5)$$

Although Singer [15] introduced the concept of reacted site probability theory into immunochemistry nearly 50 years ago, immunologists still seem reluctant to accept its validity. Confirmation of the inference that $[1 - (R_{Ag})_t/R_o]^{1/2}$ is the experimental counterpart of the fraction of unoccupied antibody sites, $[Ab']_t/[Ab']_{tot}$, in the reaction mixture has thus been provided by the following illustrative example of its application.

Relative contribution of $AbAg_2$ to fractional antibody saturation

To illustrate the reacted site probability approach, the proportion of antigen bound as $AbAg_2$ is first determined for each successive antigen attachment in the pathway to saturation of all antibody molecules in the system. For simplicity, it is assumed that $k_a[Ag]_{tot} \gg k_d$ to allow the disregard of complex dissociation (Eq. (3b)).

Consider initially a system with only five bivalent acceptor (antibody) molecules A. For the first attachment of a univalent ligand (antigen) molecule S, there are clearly 10 possible ways of forming a 1:1 complex AS, all of which are equally probable (first line of Table 1). The first opportunity for AS_2 formation occurs during attachment of the second ligand molecule. However, such formation of S–A–S is restricted to the attachment of ligand to only 1 of the 9 unoccupied acceptor sites; the alternative distribution with S attached to sites on 2 different acceptor molecules is thus 8 times more likely (Table 1). For 20% acceptor site saturation (2 sites occupied), the probability of AS_2 formation is thus 1/9 (i.e., 0.111); this probability, denoted as $P(2,2)$ in Table 1, follows a general terminology $P(i,j)$, where i refers to the total number of ligand molecules bound and j refers to the number of molecules bound as AS_2.

During the third ligand attachment, there are two more opportunities for AS_2 formation by its location on either of the 2 AS molecules in the lower distribution for second ligand attachment. Values of $P(3,j)$ for the three possible ligand distributions are listed in the penultimate column of Table 1. This availability of $P(i,j)$ values for each possible ligand distribution then allows the determination of the fraction of bound antigen present as AS_2, $F_s(AS_2)$, from the expression

$$F_s(AS_2) = \sum (j/i)P(i,j) \quad (6)$$

which has been used to calculate the value of this parameter listed in the final column of Table 1.

The results of similar considerations for the subsequent steps to saturation of the 5 acceptor molecules (10 sites) are summarized in the remaining lines of Table 1. On the grounds that those values of $F_s(AS_2)$ refer specifically to the system with 5 acceptor molecules, the whole exercise has been repeated for systems with 10, 15, 20, and 40 molecules of A in order to ascertain the likely variation of this parameter with number of acceptor sites. That variation in $F_s(AS_2)$ for 20, 40, 60, and 80% site saturation is shown in Fig.1, where the abscissa is expressed as the reciprocal of the number of acceptor sites to facilitate extrapolation to the ordinate intercept; the value in the limit of an infinite number of acceptor molecules should be more applicable to the experimental situation in KinExA studies, where the antibody concentration (typically 20–60 pM) requires the consideration of systems with 10^{13} to 10^{14} as the order of magnitude for the number of acceptor molecules. Although the extent of that extrapolation is clearly underemphasized in Fig. 1, the results conform with the conclusion that the fraction of ligand (antigen) bound as AS_2 is numerically equal to F_s, the fractional saturation of acceptor (antibody) sites.

As foreshadowed above, the inference from Fig. 1 that $F_s(AS_2)$ is a measure of F_s^2 is merely an illustrative manifestation of reacted site probability considerations [12–15] that has been included to provide reassurance that the ratio of fluorometric responses in a kinetic experiment is, indeed, monitoring the square of the fractional saturation of antibody sites—the condition incorporated into Eq. (6).

Experimental ramifications

The consequences of the above theoretical considerations are illustrated by employing Eq. (4) to calculate the time dependence of fractional site saturation at 10-min intervals for a mixture with $[Ab]_{tot}$ = 10 pM, $[Ag]_{tot}$ = 500 pM, $k_a = 3 \times 10^6$ M^{-1} s^{-1}, and $k_d = 1.5 \times 10^{-4}$ s^{-1} (an equilibrium dissociation constant K_d of 50 pM). In that regard, the restriction of $[Ab]_{tot}$ (which does not appear in the calculation) to 10 pM (20-pM sites) ensures a decrease of less than 4% in $[Ag]_t$ and thus provides reasonable support for the approximation inherent in the substitution of $[Ag]_{tot}$ for $[Ag]_t$ that renders the system amenable to pseudo-first-order kinetic analysis. Delineation of the time course for the decrease in the fraction of unoccupied antibody sites, $(1 - F_s) = [Ab']_t/[Ab']_{tot}$ (shown as open symbols in Fig. 2), allows the calculation of F_s^2 and hence the corresponding decrease in $(R_{Ag})_t/R_o$ (closed symbols in Fig. 2). The current practice [7,10] of regarding $(R_{Ag})_t/R_o$ as $(1 - F_s)$ rather than $(1 - F_s^2)$ clearly leads to underestimation of k_{obs}, or of k_a if $k_a[Ag]_{tot} \gg k_d$, as well as overestimation of K_d from the time-indepen-