Review

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Affinity capillary electrophoresis: A physical-organic tool for studying interactions in biomolecular recognition

Affinity capillary electrophoresis (ACE) is a technique that is used to measure the binding affinity of receptors to neutral and charged ligands. ACE experiments are based on differences in the values of electrophoretic mobility of free and bound receptor. Scatchard analysis of the fraction of bound receptor, at equilibrium, as a function of the concentration of free ligand yields the dissociation constant of the receptor-ligand complex. ACE experiments are most conveniently performed on fused silica capillaries using a negatively charged receptor (molecular mass < 50 kDa) and increasing concentrations of a low molecular weight, charged ligand in the running buffer. ACE experiments that involve high molecular weight receptors may require the use of running buffers containing zwitterionic additives to prevent the receptors from adsorbing appreciably to the wall of the capillary. This review emphasizes ACE experiments performed with two model systems: bovine carbonic anhydrase II (BCA II) with arylsulfonamide ligands and vancomycin (Van), a glycopeptide antibiotic, with D-Ala-D-Ala (DADA)-based peptidyl ligands. Dissociation constants determined from ACE experiments performed with charged receptors and ligands can often be rationalized using electrostatic arguments. The combination of differently charged derivatives of proteins – protein charge ladders – and ACE is a physical-organic tool that is used to investigate electrostatic effects. Variations of ACE experiments have been used to estimate the charge of Van and of proteins in solution, and to determine the effect of the association of Van to Ac_2KDADA on the value of pK_a of its *N*-terminal amino group.

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Abbreviations: **DADA,** D-Ala-D-Ala; **ACE,** affinity capillary electrophoresis; **BCA II,** bovine carbonic anhydrase II; **L,** ligand; **R,** receptor; **R-L,** receptor-ligand complex; **Van,** vancomycin

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1 Introduction

Affinity capillary electrophoresis (ACE) is a useful technique for measuring binding constants of receptors for ligands in aqueous solutions, and may be used as a physical-organic tool for estimating the physical interactions that determine these binding constants. The technique uses the resolving power of capillary electrophoresis (CE) to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand. Scatchard analysis of the fraction of bound receptor at equilibrium is generally used to estimate values of dissociation constants of receptor-ligand complexes. In this review, we describe applications of ACE, with particular emphasis to two model binding systems:

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(i) bovine carbonic anhydrase and derivatives of benzenesulfonamide and (ii) vancomycin and D-Ala-D-Ala (DADA) based peptidyl ligands. In general, ACE experiments can be rationally designed for a receptor-ligand system if the charge, mass, and valency of the receptor and its ligand are clearly defined.

1.1 Principles of ACE

The value of electrophoretic mobility, $\mu_{electro}$ of an analyte is related directly to its charge, as measured by CE, Z_{CE}, and inversely correlated to M^{α}/C_P, its coefficient of friction (Eq. 1) [1–3]. The term C_P is a proportionality constant, M is the molecular weight of the protein, and α is a constant related to the shape of the protein under the conditions of its analysis.

$$\mu_{electro} = C_P \frac{Z_{CE}}{M^{\alpha}} \tag{1}$$

The value of $\mu_{electro}$ of an analyte is expressed as its steady-state velocity, v_x, relative to that of a neutral marker, v_{nm}, per unit electric field, and is calculated using Eq. (2). In this equation, L_{tot} (m) is the total length of the capillary, L_{det} (m) is the length of the capillary from the inlet to the detector, V (V) is the voltage applied across the capillary, and t_x (s) and t_{nm} (s) are the times of migration of an analyte peak and neutral marker, respectively.

$$\mu_{electro} = \frac{(v_x - v_{nm})}{\frac{V}{L_{tot}}} = \frac{\left(\frac{L_{det}}{t_x} - \frac{L_{det}}{t_{nm}}\right)}{\frac{V}{L_{tot}}} \tag{2}$$

The dissociation constant of a receptor (R) for a ligand (L), K_d, can be measured using ACE only if the value of $\mu_{electro}$ of a receptor-ligand complex (R·L), $\mu^{R\cdot L}_{electro}$, differs significantly from that of R, $\mu^{R}_{electro}$. According to Eq. (1), there are three limiting situations where this condition will be met: (i) the bound ligand contributes significantly to the charge of the complex but not to its coefficient of friction (the binding of small, charged ligands); (ii) the bound ligand contributes significantly to the coefficient of friction of the complex, but not to its charge (the binding of large, neutral ligands), or (iii) the bound ligand contributes significantly to both the charge and coefficient of friction of the complex (the binding of large, charged ligands). We have focused on the first two cases in designing our ACE experiments.

The value of K_d is defined in terms of the equilibrium concentrations of R, L, and R·L by Eq. (3). We define R_f as the fraction of the total concentration of R ($[R]_T$) present as R·L (Eq. 4). If the value of [R·L] is expressed in terms of K_d, [R], and [L], then Eq. (4) can be rearranged to Eq. (5), the general form of the equation used in Scatchard analysis.

$$K_d = \frac{[R][L]}{[R\cdot L]} \tag{3}$$

$$R_f = \frac{[R\cdot L]}{[R] + [R\cdot L]} = \frac{[R\cdot L]}{[R]_T} \tag{4}$$

$$\frac{R_f}{[L]} = \frac{1}{K_d} - \frac{R_f}{K_d} \tag{5}$$

For a mixture of rapidly equilibrating R and R·L, a single peak is observed by CE for any given concentration of L in the running buffer. The value of $\mu_{electro}$ of this peak is the concentration-weighted average of the values of $\mu^{R}_{electro}$ and $\mu^{R\cdot L}_{electro}$, $\mu^{avg}_{electro}$ (Eq. 6). Equation (6) can be rearranged to give an expression for R_f (Eq. 7). Scatchard analysis of the

$$\mu^{avg}_{electro} = R_f \mu^{R\cdot L}_{electro} + (1 - R_f)\mu^{R}_{electro} \tag{6}$$

$$R_f = \frac{\Delta\mu^{avg}_{electro}}{\Delta\mu^{avg,\,max}_{electro}} = \left(\frac{\mu^{avg}_{electro} - \mu^{R}_{electro}}{\mu^{R\cdot L}_{electro} - \mu^{R}_{electro}}\right) \tag{7}$$

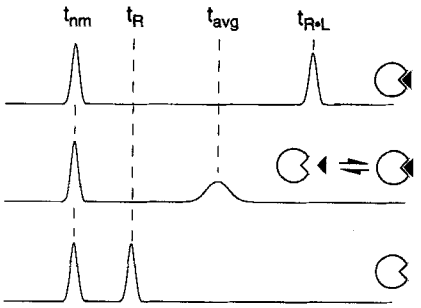
values of R_f as a function of the concentration of L in the running buffer, yields the value of K_d. Figure 1 depicts a general ACE experiment for a rapidly equilibrating system of R and R·L in which differences in values of $\mu^{R}_{electro}$ and $\mu^{R\cdot L}_{electro}$ are due to differences in the corresponding values of charge of R and R·L. When the time for a dissociation event of R·L is much longer than the time of analysis of a mixture of R and R·L, then separate peaks are observed by CE for R and R·L. Values of R_f and [L] must then be determined by analyzing equilibrated mixtures of R and L by CE. All of the examples that we describe in this review are of rapidly equilibrating systems.

For Scatchard analysis based on Eqs. (5)–(7), we make the following assumptions: (i) the concentration of R is much lower than that of the L; (ii) R and R·L are in equilibrium; (iii) the binding stoichiometry between R and L is 1:1; (iv) the interaction of R and L with the wall of the capillary does not influence the binding of R to L, and (v) the electric field does not alter the measured binding constants.

1.2 Technical issues

1.2.1 Compensating for changes in electroosmotic flow

Calculating the values of $\mu^{avg}_{electro}$ corrrects for changes in electroosmotic flow (EOF) that may result with increasing concentrations of L in the running buffer [4]. Since the value of $\mu_{electro}$ of an analyte is inversely correlated with its time of migration (Eq. 2), the spacing between two peaks along the *x*-axis, in an electropherogram plotted as absorbance *vs.* −1/time, is directly proportional to the difference in their corresponding values of $\mu_{electro}$ [5]. By aligning manually the peaks corresponding to neutral marker in stacked electropherograms plotted as a function of −1/time, we correct for changes in EOF among the different runs. Plots in the 1/time domain reveal whether the values of $\mu_{electro}$ of the protein standards – used to detect nonspecific associations with ions – change appreciably with increasing concentration of L in the running buffer, and also indicate accurately



Mass	Charge	R_f	$\mu^x_{electro}$
M + m	$Z_{CE} + z_{CE}$	1	$\mu^{R•L}_{electro} \sim \frac{C_P(Z_{CE}+z_{CE})}{M^\alpha}$
		$\frac{\Delta\mu^{avg}_{electro}}{\Delta\mu^{avg,\,max}_{electro}}$	$\mu^{avg}_{electro} = R_f\mu^{R•L}_{electro} + (1-R_f)\mu^{R}_{electro}$
M	Z_{CE}	0	$\mu^{R}_{electro} \sim \frac{C_P Z_{CE}}{M^\alpha}$

$$\mu^x_{electro} = \frac{L_{det}L_{tot}}{V}\left(\frac{1}{t_x} - \frac{1}{t_{nm}}\right) \quad x = R,\ avg,\ R•L$$

$$\Delta\mu^{avg}_{electro} = \mu^{avg}_{electro} - \mu^{R}_{electro}$$

$$\Delta\mu^{avg,\,max}_{electro} = \mu^{R•L}_{electro} - \mu^{R}_{electro}$$

Figure 1. An example of an ACE experiment involving a charged receptor (R), and a low molecular weight, charged ligand (L). The charge of the receptor-ligand complex (R·L) differs from that of R by the value of charge of bound L (z_{CE}); the bound L does not contribute significantly to the coefficient of friction of the complex. Since R and R·L are rapidly equilibrating, a single peak is seen by ACE for any concentration of L in the running buffer. The value of $\mu_{electro}$ of this peak, $\mu^{avg}_{electro}$, is the concentration-weighted average of the value of $\mu_{electro}$ of R and that of R·L. Peak broadening is observed when the time for a single dissociation event of R·L is comparable to the time of migration of the average peak observed by CE.

when a receptor is completely saturated with ligand. This information may not be determined clearly from plots in the time domain. Plots in the 1/time domain are useful for following the binding isotherms of several receptor peaks simultaneously. Changes in EOF with increasing concentration of ligand could result in the misassignment of these peaks in electropherograms plotted as a function of time.

1.2.2 Buffer additives

Proteins that have high values of molecular mass (> 100 kDa) tend to adsorb to the surface of negatively charged capillaries. To overcome this problem we have used organic zwitterions as described by Jorgenson and others [6–8]. Since these zwitterions must be used in high concentration (typically 0.5 M) to be effective, it is possible that they may influence protein-ligand interactions. We have not, however, investigated such effects.

1.2.3 ACE with positively charged proteins

The majority of proteins whose binding constants we have determined by ACE have values of p*I* lower than 8.4, the pH of the running buffer that we typically use in these experiments. These proteins have a net negative charge at pH 8.4 and interact relatively weakly with the negatively charged wall of an uncoated silica capillary. We have not studied extensively proteins that have values of p*I* > 8.4 (*i.e.*, proteins with net positive charge) at pH 8.4 by ACE. These proteins can be examined using running buffers having higher values of pH; many proteins may, however, denature at elevated values of pH. We have found that a better strategy is to use capillaries coated with Polybrene, polyethylenimine, or other positively charged polymers [9]. These polymers adhere electrostatically to the surface of the inner wall of the capillary and make the surface positively charged. ACE experiments involving positively charged proteins are then performed in reverse polarity, using buffers having biologically compatible values of pH. The problem with working with a mixture of positively and negatively charged proteins is not one that we have addressed. Some components of this system will adsorb on either positively or negatively charged capillaries; neutral, coated capillaries may provide a solution to adsorption, but at the cost of increasing the time required for analysis.

