

Review

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Immunoaffinity capillary electrophoresis: Determination of binding constant and stoichiometry for antibody-antigen interaction

Affinity capillary electrophoresis (ACE) can provide both qualitative and quantitative information on molecular interactions and affords the advantages of very low sample consumption, high mass sensitivity, short analysis time, and the use of automated instrumentation. It has been applied clinically and biochemically to the determination of the binding constant and to the measurement of the binding stoichiometry for interactions between antibodies (Ab's) and antigens (Ag's) in free solution. In many situations, the Ag molecule has two or multiple binding sites, each of which has a similar or different intrinsic affinity for binding independently to the combining site(s) on an Ab molecule. The multivalent binding reactions between Ab and Ag molecules often occur. The objective of this review is to describe the uses of ACE in the determination of binding constants and stoichiometry of Ab-Ag interactions (immunoaffinity capillary electrophoresis), focusing especially on multivalent Ab-Ag interaction modes. Five model binding systems developed recently using ACE techniques are described with principles and examples: (i) divalent mAb-monovalent Ag interaction, (ii) divalent mAb-(homo)polyvalent Ag interaction, (iii) cooperativity of two binding sites of mAb-monovalent Ag interaction, (iv) monovalent Fab-divalent Ag interaction, and (v) polyclonal Ab-monovalent Ag interaction. Finally, the determination of binding stoichiometry of Ab-Ag interactions by ACE is described.

Keywords: Affinity capillary electrophoresis / Antibodies / Antigens / Binding constant / Review
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Abbreviations: ACE, affinity capillary electrophoresis; dAg, divalent antigen; DNP, *N*-(2,4-dinitrophenyl)amino; Fab, antigen-binding fragment; hpAg, homopolyvalent antigen; HRV, human rhinovirus; IgG, immunoglobulin G; mAb, monoclonal antibody

1 Introduction

The basic principles of Ab-Ag interaction are those of any biomolecular reaction. The binding of Ag by Ab can, in general, be described by the same theories and studied by the same experimental approaches as the binding of a hormone by its receptor, of a substrate by enzyme, or of oxygen by hemoglobin. There are several major differences, however, between Ab-Ag interactions and these

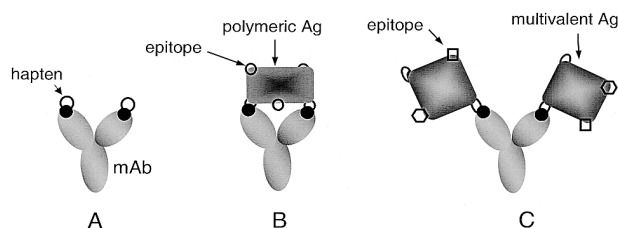


Figure 1. Schematic diagram depicting the interaction of mAb with haptens or Ag's. (A) mAb binding to two separate homologous haptens. (B) Bivalent binding of mAb to polymeric Ag. (C) mAb binding to multivalent Ags.

other situations. First, unlike most enzymes and many hormone-binding systems, Ab-Ag interactions are non-covalent interactions and therefore reversible. Second, antibodies can be raised, by design of the investigator, with specificity for almost any substance known. In each case, one can find Ab's with affinities as high as and specificities as great as those of enzymes for their substrates and receptors for their hormones. The interaction of Ab with Ag can thus be taken as a prototype for interactions of macromolecules with ligands in general.

The quantitative aspects of the interaction of Ab and Ag can be studied by the use of a variety of methods which utilize either thermodynamic or kinetic approaches. At equilibrium, the binding constant or affinity of Ab-Ag reaction may be expressed as: $K = [AbAg]/[Ab][Ag]$, where Ab represents free antibody sites, Ag the free antigen and AbAg the antibody-antigen complex. It should thus be possible to study the interaction kinetics of this reaction by conventional techniques involving addition of reactants, mixing and the subsequent determination of the concentration of the reactants or products at time intervals thereafter. Unfortunately, this simple type of approach is not suitable for the study of the reaction between Ab and complex multivalent Ag. One monoclonal antibody (mAb) molecule, immunoglobulin G (IgG), has two identical com-

binning sites and can combine with two separate homologous hapten molecules (Fig. 1A), with two identical antigenic determinants (epitopes, the regions of an Ag that bind to an Ab) on one polymeric Ag molecule (Fig. 1B), or with separate epitopes on two Ag molecules (Fig. 1C).

From the application of the law of mass action to the Ab-Ag reaction, a variety of methods have been developed which facilitate experimental calculations of binding constants for the interaction between divalent Ab and multivalent Ag. However, of the various existing methods for estimating the binding constant, most demand radioactive labeling (e.g., ammonium sulfate precipitation, radioimmunoassay) or chromophoric characteristics of the interacting compounds (e.g., fluorescence quenching, fluorescence enhancement), and these methods may require secondary reagents with an additional immobilization process (e.g., ELISA, immunosensor) for measurement at the liquid-solid interface. Capillary electrophoresis (CE) has recently become a tool for characterizing the molecular interactions in free solutions. This method not only affords the advantage of small capillary dimensions, leading to high mass sensitivity and reduced consumption of expensive affinity proteins or ligands, but also allows efficient separation and reproducible quantification by on-line detection of unlabeled components in the free solution rather than at the liquid-solid interface. The use of CE for the measurement of binding parameters for protein-ligand interactions has been reported [1–5], and several efforts have been made to use CE for observing Ab-Ag complex formation [6, 7], analyzing Ab's by affinity assays [8–12], or measuring the binding constant for slow Ab-Ag interaction [13–17].

Affinity capillary electrophoresis (ACE), a newly developed technique based on CE, has recently been introduced for the study of fast molecular interaction kinetics and the evaluation of binding constants for Ab-Ag interactions in free solution [18–26] (Table 1). In these reports, the

Table 1. Molecule interactions where equilibrium constants have been determined by mobility-shift analysis

Stoichiometry (Ag : Ab)	Interaction systems	Ref.
1 : 1	mAb against a negative charge ligand, phosphotyrosine	[18]
1 : 1	mAb specific to a double-stranded 32-mer oligonucleotide and a single-stranded oligonucleotide	[19]
1 : 2	mAb fragment specific to a divalent antigen, an 18-mer diphosphotyrosine peptide	[20]
1 : Poly	mAb against phosphitin, a phosphoprotein phosphorylated on the multiple-site serine residues	[21]
2 : 1	Charge ligands containing DNP groups to bivalent monoclonal rat anti-DNP	[22]
2 : 1 ^{a)}	Monoclonal anti-HSA and HSA	[24]
2 : 1 ^{a)}	Monoclonal anti-BSA and BSA	[25]
28 : 1 ^{a)}	A nonaggregating Ab (mAb 8F5) and HRV2	[26]
60 : 1 ^{a)}	Monovalent human very-low-density lipoprotein receptor encompassing repeats 2 and 3 and HRV2	[26]
Poly : 1	Polyclonal Ab specific to a hapten, DNP	[23]

a) Ag attaches to *n* equivalent epitopes with equal affinity.

simpler anti-(haptent)-haptent interaction can be sufficiently described by a 1:1 stoichiometry. However, the determination of the binding constant of anti-protein Ab is complicated by the multivalence (multiple epitopes) of the protein Ag. When the Ab is monoclonal, aggregates will not come up for a monomeric protein Ag. For homopolymeric protein Ag; however, aggregates can be formed depending on conditions. The problem of aggregation can be minimized by the use of a very low concentration of reactants or by performing the determination with an Ag excess [21]. In addition, in some situations, the Ag has two or multiple binding sites, each of which has a similar or different intrinsic affinity for binding independently to each of two identical combining sites on a Ab (IgG) molecule [20, 27, 28]. It is not always easy to determine each of the intrinsic affinity. The problem of the heterogeneity of affinity of multivalent Ag's still remains an obstacle to meaningful interpretation of data obtained. This review starts with the analysis of slow Ab-Ag interaction kinetics by CE and follows with an in-depth description about the quantitative expression and evaluation of binding constants for fast Ab-Ag interaction kinetics by ACE, with particular emphasis on the non-cooperativity and cooperativity of two binding sites on the divalent monoclonal Ab.

2 Slow Ab-Ag interaction kinetics

For slow on-and-off kinetics, *i.e.*, the half time of the AbAg complexes is long compared with the time for the electrophoresis, and the reactants are generally preincubated prior to CE analysis (Figs. 2A, B). To determine the binding constant for slow Ab-Ag interaction, one generally deter-

mines the equilibrium concentrations of bound and free Ag, at increasing total Ag concentrations, but at constant Ab concentration. In this case, CE is simply a tool to separate and quantitate bound and free species. The reactants of different ratios of Ab's and Ag's are generally preincubated followed by electrophoretic separation of free from complexed AbAg molecules. Peaks corresponding to free Ag in a series of incubation mixtures are integrated, and binding curves of bound Ag as a function of free or total offered Ag or the bound/free ratio as a function of bound Ag can be constructed subsequently on the basis of calibration curves of known amounts of Ag [13–17]. Alternatively, one can vary the Ab concentration, but then the analysis is slightly more complicated.

2.1 Data analysis

In the case of slow on-and-off Ab-Ag interaction kinetics, the following form of the Langmuir adsorption isotherm may be derived [29]:

$$b/[Ab]_o = r = (nK_b[Ag]) / (1 + K_b[Ag]) \quad (1)$$

where b is the concentration of bound Ag, $[Ab]_o$ is the total concentration of Ab, n is the number of binding sites of Ab (Ab valence), r is the total fraction of Ag (haptent) bound per mole of Ab ($0 \leq r \leq n$), $[Ag]$ is the free haptent concentration, and K_b is the equilibrium constant for a single binding site (Fig. 2C).

The following equation is known as the Langmuir equation:

$$1/r = 1/(nK_b[Ag]) + 1/n \quad (2)$$

Therefore, a plot of $(1/r)$ versus $1/[Ag]$ over a range of free haptent concentration allows values of antibody affinity (K_b) and antibody valence (n) to be derived (Fig. 2D). For divalent anti-haptent Ab ($n = 2$ for IgG) this equation can be used to calculate the average intrinsic equilibrium constant of the Ab for the haptent. When half the Ab binding sites are bound ($r = 1$), K_b is equal to the reciprocal of the free haptent concentration at equilibrium.

The advantage of applying this relationship to calculate the equilibrium constant is that the result is not affected by the changes in electroosmotic flow and electrophoretic mobilities of the free or bound antigens. However, a particular attention should be paid on the constant injection volume and reproducible peak area. In addition, another particular attention should be paid if the Ag is multivalent and can bind more than one Ab binding site; then Eq. (2) is valid only in Ag excess where the frequency of Ags with more than one Ab bound is very low. In this section, we are discussing only monovalent Ag's (*e.g.*, haptent) applied in CE, but this proviso must be kept in mind when the Scatchard or Langmuir analysis is applied in other CE circumstances.

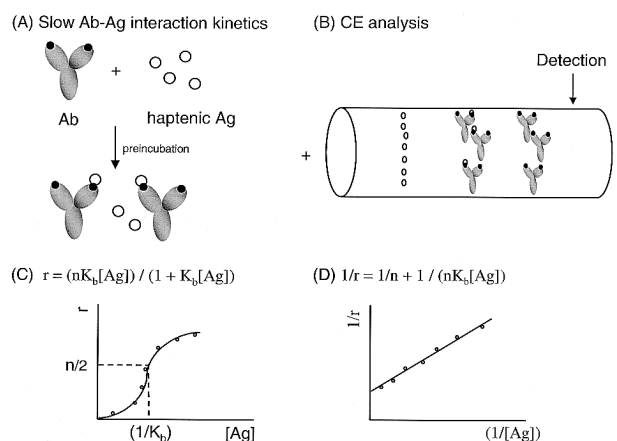


Figure 2. Principle of CE-based binding studies for slow interaction kinetics of divalent Ab with haptent Ag according to the peak area-change analysis. (A) Preincubation prior to CE analysis. (B) Schematic representation of CE running. (C), (D) Data analysis (for details, see section 2.1).