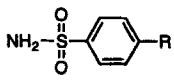
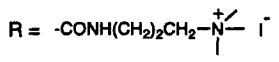
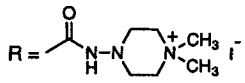
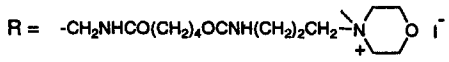
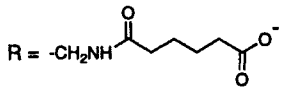
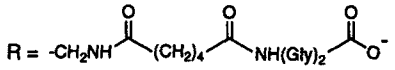
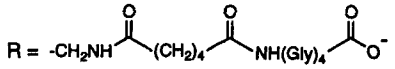
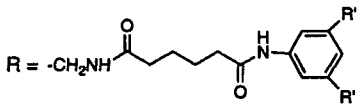
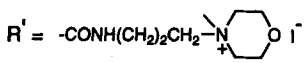
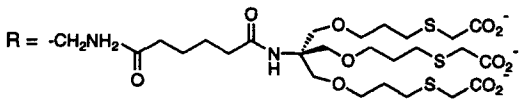
2 Model systems for ACE

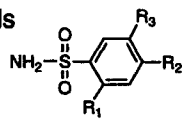
Most of our development of ACE has come from the study of two model systems: bovine carbonic anhydrase II (BCA II; EC 4.2.1.1, from bovine erythrocytes) and benzenesulfonamide ligands, and vancomycin (Van) and DADA peptidyl ligands. BCA II is a commercially available, negatively charged protein that does not adsorb significantly to the walls of fused silica capillaries. Its active site has been clearly defined from X-ray crystal structures of homologous human proteins [10, 11]. In addition, charged benzenesulfonamide inhibitors to BCA II are easy to synthesize: these inhibitors have values of K_d between 10^{-5} and 10^{-9} M (Table 1). We chose Van as a model receptor because of the large amount of information regarding its binding pocket and the specificity with which it binds to peptidyl ligands [12].

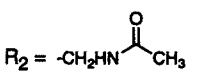
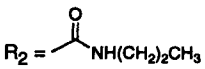
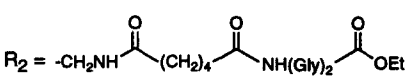
2.1 Determination of the binding affinities of BCA II to charged and neutral ligands

ACE has been used to measure directly the values of K_d of BCA II to charged benzenesulfonamide inhibitors. The ACE experiments for these systems are based on the difference in the value of Z_{CE} of a complex of BCA II with a small, charged ligand, and that of free BCA II.

Table 1. Dissociation constants (K_d) of BCA II to benzenesulfonamide ligands determined by ACE

		K_d (μM) [Reference]
Charged Ligands		
1	R = 	0.9 [4]
2	R = 	0.5 [4] 0.6 [69]
3	R = 	0.8 [4]
4	R = $-CO_2^-$	1.2 [4]
5	R = 	1.7 [4] 2.2 [69]
6	R = 	1.6 [69]
7	R = 	1.2 [69]
	R = 	
8	R' = $-CO_2^-$	2.2 [unpublished]
9	R' = 	0.5 [4]
10	R = 	6.7 [34]

Neutral Ligands 

	R_1	R_2	R_3	
11	H	H	H	0.9 [34]
12	H	CH_3	H	0.5 [34]
13	Cl	Cl	Cl	0.2 [34]
14	H	NO_2	H	0.1 [34]
15	R_2 = 		R_1, R_3 = H	0.7 [69]
16	R_2 = 		R_1, R_3 = H	0.1 [69]
17	R_2 = 		R_1, R_3 = H	1.4 [69]

Scatchard analysis of the change in the value of $\mu_{electro}^{avg}$ of BCA II as a function of the concentration of the charged ligand in the running buffer gives the value of K_d for that ligand (Eqs. 5–7). As expected, the values of $\mu_{electro}$ of complexes of BCA II to small, neutral ligands are not appreciably different from the value of free BCA II. To measure the value of K_d of BCA II to a neutral ligand (L_o), K_d^o, we perform a competitive ACE assay; we measure the value of $\mu_{electro}^{avg}$ of BCA II in the presence of a fixed concentration of a charged ligand ($L_\pm$), of known K_d, and increasing concentrations of L_o in the running buffer [4]. Since the value of $\mu_{electro}$ of the complex of BCA II with a neutral ligand, $\mu_{electro}^{R\cdot L_o}$, and the value of $\mu_{electro}^{R}$ are approximately the same, the value of $\mu_{electro}^{avg}$ that is measured in this system is expressed as the concentration-weighted average of the value of $\mu_{electro}$ of the complex of BCA II with a charged ligand ($L_\pm$), $\mu_{electro}^{R\cdot L_\pm}$, and the value of $\mu_{electro}^{R\cdot L_o}$ (Eq. 8). The value of R_f for this competitive binding system is expressed by Eq. (9). Rearrangement of Eq. (9) leads to Eq. (10), the equation used for Scatchard analysis of this competitive binding system, where $K_d^\pm$ is the known value of K_d of the charged ligand, and $[L_\pm]$ is its fixed concentration. Scatchard analysis of the value of R_f measured by ACE (Eq. 11) as a function of the concentration of L_o in the running buffer leads to the value of K_d^o. Table 1 summarizes the values of K_d of BCA II for different charged and neutral sulfonamide ligands obtained by direct and competitive methods, respectively. An example

$$\mu_{electro}^{avg} = R_f\mu_{electro}^{R\cdot L_o} + (1 - R_f)\mu_{electro}^{R\cdot L_\pm} \quad (8)$$

$$R_f = \frac{[R\cdot L_o]}{[R] + [R\cdot L_o] + [R\cdot L_\pm]} = \frac{[R\cdot L_o]}{[R]_T} \quad (9)$$

$$\frac{R_f}{[L_o]} = \frac{1 - R_f}{K_d^o\left(1 + \frac{[L_\pm]}{K_d^\pm}\right)} \quad (10)$$

$$R_f = \frac{\Delta\mu_{electro}^{avg}}{\Delta\mu_{electro}^{avg,\,max}} = \left(\frac{\mu_{electro}^{avg} - \mu_{electro}^{R\cdot L_\pm}}{\mu_{electro}^{R\cdot L_o} - \mu_{electro}^{R\cdot L_\pm}}\right) \quad (11)$$

of the direct method is shown in Fig. 2, where we simultaneously measure the affinities of several isozymes of CA (from human and bovine erythrocytes) to 5 (Table 1) [13].

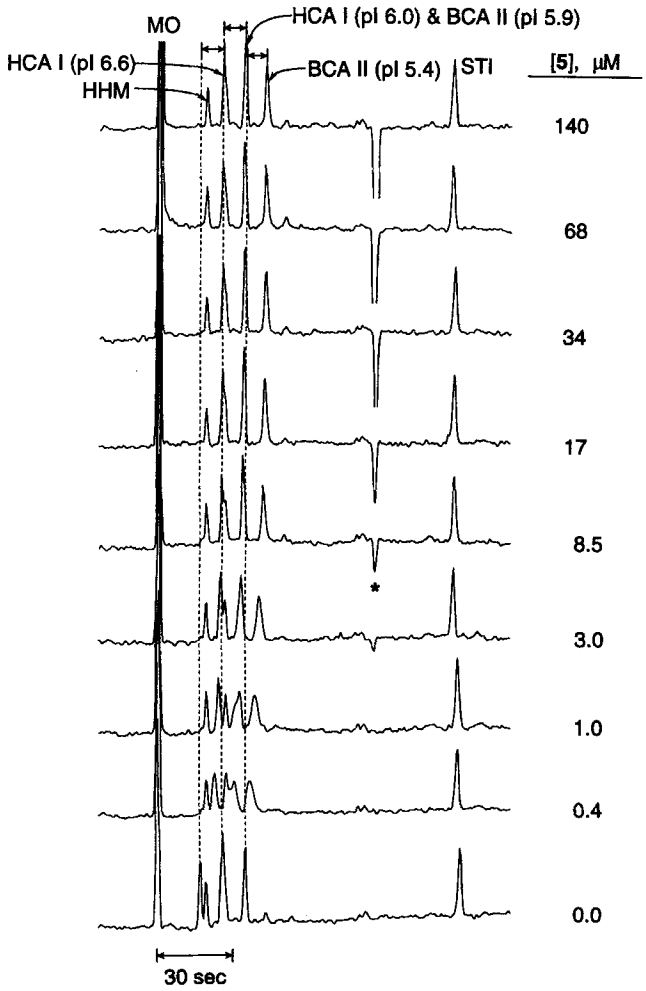


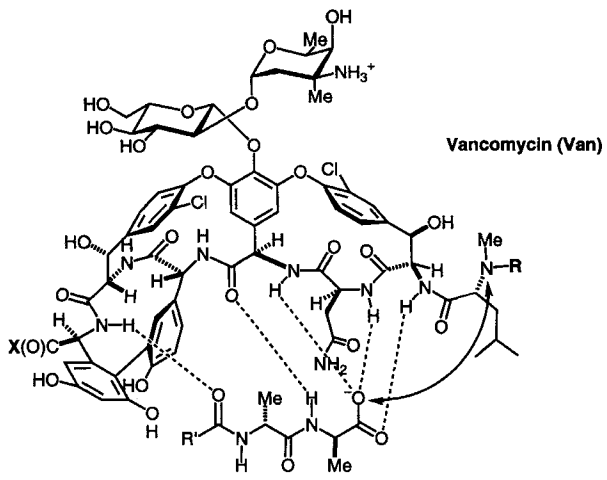
Figure 2. A set of ACE experiments of a mixture of isozymes of carbonic anhydrase: HCA I (human carbonic anhydrase, p*I* 6.6); HCA I (p*I* 6.0); BCA II (bovine carbonic anhydrase, p*I* 5.9), and BCA II (p*I* 5.4) for ligand 5 (Table 1). The electrophoresis buffer used was 25 mM Tris-192 mM Gly, pH 8.3. Mesityl oxide (MO) is used as an electrically neutral marker; horse heart myoglobin (HHM) and soybean trypsin inhibitor (STI) are protein standards. The inverted peak (*) is due to the lower amount of ligand 5 in the migrating plugs relative to the running buffer due to its binding to the isozymes of carbonic anhydrase. Reprinted from [13], with permission.

2.2 Determination of the binding affinities of Van to DADA peptidyl ligands

The binding of Van to DADA is one of the most extensively studied interactions in biomolecular recognition (Scheme 1) [14–16]. We have estimated values of K_d of Van for different peptidyl ligands by two different methods: the R_L and L_R methods (Fig. 3) [14]. The R_L method is the same as the direct method described in the previous section; in this method, we monitor the value of $\mu_{electro}^{avg}$ of Van by CE as we increase the concentration of a charged peptidyl ligand (L) in the running buffer. This method relies on the difference in the values of Z_{CE} of Van and Van·L at pH 7.1, the pH of running buffer used in the ACE experiments. In the L_R method, the value of $\mu_{electro}^{avg}$ of a ligand is followed as the concentration of Van in the running buffer is increased. This method is based on the difference in the coefficient of friction of Van and Van·L when the pH of running buffer is equal to 7.5, the pH at which Van is electrically neutral. We use a variation of this technique to screen libraries of ligands for tight-binding inhibitors, as described later.

2.3 Estimating the contribution of electrostatics to the binding of DADA to Van

Using the R_L method, we studied the effect of modification of the *C*- and *N*-termini of Van on its affinity for ligands containing the DADA group (Table 2) [17]. We coupled the *C*-terminus of Van with Asp and 1-propylamine to form the two receptors: AspNHCOVan and C_3H_7-



	X	R
Van	O^-	H_2^+
VanN(CH$_3$)Ac	O^-	$C(O)CH_3$
C_3H_7NHCOVan	$CH_3(CH_2)_2NH$	H_2^+
VanN(CH$_3$)Suc	O^-	$C(O)CH_2CH_2CO_2^-$
AspNHCOVan	$-O_2C$, NH, H, CO_2^-	H_2^+

Scheme 1. The interactions observed between Van and a bound DADA peptidyl ligand. The dashed lines indicate intermolecular hydrogen bonds; the double-headed arrow represents the electrostatic interaction between the carboxylate on the ligand and the *N*-terminal $-NH^+_2CH_3$ group on Van. Reprinted from [65] with permission.