2.2 Examples

We reported the use of a monoclonal anti-phosphoserine Ab against a negatively charged hapten, phosphoserine, as a model system to demonstrate the feasibility for the determination of the high (slow off-rates) Ab affinity in the free solution based on the change in peak areas of detectable and quantitatively recoverable haptens [13]. The slow Ab-Ag interaction kinetics led to band broadening and resulted in far lower efficiency of the separation of complexed Ab from unbound Ab (Fig. 2). However, when the run-to-run reproducibility of free phosphoserine was examined, it was found that it can be recovered quantitatively under the conditions of the electrophoresis. On the basis of measurement of peak areas at different phosphoserine concentrations, the association constant was estimated ($K_b = 5.21 \times 10^5 \text{ M}^{-1}$). The method is generally useful for the study of monoclonal antibody-hapten interaction where the kinetics is slow and where the charge/mass ratio of the unbound Ag differs from that of the complexed molecule.

3 Fast Ab-Ag interaction kinetics

Fast Ab-Ag interactions kinetics commonly characterized by a relatively short lifetime of the Ab-Ag complexes can be analyzed by ACE [18, 30–37] of one component (Ab) in the presence of the other component (Ag) added to the background electrolyte. Since the ACE was introduced by Chu and Whitesides in 1992 [30], ACE has been widespread in many fields and used for the study of Ab-Ag interactions and the evaluation of binding constants in free solution [18, 19–26] (Table 1). The basic principle lies in the measurement of a changed electrophoretic mobility (migration shift) of the complex as compared to that of free species. When the on-and-off rates of Ab-Ag interactions are sufficiently fast compared with the time of the electrophoresis, all Ab molecules will participate in approximately the same number of complexes for the same amount of time, *i.e.*, there is a dynamic equilibrium between the complexed and noncomplexed species which have different electrophoretic velocities. The resulting migration time will therefore change without change in peak shape. Estimation of binding constants for Ab-Ag interactions on the basis of migration shifts is then feasible.

3.1 Divalent mAb-monovalent Ag interaction

Interactions between divalent mAb, IgG, and monovalent Ag can be either cooperative (Section 3.3) or noncooperative (Sections 3.1 and 3.2): the binding at one site may or may not influence binding at another site. If the

combining sites on the mAb are independent (*i.e.*, display no positive or negative cooperativity for Ag binding), then for many purposes one can treat these combining sites, reacting with monovalent ligands, as if they were separate molecules. Thus, many, but not all, of the properties we shall discuss in the following two sections (Sections 3.1 and 3.2) can be analyzed in terms of the degree of saturation of the binding sites of mAb, independent of the number of such sites per mAb molecule (2 for IgG). On the other way, the simplest case in mAb-Ag interactions is that of the interaction of mAb with monovalent ligand. One may include in this category both anti-hapten mAb's reacting with truly monovalent haptenic antigens, and anti-monomeric protein mAb's, which have been fractionated to obtain a population that reacts only with a single, nonrepeating site on the monomeric protein Ag. In the latter case, the Ag behaves as if monovalent in its interaction with the particular Ab population under study. The proviso that the site recognized (antigenic determinant) be nonrepeating, *i.e.*, occur only once per Ag molecule, is critical.

3.1.1 Data analysis

The binding constant is commonly calculated from the shift of the electrophoretic mobility of the free or bound Ab (Fig. 3). The electroosmotic flow (EOF) change can be compensated and, thus, it is not necessarily constant. However, several possible factors affecting EOF have been well addressed, including protein adsorption on the capillary wall, Joule heating, pH change, *etc.* [38]. To improve the accuracy, the injected sample generally con-

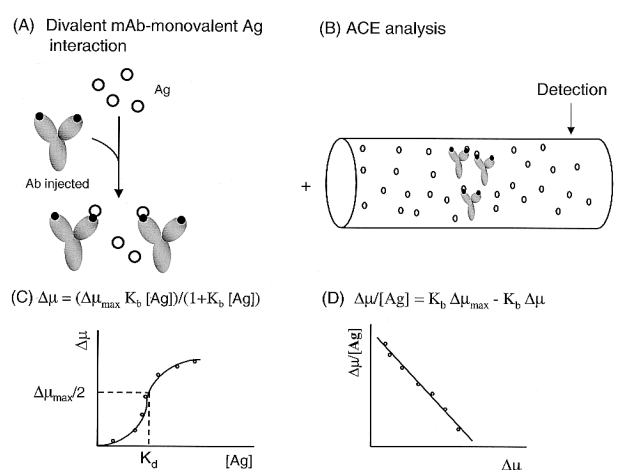


Figure 3. Principle of ACE-based binding studies for the interactions of divalent Ab's with monovalent Ag's according to the migration-shift analysis. (A) The separation buffer is filled with Ag prior to Ab injection. (B) Schematic representation of ACE running. (C), (D) Data analysis (for details, see Section 3.1.1).

tains a neutral internal standard. The correlated electrophoretic mobility ($\Delta\mu$) of the Ab can then be calculated from Eq. (3):

$$\Delta\mu = L_t L_d / V [(1/t_{Ab+Ag}) - (1/t_{N+Ag})] - [(1/t_{Ab}) - (1/t_N)] \quad (3)$$

where L_t and L_d is the total length and effective length of the capillary, respectively, V is the voltage across the capillary, t_{Ab+Ag} and t_{N+Ag} are the measured migration time of the antibody, and the neutral internal standard in the presence of antigen concentration, and t_{Ab} and t_N are the measured migration time of the antibody and the neutral internal standard in the absence of the Ag. Once the electrophoretic mobilities are corrected, a plot of $\Delta\mu/[Ag]$ versus $\Delta\mu$ is depicted, revealing a straight line with slope ($-K_b$) and intercept ($K_b\Delta\mu_{max}$) based on Eq. (4) [39]:

$$\Delta\mu/[Ag] = K_b\Delta\mu_{max} - K_b\Delta\mu \quad (4)$$

where $[Ag]$ is the concentration of Ag in the running buffer, $\Delta\mu_{max}$ is the mobility of the Ab when it is saturated with the Ag, and $\Delta\mu$ is the change in the electrophoretic mobility of the AbAg complex in the presence of various concentrations of the Ag.