NHCOVan, respectively. We concluded from the values of K_d of these two receptors for the ligand AcDADA that the charge or detailed structure at the *C*-terminus of Van does not contribute significantly to the binding of DADA ligands (Table 2). We acylated the *N*-terminus of Van using anhydrides of acetic and succinic acid to form the two derivatives VanN(CH$_3$)Ac and VanN(CH$_3$)Suc, respectively. We found that the K_d of VanN(CH$_3$)Ac for Ac$_2$KDADA at pH 7.1 was 11-fold higher than that of Van, while the corresponding value for VanN(CH$_3$)Suc was an additional factor of two higher. Decreasing the pH of the running buffer lowered the K_d of VanN(CH$_3$)Suc for Ac$_2$KDADA, while that for VanN(CH$_3$)Ac remained virtually unchanged. These results suggest that the charge on the *N*-terminal amino group of Van is important in the binding of DADA ligands to Van. When this charge is neutralized, the affinity for ligands decreases significantly. When a negative charge is placed in close proximity to the *N*-terminus of Van, the affinity is further decreased; the value of this K_d can be decreased as the pH is lowered (that is, as the pendant carboxylate group on the *N*-terminus of Van is protonated). These

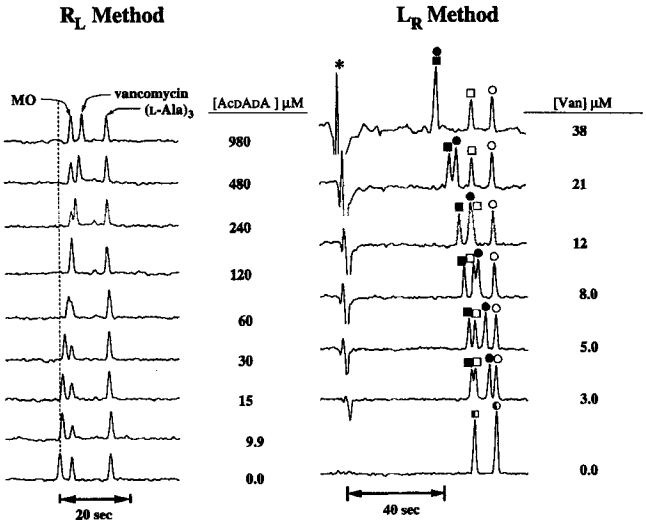


Figure 3. Method R_L: ACE of Van in 10 mM phosphate buffer, pH 7.1, containing increasing concentrations of AcDADA. The electrically neutral marker, mesityl oxide (MO), and the tripeptide L-Ala-L-Ala-L-Ala were used as internal standards. Method L_R: ACE of *N*-Fmoc-Gly-D-Ala-D-Ala, (●), *N*-Fmoc-Gly-D-Ala-D-Ala-D-Ala, (■), *N*-Fmoc-Gly-L-Ala-L-Ala, (○) and *N*-Fmoc-Gly-L-Ala-L-Ala-L-Ala, (□) in 20 mM phosphate buffer, pH 7.5, containing different concentrations of Van. The asterisk (*) indicates an unidentified neutral species carried through the capillary by EOF. Reprinted from [14], with permission.

results demonstrate the power of ACE in elucidating the contribution of electrostatics to the binding of ligands to receptors.

2.4 Screening of a peptide library

We have demonstrated that ACE can be used to screen small libraries of peptide ligands for Van [21]. The principle of the screening process is illustrated in Fig. 4. The value of $\mu^{avg}_{electro}$ of a reference ligand (L_{ref}) measured by the L_R method reflects its partitioning between free and bound states. When other ligands are introduced into the running buffer that compete for Van, the value of $\mu^{avg}_{electro}$ of L_{ref} will be closer to the value of $\mu_{electro}$ of unbound L_{ref}. This technique was used to identify a tight binding ligand for Van from a mixture of 32 equimolar ligands [21]. The reference ligand chosen for the studies has a value of K_d that is in the range of values targeted by the screening procedure. The number of compounds being screened is a function of the targeted K_d. The tighter the targeted binding, the larger the number of compounds that can be screened. We have found that up to 10% of the volume of the running buffer can comprise these peptidyl ligands for Van without changing significantly the dielectric strength and viscosity of the running buffer. Other receptor-ligand systems that we have studied in our group include: calmodulin (bovine testes) and Ca^{2+}; glucose-6-phosphate dehydrogenase and $NADP^+$ [22]; and SH3 domain and peptides [23]. Table 3 lists some of the binding systems studied by ACE.

3 ACE studies involving divalent receptors

Many biological systems interact multivalently, that is, through multiple associations of two or more receptors

Table 2. Dissociation constants of Van and its derivatives to peptidyl ligands determined by ACE

Receptor	Ligand	K_d, μM (pH) R_L method	L_R method	Literature
Van	AcDAdA	99 (7.1)[a]	208 (7.5)[b]	63 (7.0)[c]
		115 (7.1)[d]		91 (8.3)[e]
		192 (8.4)[f]		
Van	Ac_2KDAdA	2.3 (5.2)[g]		0.7 (5.1)[h,i]
		4.3 (7.1)[a]		21 (5.1)[j]
				1 (7.0)[b]
Van	Succinyl-DAdA	108 (7.1)[d]		
Van	Fmoc-Gly-DAdA	33 (7.1)[d]	53 (7.5)[b]	
Van	Fmoc-Gly-DADADA		71 (7.5)[b]	
AspNHCOVan	AcDAdA	95 (7.1)[a]		
		167 (8.4)[f]		
C_3H_7NHCOVan	AcDAdA	76 (7.1)[a]		
		107 (8.4)[f]		
VanN(Me)Ac	Ac_2KDAdA	42 (4.7)[g]		13 (5.1)[h]
		42 (5.2)[g]		
		48 (7.1)[a]		
VanN(Me)Suc	Ac_2KDAdA	64 (4.7)[g]		
		76 (5.3)[g]		
		105 (6.9)[a]		

a) ACE binding assay in 20 mM sodium phosphate buffer [17]
b) ACE binding assay in 20 mM sodium phosphate buffer [14]
c) Fluorescence binding assay in 100 mM phosphate buffer [16]
d) ACE binding assay in 10 mM sodium phosphate buffer [14]
e) CE binding assay in 50 mM Tris HCl [18]
f) ACE binding assay in 25 mM Tris – 192 mM Gly buffer [17]
g) ACE binding assay in 18 mM sodium acetate buffer [17]
h) UV difference binding assay in 20 mM sodium citrate buffer [19]
i) UV difference binding assay in 20 mM citrate [15]
j) UV difference binding assay in 20 mM sodium citrate [20]

and/or ligands [64]. We used ACE to study two systems: (i) a divalent receptor for a monovalent ligand, where we quantified the cooperativity of binding in the system, and (ii) a divalent receptor for a divalent ligand, where we quantified the increase in binding affinity, relative to the monovalent system.

3.1 Determination of the binding affinity of anti-DNP rat IgG_{2b} for ligands containing the *N*-2,4 dinitrophenyl group

We determined the binding affinity of the two binding sites of the antibody, anti-2,4-DNP rat IgG_{2b}, for *N*-2,4 dinitrophenyl (DNP) ligands using ACE. The high molecular mass of the divalent antibody (150 kDa), required that we design our ACE experiment accordingly. We used 3-quinuclidinopropanesulfonate and K_2SO_4 to minimize the adsorption of the protein to the walls of the capillary. Since the peak due to IgG was broad by CE we performed ACE experiments with a DNP ligand that had a net charge of –9 so that we could more easily discern changes in values $\mu_{electro}^{avg}$ of the antibody with increasing concentrations of ligand in the running buffer (Fig. 5A). Figure 5B shows a series of stacked electropherograms plotted as a function of –1/time. Stacking the electropherograms over the neutral marker, *p*-methoxybenzyl alcohol, compensated for changes in EOF with increasing concentration of ligand in the running buffer, and showed that the values of $\mu_{electro}$ of two protein standards, BCA (p*I* 5.9) and α-lactalbumin, did not change significantly due to interactions with ions in the running buffer.

The equilibria involved in the association of the DNP ligands with IgG are shown in Fig. 5C; K_1 and K_2 are the dissociation constants for the singly and doubly occupied protein, respectively. The relationship between K_1, K_2 and K is based on the number of sites available for association and dissociation events. The value of $\mu_{electro}^{avg}$ of IgG measured in ACE experiments is the concentration-weighted average of the values of $\mu_{electro}$ of three species: the free protein, Ig; the singly occupied protein, IgL; and the doubly occupied protein, IgL_2 (Eqs. 12, 13).

$$\mu_{electro}^{avg} = \frac{[Ig]}{[Ig_o]}\mu_{electro}^{Ig} + \frac{[IgL]}{[Ig_o]}\mu_{electro}^{IgL} + \frac{[IgL_2]}{[Ig_o]}\mu_{electro}^{IgL_2} \quad (12)$$

$$[Ig_o] = [Ig] + [IgL] + [IgL_2] \quad (13)$$

If the two binding sites are identical and noncooperative, then Scatchard analysis of values of $\mu_{electro}^{avg}$ obtained with different concentrations of ligand in the running buffer will yield a straight line; a curved line would indicate that the binding is cooperative. Since small degrees of curvature are difficult to detect in Scatchard plots, we devised a method of analysis that would yield both values of K_1 and K_2. We derived Eq. (14), the equation used in

$$\frac{\mu_{electro}^{avg} - \frac{1}{2}(\mu_{electro}^{IgL_2} + \mu_{electro}^{Ig})}{\mu_{electro}^{avg} - \mu_{electro}^{Ig}}[L] = \left(\frac{\mu_{electro}^{IgL_2} - \mu_{electro}^{avg}}{\mu_{electro}^{avg} - \mu_{electro}^{Ig}}[L]^2\right)\frac{1}{K_2} - K_1 \quad (14)$$

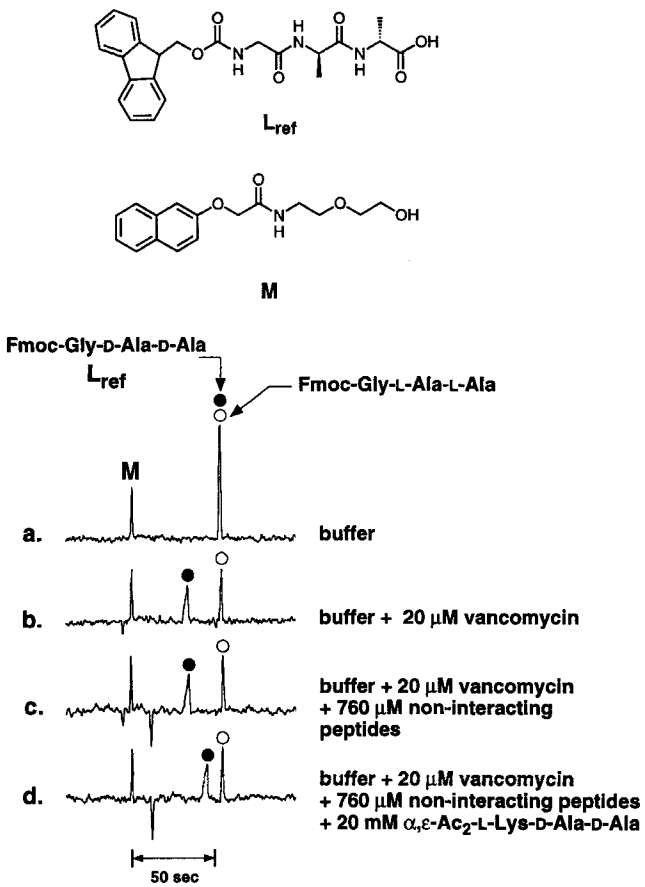


Figure 4. The concentration of Van in the electrophoresis buffer (20 mM phosphate, pH 7.4) affects the electrophoretic mobility of Fmoc-Gly-D-Ala-D-Ala (L_{ref}, •) but not of Fmoc-Gly-L-Ala-L-Ala (○). In going from (c) to (d), the change in mobility of Fmoc-Gly-D-Ala-D-Ala reflected the change, from 20 μM in (c) to ~ 10 μM in (d), in the concentration of free Van in the running buffer due to the presence of a competing ligand Ac_2KDADA in the buffer used in (d). The neutral marker M was used as an internal standard. The negative peak evident in (c) and (d) resulted from the dilution of L-Ala-L-Trp present in the electrophoresis buffer at the point of injection. Reprinted from [21] with permission.

this analysis, starting from Eqs. (12, 13), the expressions for K_1 and K_2 shown in Fig. 5C, and the assumption that the value of $\Delta\mu_{electro}^{avg,\,max}$ for the singly occupied IgG is half of that of the doubly occupied one, *i.e.*, $(\mu_{electro}^{IgL} - \mu_{electro}^{Ig}) = {}^1/_2\,(\mu_{electro}^{IgL_2} - \mu_{electro}^{Ig})$. By using Eq. (14) to analyze the values of $\mu_{electro}^{avg}$ of the antibody as a function of the concentration of L in the running buffer, we obtained values of both K_1 and K_2, found that K_2/K_1 was $\cong$ 4, and thus, established that the binding was noncooperative. This type of analysis should be useful for determining the cooperativity involved in other biological binding systems.

3.2 Determination of the binding affinity of a dimeric derivative of Van to dimeric KDADA

We developed a competitive binding assay using ACE to determine the value of K_d of a dimeric derivative of Van, Van-R_d-Van, to a dimeric form of KDADA, L–R'_d-L (Fig. 6; "d" in these structures indicates "dimer") [65]. In this assay we measured the values of $\mu_{electro}^{avg}$ of Van-R_d-Van with running buffers that contained increasing concentrations of L-R'_dL and 1 mM of a monomeric derivative of KDADA (*P*), a concentration of *P* that saturated both binding sites of Van-R_d-Van. The value of $\mu_{electro}^{avg}$ of Van-R_d-Van is the concentration-weighted average of the values of $\mu_{electro}$ of three species: P·Van-R_d-Van·P, P·Van-R_d-Van·L-R'_d-L, and $\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}$. We define ΔZ_{seq} as the calculated difference in the charge of R·L, $Z_{seq}^{R\cdot L}$, and that of R, Z_{seq}^{o} (Eq. 15). Since the values of ΔZ_{seq} and molecular weight of P·Van-R_d-Van·P and

$$\Delta Z_{seq} = Z_{seq}^{R\cdot L} - Z_{seq}^{o} \tag{15}$$

P·Van-R_d-Van·L-R'_d-L are similar (Fig. 6), the value of $\mu_{electro}^{avg}$ of Van-R_d-Van can be expressed by Eq. (16). We derived Eq. (19), the equation used in the analysis of this binding system, starting from Eqs. (17, 18) and the expressions for K_1 and K from Fig. 6.

$$\mu_{electro}^{avg} = R_f\mu_{electro}^{\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}} + (1 - R_f)\mu_{electro}^{\text{P·Van-}R_d\text{-Van·P}} \tag{16}$$

$$R_f = \frac{[\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}]}{[\text{Van-}R_d\text{-Van}]_T} \tag{17}$$

$$[\text{Van-}R_d\text{-Van}]_T = [\text{P·Van-}R_d\text{-Van·P}] + [\text{P·Van-}R_d\text{-Van·L-}R'_d\text{-L}] + [\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}] \tag{18}$$

$$\frac{R_f}{[\text{L-}R'_d\text{-L}]} = -\left(\frac{K_1}{[P]} + \frac{K}{[P]^2}\right)R_f + \frac{K}{[P]^2} \tag{19}$$

$$R_f = \left(\frac{\mu_{electro}^{avg} - \mu_{electro}^{\text{P·Van-}R_d\text{-Van·P}}}{\mu_{electro}^{avg,\,max} - \mu_{electro}^{\text{P·Van-}R_d\text{-Van·P}}}\right) \tag{20}$$

Analysis of the values of $\mu_{electro}^{avg}$ of Van-R_d-Van by using Eqs. (19, 20) gives the value of *K*, the dissociation constant of $\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}$ to P·Van-R_d-Van·P, to be 20 mM (Fig. 7). We calculated the value of K_d^c, the dissociation constant of $\overline{\text{Van-}R_d\text{-Van·L-}R'_d\text{-L}}$ to Van-R_d-Van to be 1.1 nM by substituting the calculated value of *K* and the value of K_d^m, the average value of K_{d1}^m and K_{d2}^m (4.8 μM), determined from a separate *UV* tritration experiment, into Eq. (21). The estimated value of K_d^c is equivalent

$$K = K_1K_2 = \frac{K_{d1}^mK_{d2}^m}{K_{d1}^cK_{d2}^c} = \frac{(K_d^m)^2}{K_d^c} \tag{21}$$

to a free energy of binding of −12.2 kcal/mol, 4.2 kcal/mol higher than the corresponding value for the binding of Van to KDADA [15]. Similar increases in the free energy of binding have been observed with cyclodextrin dimers and ditopic substrates [66].

Table 3. Studies on binding of ligands to receptors using ACE

	Example	Reference
(A)	Protein – protein interactions	
	Human serum albumin (HSA) and anti-HSA	[24]
	Human growth hormone (hGH) and anti-hGH (or its fragment)	[25]
	IgG with protein A	[26]
	Insulin dimerization	[27]
(B)	Protein – DNA interactions	
	*Eco*R1 and oligonucleotide; peptide and oligonucleotide	[28]
	Transcription factor-oligonucleotide	[29]
(C)	Protein – peptide interactions	
	SH3 domain and proline-rich peptides	[23]
	Antibody-antigen interaction	[30, 31]
	Hsc70 and deoxyspergualin	[32, 33]
(D)	Protein – drug interactions	
	Bovine carbonic anhydrases (CA) and arylsulfonamides; glucose-6-phosphate dehydrogenase (G6PDH) and $NADP^+$; G6PDH and NADPH; albumin and ibuprofen; IgG_{2b} and 2,4-DNP	[4, 22, 34–36]
	Bovine serum albumin (BSA), bacterial cellulase and tryptophan, benzoin, pindolol, promethazine, warfarin	[37]
	HSA and kynurenine, tryptophan, 3-indole lactic acid, 2,3-dibenzoyl-tartaric acid, 2,4-dinitrophenyl-glutamate	[38]
	Cellulase and β-blockers	[39]
	BSA and warfarin, leucovorin	[40, 41]
	Albumin-ofloxacin	[42]
(E)	Protein – metal ion interactions	
	Calmodulin, parvalbumin, thermolysin and Ca(II); carbonic anhydrase, thermolysin and Zn(II)	[43, 44]
	C-reactive protein and Ca(II)	[45]
	RNases, cytochrome c, chymotrypsin and kallikrein with iminodiacetate-Cu(II)	[46]
(F)	Protein – carbohydrate interactions	
	Concanavalin A-monosaccharides	[47]
	Lectin-carbohydrate	[48–50]
	Human lactoferrin and heparin	[51]
(G)	Glycopeptide – peptide interactions	
	Vancomycin and peptides	[14, 18, 52–55]
(H)	Peptide – carbohydrate interactions	
	Synthetic peptide and anionic carbohydrates	[56]
	Peptide fragments from amyloid P component and heparin	[57]
(I)	Peptide – dye interactions	
	Synthetic peptide and Congo Red	[58]
(J)	Carbohydrate – drug interactions	
	Methyl-β-cyclodextrin and propranolol	[59]
	β-cyclodextrin and salbutamol	[60]
(K)	Oligonucleotide – oligonucleotide interactions	
	$d(A)_n/d(T)_n$	[61–63]

4 Using ACE to estimate the charge on proteins and smaller molecules

4.1 Estimating the charge of BCA II

The charge on BCA II, at a given value of pH, can be estimated by measuring the values of $\mu_{electro}$ of complexes of BCA II with charged ligands differing in known values of charge (Fig. 8A) [67]. If we assume that the coefficient of friction of the protein does not change significantly upon association with each ligand then the value of $\Delta\mu_{electro}$, the difference in the value of $\mu_{electro}$ of a BCA II-ligand complex, $\mu^{R\cdot L}_{electro}$, and that of free BCA II, $\mu^{o}_{electro}$, is related to the value of ΔZ_{CE}, the difference in the values of charge of the protein-ligand complex, $Z^{R\cdot L}_{CE}$, and that of the free protein, Z^{o}_{CE} (Eq. 22). The value of ΔZ_{CE} can be estimated by the value of ΔZ_{seq}, the difference in the value of calculated charge of the complex, $Z^{R\cdot L}_{seq}$, and that of free protein, Z^{o}_{seq}, based on the sequence of the protein, and the structure of the ligand (Eq. 23).

$$\Delta\mu_{electro} = \left(\frac{C_P}{M^{\alpha}}\right)\Delta Z_{CE} \quad (22)$$

$$\Delta Z_{CE} \cong \Delta Z_{seq} = Z^{R\cdot L}_{seq} - Z^{o}_{seq} \quad (23)$$

We estimated the values of Z^{o}_{CE} of the two isozymes of BCA II of p*I* 5.9 and 5.4, in a buffer of 25 mM Tris-192 mM Gly, pH 8.3, to be –3.7 and –5.0 units of charge, respectively, by analyzing the values of $\mu_{electro}$ of the free isozymes and those of their complexes with ligands 2, 5, and 8 (Table 1) according to Eq. (24) (Fig. 8C) [67].

$$\Delta Z_{seq} \cong Z^{o}_{CE}\left(\frac{\Delta\mu_{electro}}{\mu^{o}_{electro}}\right) \quad (24)$$

As a check on this procedure we covalently modified the Lys ε-NH_3^+ groups of the two isozymes of BCA II with 4-sulfophenyl isothiocyanate to generate three sets of derivatives of the protein that differed in approximately