3.1.2 Examples

Heegaard [18] used a mAb (anti-phosphotyrosine) against a negatively charge Ag (hapten), phosphotyrosine, as a model system to test the use of ACE for the study of Ab-Ag interaction. Monoclonal anti-phosphotyrosine Ab with sufficiently fast interaction kinetics to be amenable to binding constant determination by this migration-shift method was used. On the assumption that the combining sites on the Ab are independent and identical, micromolar dissociation constants were calculated from the measurement of migration shift in the presence of various concentrations of the hapten. They also showed similar method for determination of dissociation constants between monoclonal Ab and DNA by migration-shift immuno-ACE method [19]. By ACE it was shown that the binding of the monoclonal anti-DNA Ab to DNA is highly dependent on the ionic strength conditions and that the binding is pH-dependent with a decrease in the dissociation constant when decreasing the pH. The dissociation constant estimated for the Ab binding of a single-strand 32-mer oligonucleotide was $0.62 \mu\text{M}$ at pH 7.90.

3.2 Divalent Ab-(homo)polyvalent Ag interaction

So far, we have discussed only situations in which the Ag is monovalent, or effectively monovalent with respect to the particular mAb under study. However, in many situa-

tions the protein Ag has multiple repeating identical antigenic determinants (epitopes) [21], as in the case of homopolymers, each of which can bind independently to the identical combining sites on a divalent mAb (Fig. 1B). The determination of binding constant for anti-protein mAb will be complicated by the multivalence (multiple epitopes) of the Ag. Although the intrinsic affinity for the interaction of any single mAb combining site with any single epitope may be the same, the apparent or effective affinity may be much higher, due to the ability of a single mAb molecule to bind more than one identical epitope of an Ag molecule.

3.2.1 Data analysis

One can assume that the two combining sites of a divalent mAb, IgG, are equivalent and neither is affected by events at the other. A divalent anti-(homopolyvalent protein) mAb can therefore be regarded as a monovalent molecule. The determination of the binding constant of a monovalent mAb is complicated by the homopolyvalence (multiple identical epitopes) of the Ag; however, in Ag excess, the effect of the multiplicity of epitopes can be reduced (Fig. 4). Under these conditions, the likelihood of both mAb molecules binding to a single Ag molecule (Fig. 4C, Ab_2Ag_1) will be minimal. At the concentration of Ag around K_d , the presence of 1:1 complexes as Ab_1Ag_1' (Fig. 4A) or as a singly bonded species Ab_1Ag_1'' (Fig. 4B) can not be neglected. However, some conformational restraint of Ag may inhibit the divalent interaction in the complex Ab_1Ag_1' (Fig. 4A) but the complex Ab_1Ag_1''

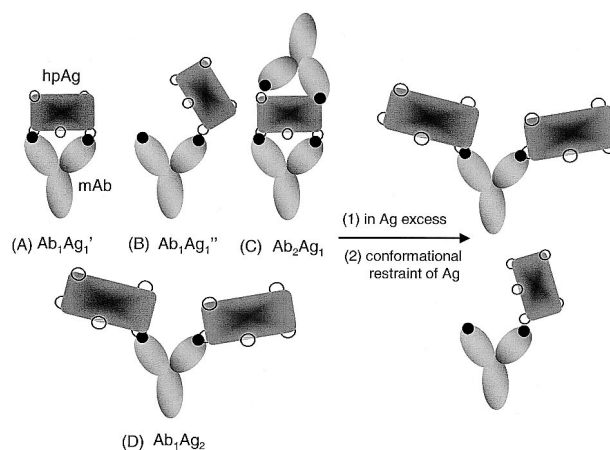


Figure 4. Scheme of the binding of divalent mAb to hpAg's. (A) One mAb molecule binding to two separate identical epitopes on an hpAg to form an Ab_1Ag_1' complex. (B) One mAb molecule binding to one epitope on an hpAg to form an Ab_1Ag_1'' complex. (C) One hpAg bound by two mAb's on separate epitopes (Ab_2Ag_1 complex). (D) One mAb molecule binding to separate epitopes on two hpAg's (Ab_1Ag_2 complex).

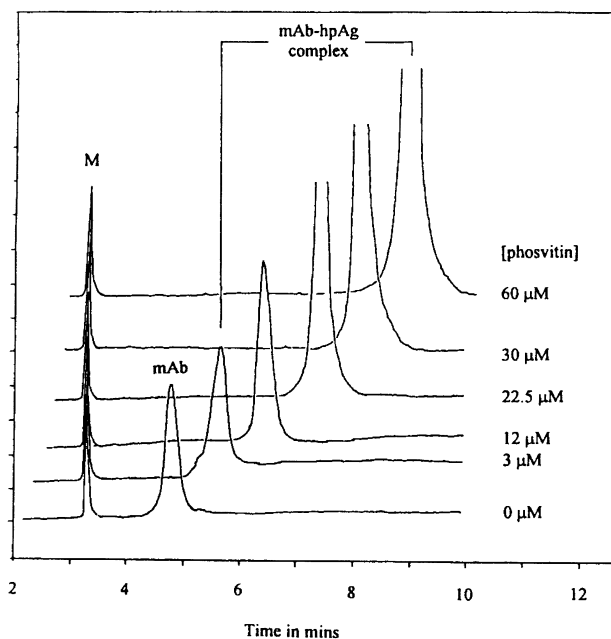


Figure 5. Evaluation of phosvitin binding to monoclonal anti-phosphoserine antibody by ACE. Peaks: M, internal peptide marker; mAb, free monoclonal anti-phosphoserine antibody; mAb-hpAg complex, monoclonal anti-phosphoserine antibody complexed with homopolyvalent phosvitin antigen (hpAg). Buffer, 65 mM boric acid, 15 mM sodium tetraborate (pH 8.3) containing mAb with phosvitin concentrations ranging from 0 to 60 μM ; CE apparatus, Beckman P/ACE 5500; uncoated capillary, 57 cm (50 cm effective separation length) \times 75 μm ID; sample, mixture of mAb (0.3 μM) and peptide marker (0.33 mM) pressure-injected at 5 s; absorbance, UV 200 nm; temperature, 22°C; operating voltage, 15 kV. Reprinted from [21], with permission.