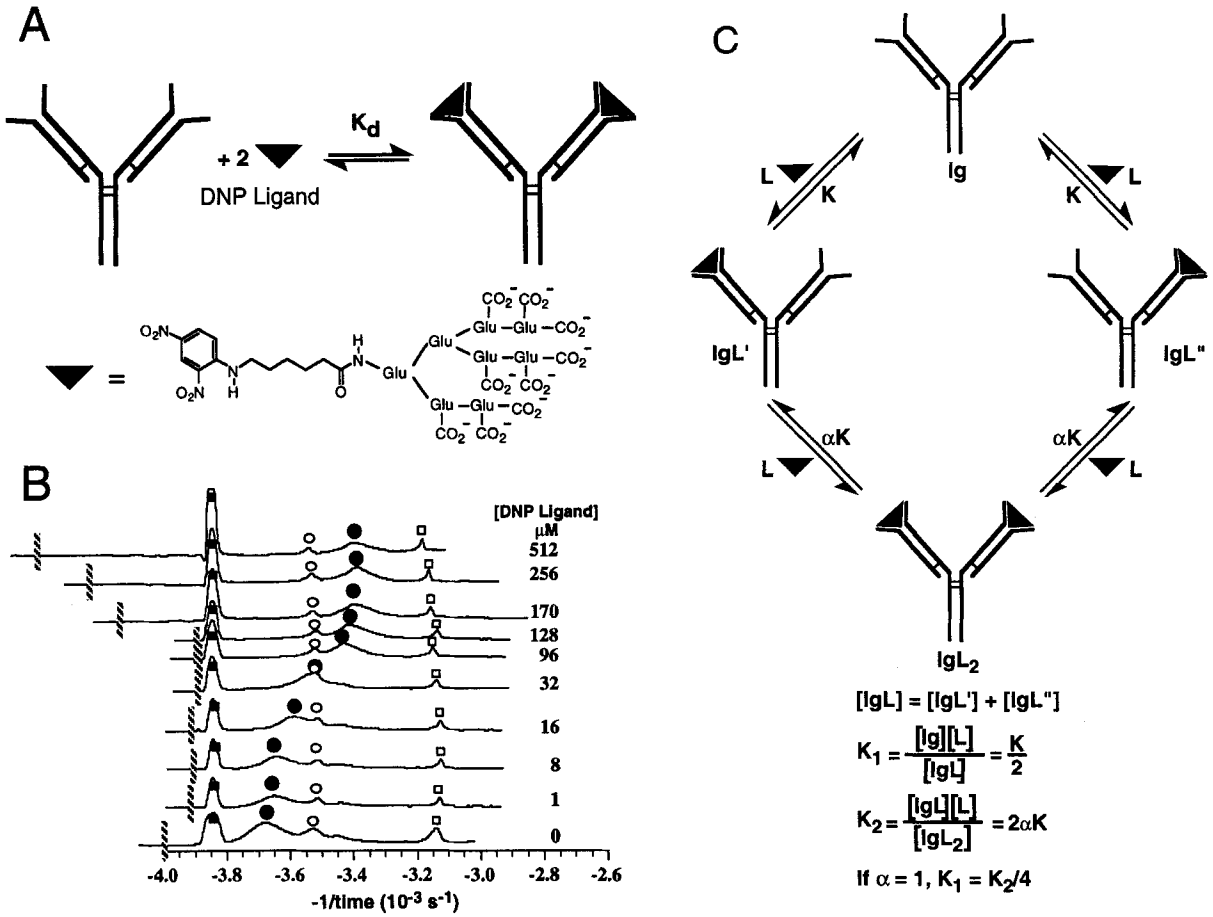


Figure 5. (A) An ACE experiment involving an anti-2,4-DNP IgG antibody and a ligand presenting a 2,4-DNP group having a charge of approximately −9 (DNP). (B) Electropherograms from this ACE experiment expressed in the 1/time domain. The vertical hatch marks indicate the relative position of each electropherogram at -4.1×10^{-3} s^{-1} in the 1/time domain. Bovine carbonic anhydrase II, p*I* 5.9 (○), and α-lactalbumin (□) are used as protein standards. Electropherograms of IgG_{2b}, (●), are obtained as a function of the concentration of DNP in the running buffer. Mesityl oxide (■) was used as an electrically neutral marker. The buffer used in these experiments was 25 mM Tris- 192 mM Gly, pH 8.3, with 0.5 M. 3-quinuclidinopropanesulfonate and 10 mM K_2SO_4 added to reduce adsorption of protein to the capillary wall. (C) Equilibria involved in Ig-antigen interactions. K_1 is the dissociation constant between the singly occupied Ig and the free Ig. K_2 is the dissociation constant between the doubly bound Ig and the singly occupied Ig. The degree of cooperativity between K_1 and K_2 is denoted as α. Reprinted from [36] with permission.

integral units of charge (Fig. 8B). The calculated values of ΔZ_{seq} are now based on Eq. (25), where Z^n_{seq} is the calculated

$$\Delta Z_{seq} = Z^n_{seq} - Z^o_{seq} \quad (25)$$

value of charge of the protein following *n* number of acylations; the value of $\Delta\mu_{electro}$ is based on Eq. (26), where $\mu^n_{electro}$ is the value of $\mu_{electro}$ of the derivative of BCA II

$$\Delta\mu_{electro} = \mu^n_{electro} - \mu^o_{electro} \quad (26)$$

having *n* number of acylations. Under electrophoretic conditions that were similar to those used in the ACE experiments, we estimated that the isozymes of BCA II of p*I* 5.9 and 5.4 had values of Z^o_{CE} of −3.5, and −4.5, respectively. We have estimated the charge of 25 native proteins by analyzing their corresponding charge ladders, made through covalent modification of their Lys ε-NH_3^+ groups, in a similar manner [68]. Many high molecular weight proteins give broad, unresolved peaks upon modification of their Lys ε-NH_2 groups, even when modified with acylating agents that impart 3–6 units of charge per modification [68]. In these cases, the ACE-based method of determining values of Z^o_{CE} is preferred. For example, we used the ACE-based method to determine the value of Z^o_{CE} of IgG_{2b} (molecular mass 150 kDa) to be −8.0 at pH 8.3 [36].

4.2 Estimation of the charge of Van and Van complexed to DADA

ACE experiments revealed that the charge on the *N*-terminal $-NH^+_2CH_3$ group of Van is important in the binding of DADA peptidyl ligands – that is, that electrostatics had a significant role in binding. These results suggested that the value of pK_a of the *N*-terminal $-NH^+_2CH_3$ group of Van may be influenced by the charge on the proximal *C*-terminal carboxylate of bound DADA ligand. To investigate this possibility we determined the charge on Van in both the free and bound state by using a method that is similar to the one described above [17]. We plotted the values of $\mu_{electro}$ of Van, AspNHCOVan and $C_3H_7NHCOVan$ as a function of ΔZ_{seq} (Fig. 9). The values of ΔZ_{seq} of these molecules are

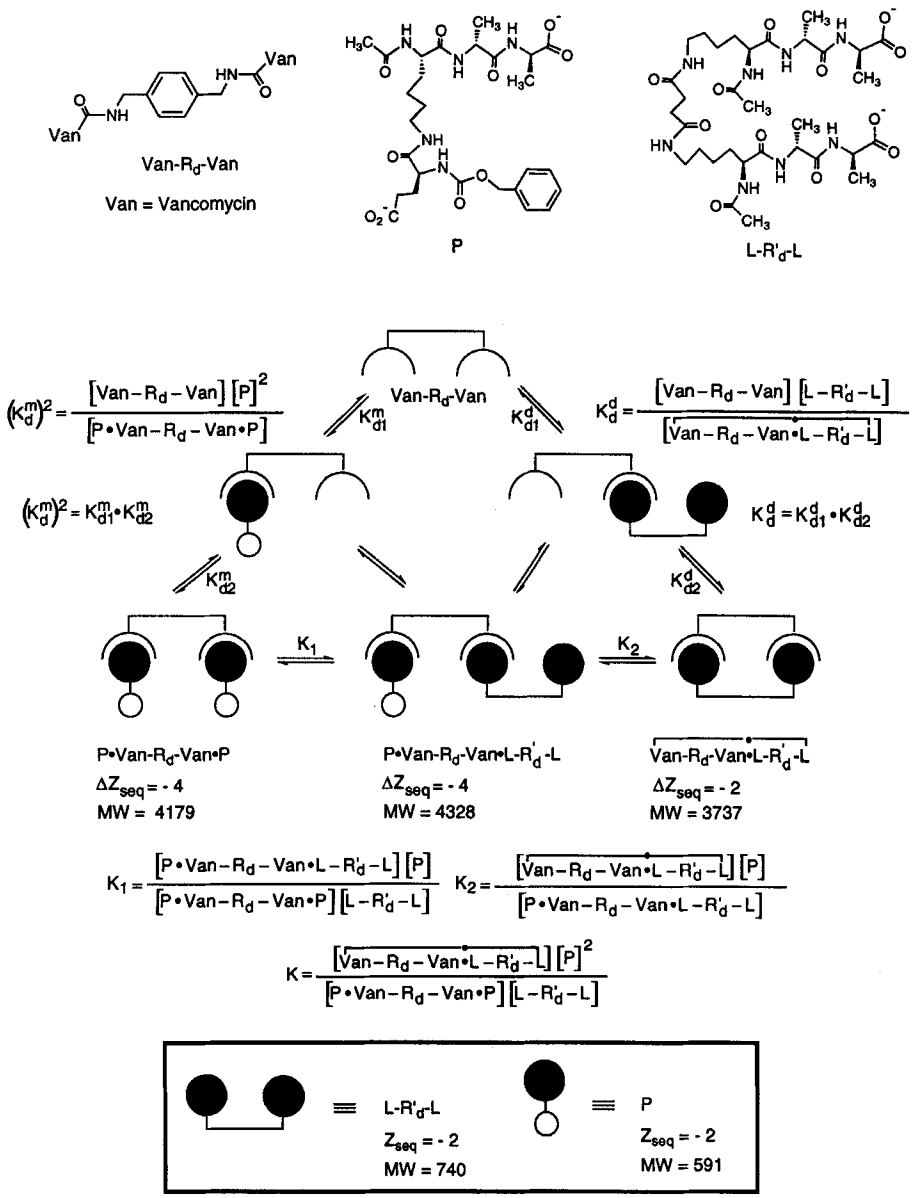


Figure 6. Equilibria involved among the different species present in the competitive assay for Van-R_d-Van. The terms K_{d1}^m and K_{d2}^m are the two dissociation constants for the complex P·Van-R_d-Van-P; K_{d1}^d and K_{d2}^d are the two dissociation constants for the complex Van-R_d-Van·L-R'_d-L. The equilibrium constant K_1 defines the exchange between P·Van-R_d-Van-P and P·Van-R_d-Van·L-R'_d-L; K_2 is the equilibrium constant for the exchange between P·Van-R_d-Van·L-R'_d-L and Van-R_d-Van·L-R'_d-L. The term ΔZ_{seq} is the difference in the calculated charge of a receptor-ligand complex and that of Van-R_d-Van. Reprinted from [65] with permission.

0, −1 and +1, respectively, based on the number of carboxylate groups that they comprise. We next fit the data by the method of linear least squares to a line described by Eq. (27), where the terms $\mu^o_{electro}$ and $\mu^n_{electro}$ are the values of $\mu_{electro}$ of native Van and

$$\mu^n_{electro} = \left(\frac{C_P}{M^\alpha}\right)\Delta Z_{seq} + \mu^o_{electro} \quad (27)$$

its derivative, respectively, and (C_P/M^α) is the slope of the line. Our analysis assumed that the coefficient of friction of each derivative of Van was approximately the same as that of Van. Extrapolation to the *x*-axis intercept (where $Z^n_{CE} = Z^{nm}_{CE} = 0$ and $\mu^n_{electro} = \mu^{nm}_{electro} = 0$, nm = neutral marker) yields the value of Z^o_{CE} of Van to be 0.30 at pH 7.1 (Eq. 28).

$$Z^o_{CE} = \frac{\mu^o_{electro}}{\left(\frac{C_P}{M^\alpha}\right)} \quad (28)$$

We similarly estimated a value of Z^o_{CE} of −0.25 for the complex of Van and AcDAdA, Van·AcDAdA, at pH 7.1, by analyzing the values of $\mu_{electro}$ of Van·AcDAdA and the corresponding complexes of AspNHCOVan and C_3H_7NHCOVan with AcDAdA, at pH 7.1 (Fig. 9).

To estimate the contribution of bound Van – that is, Van in Van·AcDAdA – to the value of Z^o_{CE} of Van·AcDAdA, we initially corrected the value of $\mu_{electro}$ of each complex, $\mu^{R\cdot L}_{electro}$, for the influence of the negative charge from bound AcDAdA. The value of $\mu^{R\cdot L}_{electro}$ of each complex can be written as the product of C_P/M^α and the sum of the charge of R in R·L (R*), $Z^{R^*}_{CE}$, and that of L in R·L (L*), $Z^{L^*}_{CE}$, based on the assumption that the coefficient of friction of R·L and R* are approximately the same (Eq. 29). If we assume that the value of $Z^{L^*}_{CE}$ is −1 at pH 7.1, then the contribution of R* to the value of $\mu^{R\cdot L}_{electro}$, $\mu^{R^*}_{electro}$, can be estimated by Eq. (30). We determined the values of $\mu^{R^*}_{electro}$ for each of the

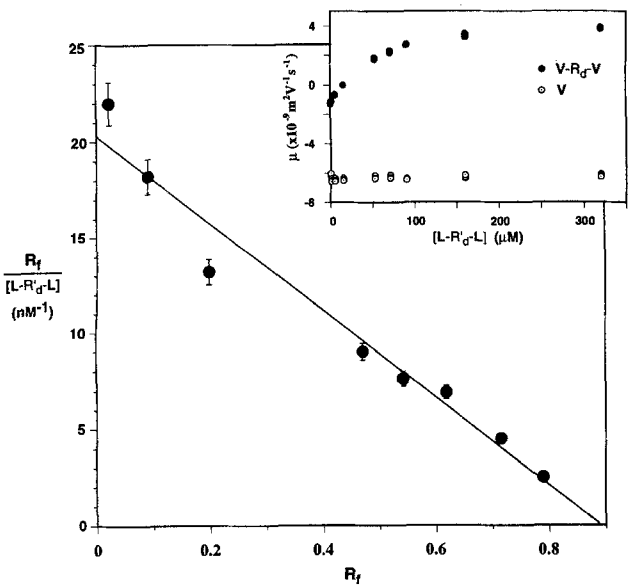


Figure 7. Plot derived from an analysis of the values of $\mu^{avg}_{electro}$ of Van-R_d-Van by using Eqs. (19, 20). The y-intercept of the best-fit line indicates that $K/[P]^2$ has a value of 20 nM^{-1}, and that the value of K is equal to 20 mM (20 nM^{-1} × [P]^2). The inset plots the values of $\mu_{electro}$ of Van-R_d-Van and of Van as a function of the concentration of L-R'$_d$-L in the running buffer. The value of $\mu_{electro}$ of Van does not change appreciably upon its association with L-R'$_d$-L, indicating that the difference in the values of $\mu_{electro}$ of Van-R_d-Van and of Van-R_d-Van·L-R'$_d$-L is due predominantly to the charge of bound L-R'$_d$-L and not due to its contribution to the coefficient of friction of the complex. Reprinted from [65] with permission.

$$\mu^{R\cdot L}_{electro} \cong \left(\frac{C_P}{M^\alpha}\right) Z^{R\cdot L}_{CE} \cong \left(\frac{C_P}{M^\alpha}\right)(Z^{R^*}_{CE} + Z^{L^*}_{CE}) \cong \cong \mu^{R^*}_{electro} - \left(\frac{C_P}{M^\alpha}\right) \quad (29)$$

$$\mu^{R^*}_{electro} \cong \mu^{R\cdot L}_{electro} + \left(\frac{C_P}{M^\alpha}\right) \quad (30)$$

three complexes and then analyzed the resulting data to estimate the value of Z^o_{CE} of Van in Van·AcDADA to be 0.75, at pH 7.1. A similar analysis conducted using pH 8.4 running buffer revealed that the value of Z^o_{CE} of Van changes from –0.59 when free to 0.03 after it binds AcDADA.

4.3 Estimation of the pK_a of the *N*-terminal $NH^+_2CH_3$ group of Van when free or in a complex with KDADA

Native Van has six ionizable residues: a *C*-terminal carboxylic acid (pK_a 2.9), an *N*-terminal secondary amino group (pK_a 7.2), a primary sugar amino group, located far from the binding site (pK_a 8.6) and three phenolic groups situated near the *C*-terminus of the molecule (values of pK_a 9.6, 10.5, 11.7) [15]. Based on the values of pK_a of these residues, we concluded that a shift in value of pK_a of the *N*-terminal $NH^+_2CH_3$ group of Van contributes to the observed differences in the values of Z^o_{CE} of free and bound Van at values of pH of 7.1 and 8.4. To quantitate the value of the shift in pK_a, we first determined the pK_a of the *N*-terminal $NH^+_2CH_3$ of both free Van and Van bound to Ac_2KDADA. To determine the pK_a of the *N*-terminal $NH^+_2CH_3$ of free Van we first removed the contributions of charge from the other ionizable groups on Van from the values of $\mu_{electro}$ of Van, $\mu^{Van}_{electro}$, obtained at different values of pH: we subtracted the values of $\mu_{electro}$ of VanN(CH$_3$)Ac, $\mu^{VanN(CH_3)Ac}_{electro}$, from those of $\mu^{Van}_{electro}$, as a function of pH. The resulting data are the effective values of $\mu_{electro}$ of Van due only to its coefficient of friction and the charge of its *N*-terminal $-NH^+_2CH_3$ group. These data were fit to Eq. (31) by

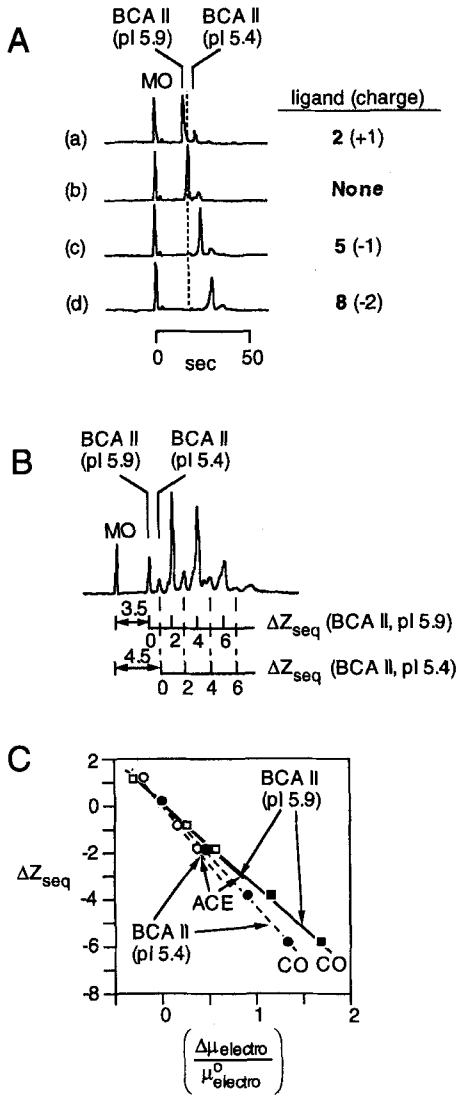


Figure 8. Estimation of the values of charge of the isozymes of BCA II. The commercial sample of BCA II contains two isozymes of p*I* 5.4 and 5.9 in about a 1:10 ratio; these isozymes differ by one unit of charge. (A) Association of BCA II with charged ligands: (a) [2] = 1.0 mM (b) buffer, (c) [5] = 1.0 mM, and (d) [8] = 0.5 mM. See Table 1 for structures of 2, 5, and 8. (B) Covalent modification of the Lys ε-NH^+_3 groups on the isozymes of BCA II using 4-sulfophenyl isothiocyanate generated two separate groups of covalent derivatives (buffer = 25 mM Tris-192 mM Gly, pH 8.3). (C) Estimation of charge from the analysis of the values of $\mu_{electro}$ of the protein-ligand complexes or the covalent derivatives using Eq. (24). The data shown for the two isozymes of BCA II were collected using ACE and covalent (CO) methods. Adapted from reference [67].

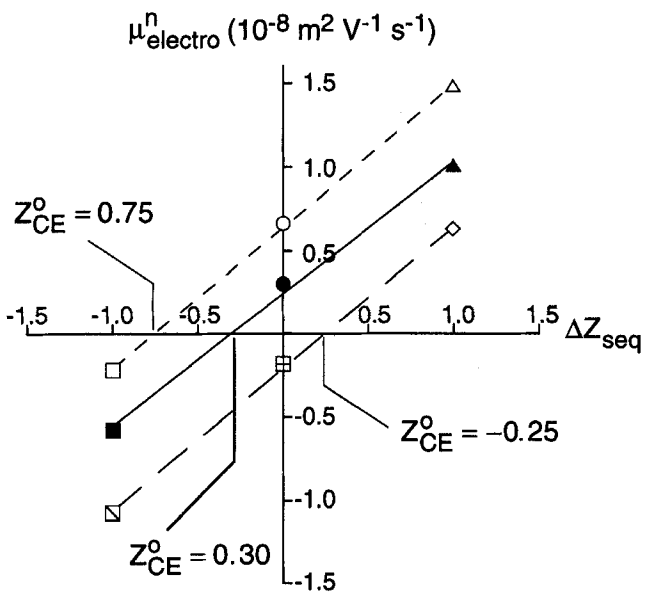


Figure 9. Estimation of the values of Z^{o}_{CE} of free Van and of Van in a complex with AcDADA, at pH 7.1. The values of $\mu_{electro}$ of Van and its covalent derivatives were plotted as a function of ΔZ_{seq}; the *x*-intercept of the best-fit line gave a value of Z^{o}_{CE} of 0.30 for free Van. A similar analysis of the values of $\mu_{electro}$ of the complexes of Van and of its derivatives with AcDADA revealed that the value of Z^{o}_{CE} of Van·AcDADA was −0.25 at pH 7.1. We determined the values of $\mu_{electro}$ of Van in Van·AcDADA and the corresponding values of each of the derivatives of Van in their respective complexes by using Eq. (30). See Section 3.2 for details. An analysis of these values of $\mu_{electro}$ revealed that the value of Z^{o}_{CE} of Van in Van·AcDADA was equal to 0.75 at pH 7.1. Reprinted from [17] with permission.

the method of nonlinear least squares; this equation relates the value of $\mu^{avg(Van^+/Van)}_{electro}$, the concentration-weighted average of the values of $\mu_{electro}$ of the

$$\mu^{avg(van^+/Van)}_{electro} = \theta\,\mu^{RNH_2^+CH_3}_{electro} + (1-\theta)\mu^{RNHCH_3}_{electro} = \left(\frac{C_P}{M^o}\right)\frac{1}{1+10^{pH-pK_a}} \quad (31)$$

$$\theta = \frac{[RNH_2^+CH_3]}{[RNH_2^+CH_3]+[RNHCH_3]} \quad (32)$$

protonated and free forms of Van (θ = mole fraction of protonated Van; Eq. 32), to the pH of the running buffer, and the pK_a of the $-NH_2^+CH_3$ group. Analysis of this titration curve gave a pK_a of 7.1 for the *N*-terminal $-NH_2^+CH_3$ group (Fig. 10).

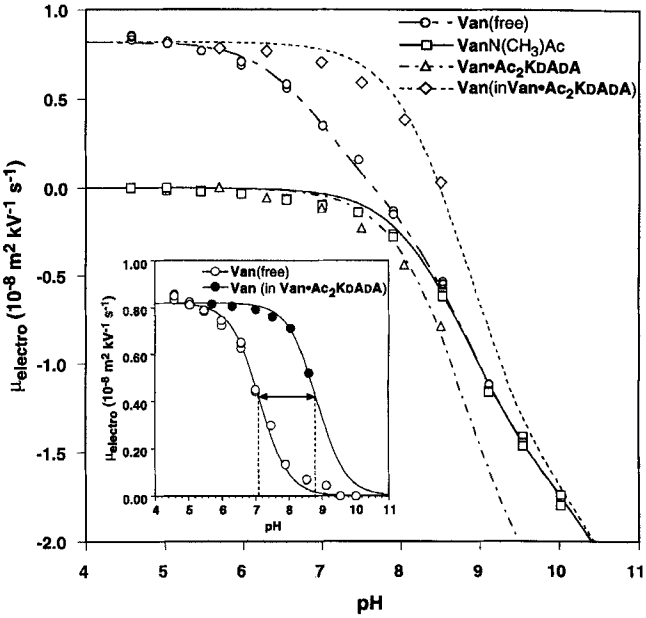


Figure 10. The values of $\mu_{electro}$ of Van, VanN(CH$_3$)Ac, and Van·Ac$_2$K-DADA, as a function of pH. The values of $\mu_{electro}$ of Van in Van·Ac$_2$K-DADA were obtained from those of Van·Ac$_2$KDADA by using Eq. (30). This inset shows the values of $\mu_{electro}$ obtained by subtracting the values of $\mu_{electro}$ of VanN(CH$_3$)Ac from those of Van, and those of Van in Van·Ac$_2$KDADA, respectively, as a function of pH. These data represent effectively the charge of the *N*-terminal $-NH_2^+CH_3$ group of Van, and that of Van in Van·Ac$_2$KDADA, as a function of pH. These data were fit to Eq. (31), to give values of pK_a of 7.1 and of 8.8 for the $-NH_2^+CH_3$ group of Van, and that of Van in Van·Ac$_2$KDADA, respectively. Reprinted from [17], with permission.