(Fig. 4B) linked by monovalent interaction should still exist. In that case, the situation is the same as that treated in Section 3.1 and Eq. (4) is valid for the analysis.

3.2.2 Examples

We used ACE to study the interaction of a divalent anti-phosphoserine mAb to a homopolyvalent antigen (hpAg), phosvitin [21]. A model system, which allows the measurement of the true dissociation constant in Ag excess based on measurement of migration shifts of mAb-hpAg complexes at different Ag concentrations in solution, was presented for the study of the interactions between an Ab and an Ag that have identical determinants. The Ab complexed with phosvitin moved much more slowly than the free Ab because phosvitin (pI 2.1) exhibits more negative charge than Ab (pI 6.8 ~ 7.6) at pH 8.3 buffer (Fig. 5). When a 1– to 40-fold concentration of Ag over Ab binding sites was conducted with this Ab, no linear plot was

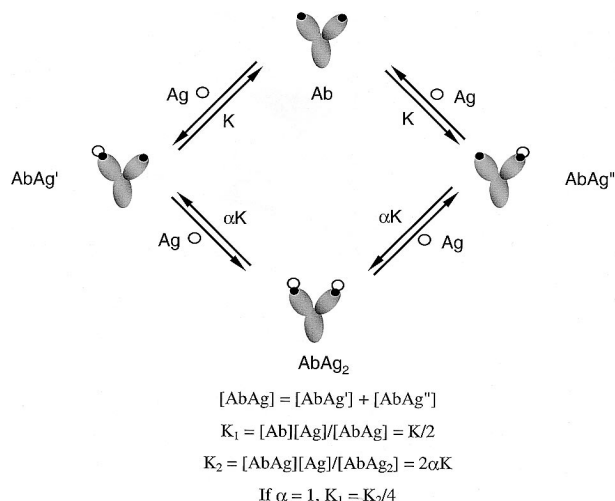


Figure 6. Equilibria involved in Ab-Ag interactions. K_1 is the dissociation constant between the single occupied Ab and the occupied Ab. K_2 is the dissociation constant between the double occupied Ab and the single occupied Ab. The degree of cooperativity between K_1 and K_2 is denoted as α . The relationship between K_1 , K_2 and K is based on the number of sites available for association and dissociation events. Adapted from [22].

observed. However, at large Ag excess (50– to 200-fold concentration excess), a linear plot was obtained. The experimental value of K_d (22.4 μM) obtained by ACE was also shown to be in close agreement with the value (17.8 μM) obtained by the conventional immunoassay based on indirect competition enzyme-linked immunosorbent assay.

3.3 Cooperativity of two binding sites of divalent mAb

There were several reports of positive cooperativity between the binding sites of Ab that bind monovalent Ag [40–42]. Cooperativity would yield a curved plot in Scatchard analysis, and values for two dissociation constants are not readily extracted from such a plot. Scatchard analysis, which was acceptable in a monovalent system by ACE as discussed in Sections 3.1 and 3.2, is thus not useful for a divalent system.

3.3.1 Data analysis

Mammen and co-workers [22] defined two dissociation constants, K_1 and K_2 , for the complex between divalent Ab (IgG) and two haptenic Ag's [22]. When intermediate concentration of the Ag is present, divalent Ab exist in three forms: unoccupied (Ab), singly occupied (AbAg), and doubly occupied (AbAg₂) (Fig. 6). In this system, they

assume that the dissociation rates (k_{off}) are sufficiently fast ($> 0.1 \text{ s}^{-1}$) that they observed a concentration-weighted average electrophoretic mobility for all three species, *i.e.*, one peak at all concentrations of Ag was detected. The electrophoretic mobility, μ^{electro} , is the weighted mobility of all three species.

$$\mu^{\text{electro}} = ([\text{Ab}]/C)\mu_{\text{Ab}} + ([\text{AbAg}]/C)\mu_{\text{AbAg}} + ([\text{AbAg}_2]/C)\mu_{\text{AbAg}_2} \quad (5)$$

$$C = [\text{Ab}] + [\text{AbAg}] + [\text{AbAg}_2] \quad (6)$$

When the two binding sites are identical and noncooperative, Scatchard analysis of values of μ^{electro} yield a straight line. In all other cases (positively or negatively cooperative binding), Scatchard plots are curved. As it is difficult to detect curves in a Scatchard plot, they derive a form analysis (Eq. 7) which yields both values of K_1 and K_2 .

$$\begin{aligned} & [\mu^{\text{electro}} - 0.5(\mu_{\text{AbAg}_2} + \mu_{\text{Ab}})[\text{Ag}]] / (\mu^{\text{electro}} - \mu_{\text{Ab}}) \\ &= [(\mu_{\text{AbAg}_2} - \mu^{\text{electro}})[\text{Ag}]^2 / (\mu^{\text{electro}} - \mu_{\text{Ab}})] \times 1/K_2 - K_1 \quad (7) \end{aligned}$$

Plotting the left-hand side of Eq. (7) against the coefficient of the term $1/K_2$ gives a line with a slope equal to $1/K_2$ and a *y*-intercept equal to $-K_1$.

3.3.2 Examples

Mammen and co-workers [22] chose charge ligands containing *N*-(2, 4-dinitrophenyl)amino (DNP) to divalent monoclonal rat anti-DNP, as a model for determination of the two dissociation constants of the AbAg complex. In this paper, 3-quinuclidinopropanesulfonate and K_2SO_4 were used to minimize the adsorption of the protein on the capillary wall. The value μ^{electro} of anti-DNP changed by increasing the concentration of the ligand. However, the value of μ^{electro} of two proteins standards, bovine carbonic anhydrase and α -lactalbumin, did not change significantly due to interactions with ions in the running buffer. They assume that the maximum value of $\Delta\mu^{\text{electro}}$ for the singly occupied Ab is half of that of the occupied one, *i.e.*, $(\mu_{\text{AbAg}} - \mu_{\text{Ab}}) = 0.5 (\mu_{\text{AbAg}_2} - \mu_{\text{Ab}})$. By using Eq. (7) to analyze the values of μ^{electro} of the Ab as a function of the concentration of ligand in the running buffer, they obtained values of both K_1 and K_2 and found that K_2/K_1 was ≈ 4 , and thus concluded that binding of Ab to ligands was noncooperative. This analysis method developed in this paper is more useful than Scatchard analysis for certain multivalent systems where cooperativity of binding is in question, but where it is also possible to make plausible assumptions about electrophoretic mobilities of protein and protein-ligand complexes. Thus, the absence of cooperativity can also be presented by a good fit of experimental data to Eq. (4), which is valid for the analysis of the interactions between bivalent binding proteins and haptenic ligand without cooperativity.

3.4 Monovalent Fab-divalent Ag interaction

In many situations [27, 28], the ligand molecule has two or multiple copies of epitope, each of which has a similar or different intrinsic affinity for binding independently of the only combining site on a receptor molecule. The multivalent binding reactions between two molecules often occur in the Ab-Ag interactions. The antigen-binding fragment (Fab) from its monoclonal IgG preparation is monovalent. Antigens can be di- or multivalent either because they contain two or multiple copies of the same epitope [43]. It is not always easy to determine each of the intrinsic affinities or to predict the effects of di- or multivalency either by the use of ACE [21] or immunochemical methods [44] or by the kinetic analysis with biosensors [45]. However, although Ag's show a heterogeneity of affinity, determination of the average affinity (the average value of these intrinsic affinities) at equilibrium is helpful for supplying information on understanding the quality of Ab molecules and the binding characteristics of Ab to di- or multivalent Ag in solution [46–48].

3.4.1 Data analysis

A monovalent Fab from its monoclonal IgG preparation has a binding site which can combine with any of two same epitopes on the divalent Ag (dAg) (Figs. 7A and B). Alternately, the two epitopes on an Ag molecule can react with the Fab molecule, respectively (Fig. 7C). However, at a high degree of Ag excess, the Fab_2Ag complex shown in Fig. 7C may not exist, based on statistical considera-

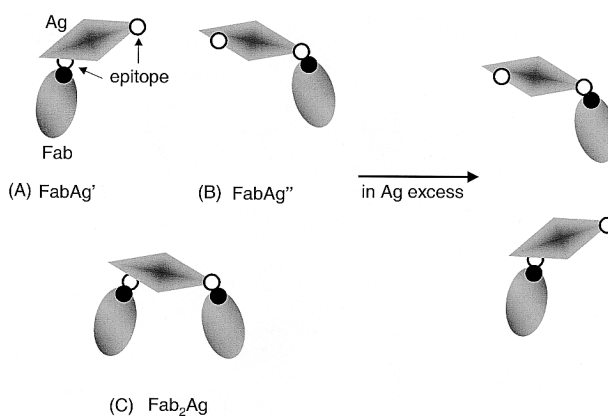


Figure 7. Schematic illustration showing the interactions of monovalent Fab molecule with its dAg which has two copies of epitopes. The Fab molecule can combine with one of two epitopes on the Ag. (A) One of two epitopes located on one Ag binding to the combining site of Fab to form FabAg' complex. (B) Another of two epitopes on the Ag binding to the combining site of Fab (FabAg'' complex). (C) Two separate Fabs combining with separate epitopes on one Ag.

tions [49–51]. At equilibrium, free Ag, free Fab, FabAg' complex, and FabAg'' complex will exist in the free solution. In Ag excess [20], Eq. [3] can be rearranged to give Eqs. (8) and (9):

$$\Delta\mu_1/\Delta\mu_1^{\max} = K_1[\text{Ag}]/(1+K_1[\text{Ag}]) \quad (8)$$

$$\Delta\mu_2/\Delta\mu_2^{\max} = K_2[\text{Ag}]/(1+K_2[\text{Ag}]) \quad (9)$$

Equation (8) plus Eq. (9) become

$$(\Delta\mu_1^{\max}/\Delta\mu_1) + (\Delta\mu_2^{\max}/\Delta\mu_2) = (K_{d1} + K_{d2})(1/[\text{Ag}]) + 2 \quad (10)$$

where K_{d1} and K_{d2} the reciprocals of the association constants K_1 and K_2 , respectively, are the dissociation constants of the Fab for both epitopes on the Ag molecule, respectively. It is proposed that the FabAg' complex has the same electrophoretic mobility (μ_1) as that of the FabAg'' complex (μ_2), *i.e.*,

$$\Delta\mu_1^{\max}/\Delta\mu_1 = \Delta\mu_2^{\max}/\Delta\mu_2 = \Delta\mu_m/\Delta\mu_c \quad (11)$$

where $\Delta\mu_c$ is the change in the electrophoretic mobility due to the presence of the concentration c of charged ligand (Ag) in the electrophoresis buffer, and $\Delta\mu_m$ is the value of $\Delta\mu_c$ at saturating concentrations of charged ligand Ag. Thus, Eq. (11) can be rewritten as

$$1/\Delta\mu_c = (1/\Delta\mu_m) + (1/\Delta\mu_m)(\overline{K_d})(1/[\text{Ag}]) \quad (12)$$

where $\overline{K_d}$, equals $(K_{d1} + K_{d2}) / 2$, is the arithmetical average value of the dissociation constants K_{d1} and K_{d2} . If the total amount of Ag available for binding is much higher than the amount of Fab, *i.e.*, if there is an Ag excess, then

$$1/\Delta\mu_c = (1/\Delta\mu_m) + (1/\Delta\mu_m)(\overline{K_d})(1/c) \quad (13)$$

where c is the total Ag concentration, and $\Delta\mu$ can be calculated from migration time.