To determine the pK_a of Van in Van·Ac$_2$KDADA, we first determined the $\mu_{electro}$ of Van·Ac$_2$KDADA, $\mu^{Van\cdot Ac_2KDADA}_{electro}$, as a function of pH; we titrated Van by CE using running buffers that varied from pH 5.7 to 8.5 and contained a concentration of Ac$_2$KDADA that saturated the binding site of Van. From these values of $\mu^{Van\cdot Ac_2KDADA}_{electro}$ we estimated the values of $\mu_{electro}$ of Van in Van·Ac$_2$KDADA ($\mu^{Van\ in\ Van\cdot Ac_2KDADA}_{electro}$) by using Eq. (30). We removed the contributions of charge from ionizable residues other than the *N*-terminal $-NH_2^+CH_3$ group of bound Van from the values of $\mu^{Van\ in\ Van\cdot Ac_2KDADA}_{electro}$, by subtracting the values of $\mu^{VanN(CH_3)Ac}_{electro}$ from those of $\mu^{Van\ in\ Van\cdot Ac_2KDADA}_{electro}$ as a function of pH. By fitting these data to Eq. (31), we determined the pK_a of the *N*-terminal $-NH_2^+CH_3$ group of bound Van to be 8.8 – a shift of 1.7 units from free Van. We concluded that the electrostatic interaction between the *C*-terminus of Ac$_2$KDADA and the *N*-terminus of Van in the complex was responsible for the shift in pK_a of the *N*-terminal $-NH_2^+CH_3$ group.

5 The contribution of electrostatics to the free energy of binding of the rungs of the charge ladder of BCA II to benzenesulfonamide ligands

The acetylation of the Lys ε-NH_3^+ groups of BCA II produces sets of derivatives that differ incrementally in the number of residues modified, and can be separated by CE into an array of distinct peaks that we call a "charge ladder" [67, 68]. Each peak or "rung" of this charge ladder comprises proteins that have the same number of acety-

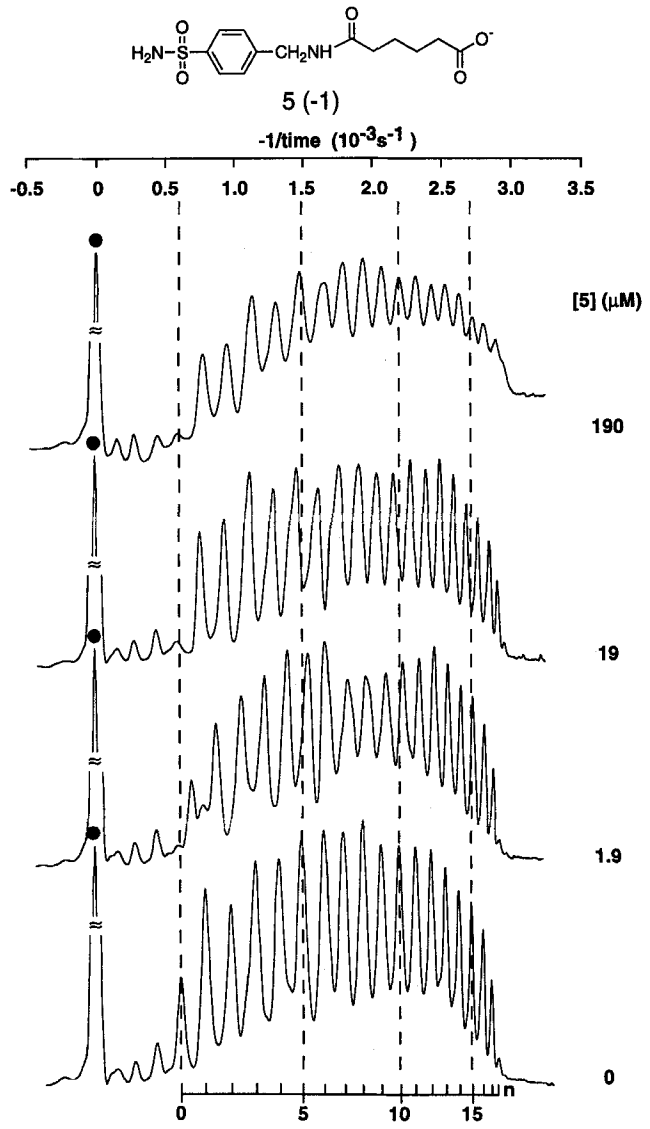


Figure 11. Electropherograms showing the changes in the values of $\mu_{electro}$ of the rungs of the charge ladder of BCA II due to the binding of ligand 5 (Table 1). The electrically neutral marker, *p*-methoxybenzyl alcohol, is indicated by the filled circle (•). The number of acetylated Lys ε-amino groups (n) is indicated by the scale at the bottom of the electropherograms. The −1/time scale shown at the top of the electropherograms is proportional to the values of $\mu_{electro}$ of the rungs of the charge ladders, relative to that of the neutral marker. Adapted from [69].

lated Lys ε-NH_3^+ groups and consequently the same approximate charge. ACE can determine the binding affinity of all of the proteins present in each rung of a charge ladder for a common ligand in the running buffer in one set of experiments. We have carried out ACE experiments using charge ladders with neutral and charged benzenesulfonamide ligands to determine the contribution of electrostatics to the values of measured binding affinities [69]. Figure 11 shows some of the electropherograms from an ACE experiment carried out with the charge ladder of BCA II and negatively charged ligand 5 (Table 1).

The difference in values of $\mu_{electro}$ of adjacent rungs, $\Delta\mu_{electro}$, is approximately uniform for the first four pairs. This result implies that the corresponding difference in values of Z_{CE} of adjacent rungs, ΔZ_{CE}, is also approximately constant for the first four pairs. We assume that the value of ΔZ_{CE} of each of these pairs of adjacent rungs is approximately equal to the corresponding value of charge calculated from the sequence of the protein, ΔZ_{seq}; at pH 8.4, the pH of the running buffer, the value of ΔZ_{seq} is equal to one unit of charge, based on a standard pK_a of 10.7 for each Lys ε-NH^+_3 group. A plot of $\mu^n_{electro}$ *vs.* $n\Delta Z_{seq}$ (n = number of acetylations) for the first five rungs of the charge ladder, is, therefore, linear with a slope equal to C_P/M^α. We estimate the value of Z_{CE} of the nth rung of the charge ladder, Z^n_{CE}, by dividing the value of $\mu_{electro}$ of the nth rung by the value of C_P/M^α (Eq. 33).

$$Z^n_{CE} = \frac{\mu^n_{electro}}{\left(\frac{C_P}{M^\alpha}\right)} \tag{33}$$

To study the effect of electrostatic interactions on binding, we plotted the free energies of binding of the rungs of the charge ladder of BCA II for its differently charged ligands as a function of Z^n_{CE}. For neutral ligands, (Table 1: 15–17), the free energy of binding, ΔG_b, does not change significantly as a function of the value of Z^n_{CE}; that is, $\Delta\Delta G_b/\Delta Z^n_{CE} \sim 0$ (Fig. 12). The affinity of BCA II for the positively charged ligand, 2, increases with increasing charge of BCA II ($\Delta\Delta G_b/\Delta Z^n_{CE} = -0.07 \pm 0.01$ kcal mol^{-1} $charge^{-1}$) while that for the negatively charged ligand, 5, decreases with increasing degree of acetylation ($\Delta\Delta G_b/\Delta Z^n_{CE} = 0.13 \pm 0.01$ kcal mol^{-1} $charge^{-1}$; Fig. 12). The magnitude of $\Delta\Delta G_b/\Delta Z^n_{CE}$ decreases as the number of bonds separating the charged group and sulfonamide group on the ligand increases; the value of $\Delta\Delta G_b/\Delta Z^n_{CE}$ for ligands 6 and 7 are 0.09 ± 0.02, and 0.03 ± 0.02 kcal mol^{-1} $charge^{-1}$, respectively. We draw the following conclusions from these results: (i) the electrostatic interaction of charged residues outside the active site influences the binding of benzenesulfonamide ligands; (ii) all the regioisomeric derivatives that compose a single rung of the charge ladder have similar values of affinity for benzenesulfonamide ligands, and (iii) the magnitude of the binding affinity for a rung of the charge ladder is dependent on the charge of the rung of the charge ladder and the distance between the charged group on the ligand and the active site of the enzyme.

6 Concluding remarks

ACE is a powerful technique that uses the resolving power of CE for determining binding constants of receptors to charged and neutral ligands. It is analytically practical since it uses only small amounts of a receptor and its ligands. The technique does not require that a receptor be pure for analysis; the migration of a single peak corresponding to the receptor can be followed accurately in a mixture, as a function of increasing concentration of ligand, by using stacked electropherograms represented in the 1/time domain. ACE is, therefore, suited for cases where purification of a receptor may lead to its inactivation. ACE is not limited to measuring a

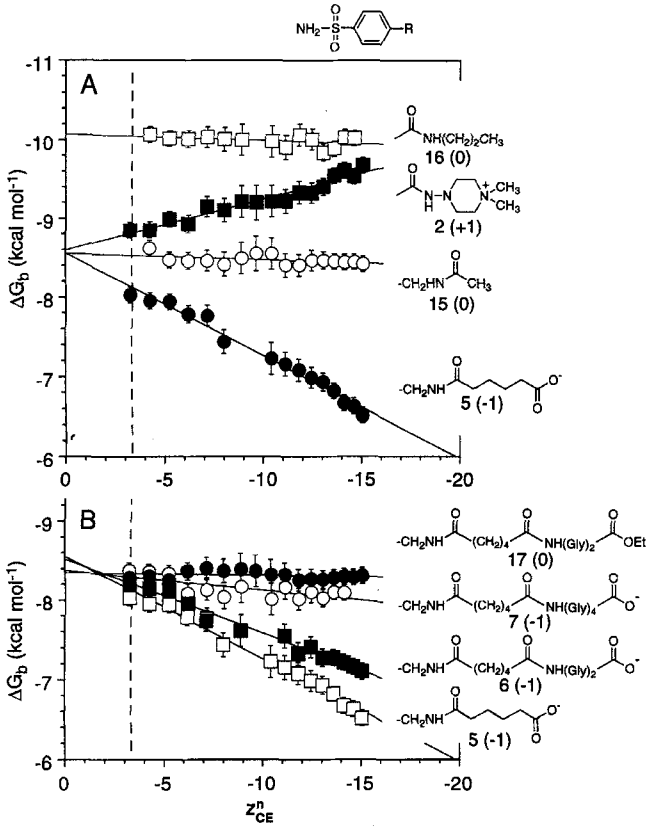


Figure 12. Dependence of the free energy of binding (ΔG_b) on the values of Z^n_{CE} of the rungs of the charge ladder of BCA II, and on the charge on the ligands. The binding affinity of each rung of the charge ladder to ligands 2, 5–7, and 15–17 (Table 1) was measured by ACE in 25 mM Tris-192 mM Gly, pH 8.3. Due to slight broadening of peaks near the center of the charge ladder (n = 6–12), the uncertainties in the values of ΔG_b for these derivatives are larger than those for the other parts of the charge ladder (for some rungs of the charge ladder, the peak broadening resulted in missing data). The slopes ($\Delta\Delta G_b/\Delta Z^n_{CE}$) from the linear regression analyses of ΔG_b *vs.* Z^n_{CE} yielded the magnitudes of influence of charges on BCA II-ligand interactions. The values of $\Delta\Delta G_b/\Delta Z^n_{CE}$ (kcal mol^{-1} charge^{-1}) for these ligands are: (2) −0.07 ± 0.01; (5) 0.13 ± 0.01; (6) 0.09 ± 0.02; (7) 0.03 ± 0.02; (15) 0.01 ± 0.01; (16) 0.01 ± 0.01; (17) 0 ± 0.01. Adapted from [69].

single binding constant at a time, but can determine simultaneously the affinity of several receptors (different isozymes or different covalent derivatives of a receptor) to a common ligand present in the running buffer [66]. This procedure can minimize variations in experimental conditions that may be observed when separate experiments are used to measure the binding affinity of a common ligand to different receptors. A variation of an ACE experiment can be used to screen a library of compounds for ligands that bind tightly to a receptor [21, 52]. UV- or fluorescence-based assays require changes in the absorption or emission spectra of a receptor upon binding a ligand. In cases where these changes are not observed, alternative experiments can be designed using ACE.

A second, related capability of ACE is that it can be used to study electrostatic interactions that influence the binding constants of receptor-ligand systems [17, 69], and to estimate the charge of proteins in solution [67] without accurately knowing their amino acid sequence. ACE has also been used to determine the binding stoichiometry in several systems [24] and to estimate the kinetic parameters for the binding of BCA II with ligand 10 (Table 1) by comparing the peak widths of intermediate receptor-ligand complexes to those simulated by computer [34]. ACE becomes problematic when applied to proteins that have high values of molecular mass (> 100 kDa) or pI (pI > 8.4). These proteins may adsorb electrostatically to the surface of fused silica capillaries. The use of zwitterions in the running buffer lowers the degree of adsorption of high molecular mass proteins; capillaries coated with cationic polymers allow the study of proteins having high values of pI by CE [9]. We hope to use coated capillaries to expand the range of binding systems to which ACE can be applied.

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7 References

[1] Kuhr, W. G., *Anal. Chem.* 1990, *62*, 403R–414R.
[2] Novotny, M. V., Cobb, K. A., Liu, J. P., *Electrophoresis* 1990, *11*, 735–749.
[3] Gordon, M. J., Huang, X., Pentoney, S. L., Jr., Zare, R. N., *Science* 1988, *242*, 224–228.
[4] Gomez, F. A., Avila, L. Z., Chu, Y. H., Whitesides, G. M., *Anal. Chem.* 1994, *66*, 1785–1791.
[5] Mammen, M., Colton, I. J., Carbeck, J. D., Bradley, R., Whitesides, G. M., *Anal. Chem.* 1997, *69*, 2165–2170.
[6] Bushey, M. M., Jorgenson, J. W., *J. Chromatogr.* 1989, *480*, 301–310.
[7] Krueger, R. J., Hobbs, T. R., Mihal, K. A., Tehrani, J., Zeece, M. G., *J. Chromatogr.* 1991, *543*, 451–461.
[8] Arentoft, A. M., Frokiaer, H., Michaelsen, S., Sorensen, H., Sorensen, S., *J. Chromatogr. A* 1993, *652*, 189–198.
[9] Córdova, E., Gao, J., Whitesides, G. M., *Anal. Chem.* 1997, *69*, 1370–1379.
[10] Liljas, A., Hakansson, K., Jonsson, B. H., Xue, Y., *Eur. J. Biochem.* 1994, *219*, 1–10.
[11] Hakansson, K., Liljas, A., *FEBS Lett.* 1994, *350*, 319–322.
[12] Williams, D. H., Waltho, J. P., *Biochem. Pharmacol.* 1988, *37*, 133–141.
[13] Chu, Y.-H., Avila, L. Z., Gao, J., Whitesides, G. M., *Acc. Chem. Res.* 1995, *28*, 461–468.
[14] Chu, Y.-H., Whitesides, G. M., *J. Org. Chem.* 1992, *57*, 3524–3525.
[15] Nieto, M., Perkins, H. R., *Biochem. J.* 1971, *123*, 773–787.
[16] Popienick, P. H., Pratt, R. F., *Anal. Biochem.* 1987, *165*, 108–113.
[17] Rao, J., Colton, I. J., Whitesides, G. M., *J. Am. Chem. Soc.* 1997, *119*, 9336–9340.
[18] Carpenter, J. L., Camilleri, P., Dhanak, D., Goodall, D., *J. Chem. Soc. Chem. Commun.* 1992, 804–806.
[19] Kannan, R., Harris, C. M., Harris, T. M., Waltho, G. P., Skelton, N. J., Williams, D. H., *J. Am. Chem. Soc.* 1988, *110*, 2946–2953.
[20] Bugg, T. D. H., Wright, G. D., Dutka-Malen, S., Arthur, M., Courvalin, P., Walsh, C. T., *Biochemistry* 1991, *30*, 10408–10415.
[21] Chu, Y.-H., Avila, L. Z., Biebuyck, H. A., Whitesides, G. M., *J. Org. Chem.* 1993, *58*, 648–652.
[22] Chu, Y.-H., Avila, L. Z., Biebuyck, H. A., Whitesides, G. M., *J. Med. Chem.* 1992, *35*, 2915–2917.
[23] Gomez, F. A., Chen, J. K., Tanaka, A., Schreiber, S. L., Whitesides, G. M., *J. Org. Chem.* 1994, *59*, 2885–2886.
[24] Chu, Y.-H., Lees, W. J., Stassinopoulos, A., Walsh, C. T., *Biochemistry* 1994, *33*, 10616–10621.
[25] Shimura, K., Karger, B. L., *Anal. Chem.* 1994, *66*, 9–15.
[26] Lausch, R., Reif, O.-W., Riechel, P., Scheper, T., *Electrophoresis* 1995, *16*, 636–641.
[27] Gao, J., Mrksich, M., Gomez, F. A., Whitesides, G. M., *Anal. Chem.* 1995, *67*, 3093–3100.

[28] Heegaard, N. H. H., Robey, F. A., *Am. Lab.* 1994, *26*, 28T–28X.
[29] Xian, J., Harrington, M. G., Davidson, E. H., *Proc. Natl. Acad. Sci. USA* 1996, *93*, 86–90.
[30] Heegaard, N. H. H., *J. Chromatogr. A* 1994, *680*, 405–412.
[31] Chen, F.-T. A., *J. Chromatogr. A* 1994, *680*, 419–423.
[32] Nadeau, K., Nadler, S. G., Saulnier, M., Tepper, M. A., Walsh, C. T., *Biochemistry* 1994, *33*, 2561–2567.
[33] Liu, J., Volk, K. J., Lee, M. S., Kerns, E. H., Rosenberg, I. E., *J. Chromatogr. A* 1994, *680*, 395–403.
[34] Avila, L. Z., Chu, Y.-H., Blossey, E. C., Whitesides, G. M., *J. Med. Chem.* 1993, *36*, 126–133.
[35] Sun, P., Hoops, A., Hartwick, R. A., *J. Chromatogr. B* 1994, *661*, 335–340.
[36] Mammen, M., Gomez, F. A., Whitesides, G. M., *Anal. Chem.* 1995, *67*, 3526–3535.
[37] Busch, S., Kraak, J. C., Poppe, H., *J. Chromatogr.* 1993, *635*, 119–126.
[38] Valtcheva, L., Mohammad, J., Petterson, G., Hjertén, S. J., *J. Chromatogr.* 1993, *638*, 263–267.
[39] Vespaleck, R., Sustaček, V., Boček, P., *J. Chromatogr.* 1993, *638*, 255–261.
[40] Kraak, J. C., Busch, S., Poppe, H., *J. Chromatogr.* 1992, *608*, 257–264.
[41] Barker, G. E., Russo, P., Hartwick, R. A., *Anal. Chem.* 1992, *64*, 3024–3028.
[42] Arai, T., Nimura, N., Kinoshita, T., *Biomed. Chromatogr.* 1995, *9*, 68–74.
[43] Kajiwara, H., Hirano, H., Oono, K., *J. Biochem. Biophys. Methods* 1991, *22*, 263–268.
[44] Kajiwara, H., *J. Chromatogr.* 1991, *559*, 345–356.
[45] Heegaard, N. H. H., Robey, F. A., *J. Immunol. Methods* 1993, *166*, 103–110.
[46] Haupt, K., Roy, F., Vijayalakshmi, M. A., *Anal. Biochem.* 1996, *234*, 149–154.
[47] Shimura, K., Kasai, K.-i., *Anal. Biochem.* 1995, *227*, 186–194.
[48] Linhardt, R. J., Han, X.-J., Fromm, J. R., *Mol. Biotechnol.* 1995, *3*, 191–197.
[49] Kuhn, R., Frei, R., Christen, M., *Anal. Biochem.* 1994, *218*, 131–135.
[50] Honda, S., Taga, A., Suzuki, K., Suzuki, S., Kakehi, K., *J. Chromatogr.* 1992, *597*, 377–382.
[51] Heegaard, N. H. H., Brimnes, J., *Electrophoresis* 1996, *17*, 1916–1920.
[52] Chu, Y.-H., Dunayevskiy, Y. M., Kirby, D. P., Vouros, P., Karger, B. L., *J. Am. Chem. Soc.* 1996, *118*, 7827–7835.
[53] Liu, J., Volk, K. J., Lee, M. S., Pucci, M., Handwerger, S., *Anal. Chem.* 1994, *66*, 2412–2416.
[54] Handwerger, S., Pucci, M. J., Volk, K. J., Liu, J., Lee, M. S., *J. Bacteriol.* 1994, *176*, 260–264.
[55] Goodall, D. M., *Biochem. Soc. Trans.* 1993, *21*, 125–129.
[56] Heegaard, N. H. H., Robey, F. A., *Anal. Chem.* 1992, *64*, 2479–2482.
[57] Heegaard, N. H. H., Mortensen, H. D., Roepstorff, P., *J. Chromatogr. A* 1995, *717*, 83–90.
[58] Biehler, R., Jacobs, A., *Capillary Electrophoresis Technical Information*, Beckman Instruments, Inc., Fullerton, CA 1993; A-1727.
[59] Wren, S. A. C., Rowe, R. C., *J. Chromatogr.* 1992, *603*, 235–241.
[60] Lemesle-Lamache, V., Taverna, M., Wouessidjewe, D., Duchene, D., Ferrier, D., *J. Chromatogr. A* 1996, *735*, 321–331.
[61] Baba, Y., Inoue, H., Tsuhako, M., Sawa, T., Kishida, A., Akashi, M., *Anal. Sci.* 1994, *10*, 967–969.
[62] Baba, Y., Tsuhako, M., Sawa, T., Akashi, M., *J. Chromatogr.* 1993, *632*, 137–142.
[63] Baba, Y., Tsuhako, M., Sawa, T., Akashi, M., Yashima, E., *Anal. Chem.* 1992, *64*, 1920–1925.
[64] Mammen, M., Choi, S.-K., Whitesides, G. M., *Angew. Chem.* 1997, in press.
[65] Rao, J., Whitesides, G. M., *J. Am. Chem. Soc.* 1997, *119*, 10286–10290.
[66] Zhang, B., Breslow, R., *J. Am. Chem. Soc.* 1993, *115*, 9353–9354.
[67] Gao, J., Gomez, F. A., Härter, R., Whitesides, G. M., *Proc. Natl. Acad. Sci. USA* 1994, *91*, 12027–12030.
[68] Colton, I. J., Anderson, J. R., Gao, J., Chapman, R. G., Isaacs, L., Whitesides, G. M., *J. Am. Chem. Soc.* 1997, *119*, 12701–12709.
[69] Gao, J., Mammen, M., Whitesides, G. M., *Science* 1996, *272*, 535–537.