3.4.2 Examples

We choose a Fab, prepared from its monoclonal anti-phosphotyrosine IgG, specific to a divalent antigen (dAg), an 18-mer diphosphotyrosine peptide, as a model to evaluate the potential of ACE for the determination of the average affinity [20]. The mobility-shift behavior of Fab in electrophoretic solution was observed (Fig. 8) and the quantitative expression was presented to estimate the arithmetical average value of the intrinsic affinities for two epitopes on the dAg with the Ag-binding site on the Fab. In dAg excess, based on measurement of mobility changes of Fab analyte at different dAg concentrations, the experimental average dissociation constant ($\overline{K_d} = 27.7 \mu\text{M}$) was calculated from Eq. (13) and found to be useful for estimat-

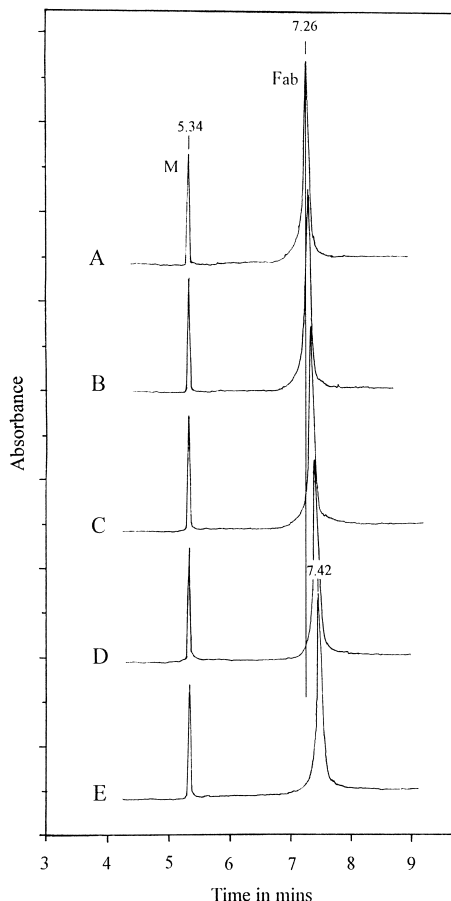


Figure 8. Interaction of divalent antigens (dAg's), 18-mer diphosphotyrosine peptides, with monovalent anti-phosphotyrosine Fab in ACE. CE apparatus, Beckman P/ACE 5500; uncoated capillary, 57 cm (50 cm effective separation length) \times 75 μm ID; sample, mixture (35 μL) of Fab (0.6 μM) in borate buffer, pH 8.2, and internal peptide marker (M) (0.1 mM) pressure-injected at 5 s followed by electrophoresis in the presence of (A) 0 M, (B) 18 μM , (C) 45 μM , (D) 90 μM , and (E) 120 μM dAg. Absorbance, UV 200 nm; temperature, 22°C; operating voltage, 15 kV. The vertical line indicates the migration time for the Fab in the control experiment (in the absence of dAg). Reprinted from [20], with permission.

ing the intrinsic affinity levels of the two different epitopes on the dAg. It was also found that the structural variation of the two epitopes for binding specificity to the Ag-binding domain of Fab is not apparent.

3.5 Polyclonal Ab-monovalent Ag interaction

The monoclonal Ab preparation only contains one epitope specificity. However, in many situations, the polyclonal Ab from immunoserum contains a heterogeneous population of Ab's of different affinity for the immunogen.

3.5.1 Data analysis

The next level of complexity arises when one is dealing with a mixture of Ab's of varying affinity. More often a Scatchard plot, based on the ratio of the bound fraction to the concentration of the free Ag at various concentrations against the concentration of the Ag, is established to evaluate the binding constant according to Eq. (14):

$$b = \frac{\sum \{n_i K_{bi} [Ab]\}}{1 + \sum K_{bi} [Ab]} \quad (14)$$

where b represents the total fraction of the Ag bound per Ab, n_i is the moles of binding site i per mole of the Ab, and K_{bi} is the binding constant for the Ag at the specific site. The Scatchard plot is linear for a homogeneous monoclonal Ab, but the Scatchard plot for heterogeneous polyclonal Ab is curved. Taking the limits as $[Ab] \rightarrow 0$ and $[Ab] \rightarrow \infty$, the intercept on the abscissa can be calculated as $\sum n_i$ and the intercept on the ordinate $\sum K_{bi} n_i$. Therefore, one can still obtain the total Ab site concentration from the intercept on the abscissa. The problem is in obtaining the different affinity, $\sum K_{bi}$, and the concentration of the individual Ab subpopulation, $\sum n_i$. It is difficult to determine the different intrinsic binding constants between polyclonal Ab and monovalent Ag by Scatchard plot.

3.5.2 Examples

We chose a polyclonal Ab specific to a hapten, DNP, as a model to evaluate the potential of ACE for detecting anti-DNP Ab subpopulation by studying the migration-shift pattern of complexes [23]. When 1- to 32-fold concentration of DNP over polyclonal Ab binding sites is conducted with this polyclonal anti-DNP Ab, the DNP-binding polyclonal Ab does not produce any apparent change in the peak shape or migration time. However, at large DNP excess (32- to 1024- fold concentration excess) the polyclonal Ab gradually splits in two subpopulations (Fig. 9). The minor peak was observed to migrate at a constant time with increasing amounts of DNP, while when the concentrations of DNP were increased continuously, the major subpopulation gradually shifted away from the minor peak. This observation can be attributed to the fact that DNP is anionic at pH 8.35 and the negative charges change the charge on the complex compared to the free Ab, resulting in a change in the migration time of the complex. We propose that the major and the minor peak, in fact, represent a subpopulation of higher-affinity Ab's and of low- or very-low-affinity Ab's, individually. A conclusion would be that very few or no higher-affinity Ab's exist in the preparation but that they are all in fact of low to moderate affinity.

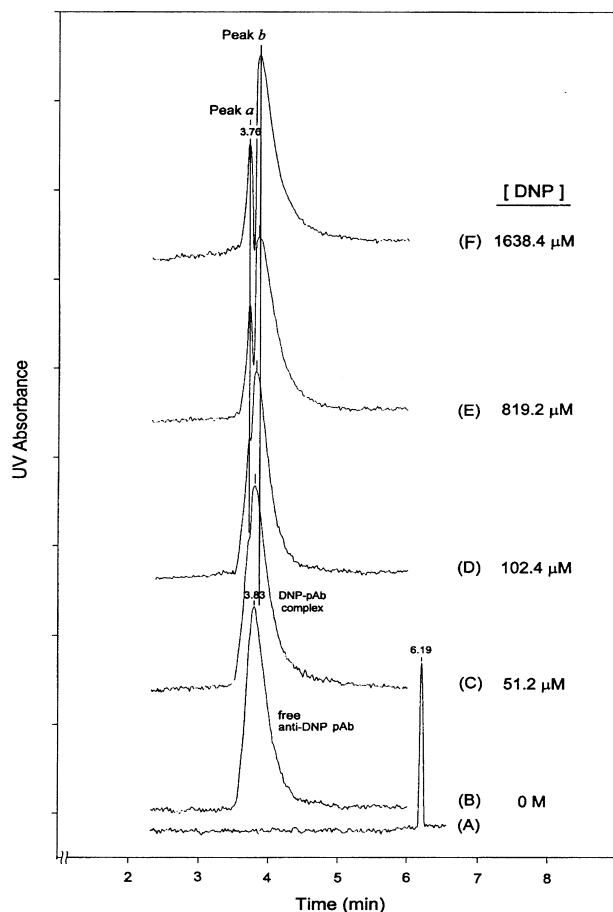


Figure 9. Electropherograms showing the binding migration-shift pattern of polyclonal anti-DNP antibody (pAb) to the DNP in ACE. CE apparatus, Beckman P/ACE System MDQ; uncoated capillary, 57 cm (50 cm effective separation length) \times 50 μ m ID; sample, mixture (35 μ L) of anti-DNP pAb (1.6 μ M maximum available binding sites) in 65 mM boric acid/15 mM sodium tetraborate buffer, pH 8.35, pressure-injected at 5 s followed by electrophoresis in the presence of (B) 0 M, (C) 51.2 μ M, (D) 102.4 μ M, (E) 819.2 μ M, and (F) 1638.4 μ M DNP. Absorbance, UV 200 nm; temperature, 22°C; operating voltage, 20 kV. In control experiments, the pAb has a migration time of approximately 3.83 min (B), whereas the DNP has a migration time of approximately 6.19 min (A) under the same conditions. Two vertical lines indicate the migration time for the peak a in (D)–(F) and peak b in (F), respectively. Reprinted from [23], with permission.

4 Determination of binding stoichiometry

ACE was primarily used to determine the binding constant in biomolecular recognition. In addition, ACE can be easily adapted to the determination of binding stoichiometry. Information obtained from binding stoichiometries allows one to measure the concentration of biologically active proteins and to probe specificity.

4.1 Data analysis

One can assume that all sites on the Ab are equivalent with respect to their interaction with the Ag. *i.e.*, Ag attaches to n equivalent paratopes with equal affinity. In the case of a high affinity system, for each mole of the Ab in the sample solution the addition of n moles of Ag results in the formation of exactly one mole of the complex. Sample containing a fixed Ab concentration and various Ag concentration are prepared and a capillary only contains the running buffer. When the concentration ratio $[Ag]_{total}/[Ab]$ increases, the concentration of the complex peaks increases until $[Ag]_{total}/[Ab] = n$. The Ab has been fully associated with Ag beyond the n value. An abrupt slope change in a plot of integrated free Ag *versus* the ratio of $[Ag]_{total}/[Ab] = n$ in samples correspond to the n value [24].

4.2 Examples

Chu and co-workers [24] reported the use of ACE as a method to determine binding stoichiometry between a monoclonal anti-human serum albumin (anti-HSA) and its Ag (HSA). The two complex peaks, which have different electrophoretic mobilities, are likely to represent the binding of one and two HSA molecules. The signal strength of two complex increases as the amount of HSA was increased. A plot of the area of normalized free HSA peaks *versus* the ratio of $[HSA]/[anti-HSA]$, with the concentration of anti-HSA held constant, shows a sharp break at a point corresponding to the binding stoichiometry 2 and rises steeply as a straight line. They show the same result for the determination of binding stoichiometry between a monoclonal anti-(bovine serum albumin) (anti-BSA) Ab and its Ag (BSA) [25]. The estimate for molecular masses of BSA, anti-BSA Ab and two complex peaks was 64, 147, 209 and 258 kDa, respectively, by using nondenaturing SDS capillary gel electrophoresis with UV absorbance.

Okun and co-workers [26] also reported on binding parameter determination for human rhinovirus serotype 2 (HRV2)-binding Ab or soluble receptor Fab's using ACE [26]. The stoichiometry largely exceeds that of molecules analyzed so far with a similar method and can theoretically be within the range of 1:12 and 1:60 ($[virus]/[Ab]$) with 12, 24, 30, and 60 symmetry-related equivalent sites present on an icosahedral surface. Binding of these compounds to their recognition sites on the viral surface is assumed to occur independently of the other symmetry-related equivalent sites (noncooperative binding). Plots based on different numerical values for the dissociation constant and the initial concentrations of the reactants resulted in curves of characteristic shapes, which depend on the relation between the dissociation constant and the analyte concentration. For a high-affinity system, the

curve approximates a decaying straight line and the binding stoichiometry can be accurately determined from the intercept with the x -axis. For intermediate-affinity systems, the binding stoichiometry can also be derived, however, with less accuracy dictated by the affinity and the initial concentrations of the analytes. For the low-affinity case, obviously no stoichiometry can be derived. The stoichiometry for the complex of a nonaggregating Ab (mAb 8F5) and of monovalent human very-low-density lipoprotein receptor encompassing repeats 2 and 3 with HRV2 was 1:28 and 1:60, respectively.

5 Concluding remarks

Essentially, the monoclonal Ab preparation employed in these immuno-ACE studies, at least in theory, contains a single, homogeneous population of Ab molecules specific to an epitope. However, in many situations the Ab preparation assayed contains a heterogeneous population of Ab's (polyclonal Ab's) of different affinity for the immunogen. Observation of hapten-specific Ab subpopulations requires the ability to detect and discriminate between the hapten-anti-hapten complex and the free species. Immuno-ACE has now been investigated for quite some time. The experimental method by immuno-ACE has been reported to observe Ab affinity distributions in Ab-Ag reactions and to assess the heterogeneity of affinity [23]. However, there is to date no proof of concept that immuno-ACE can be performed in estimating the intrinsic affinities or defining exactly how many subpopulations of different affinity exist in a polyclonal Ab. Nevertheless, it will be interesting to follow over the coming years how ACE will advance in this field of immunobiotechnology.

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