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Determination of antigen–antibody affinity by immuno-capillary electrophoresis

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Abstract

The use of affinity capillary electrophoresis for the characterization of antigen–antibody interactions (immuno-capillary electrophoresis) is shown using monoclonal antibodies against phosphotyrosine as a model system. The influence of the interaction kinetics on the peak profiles was demonstrated in experiments with addition of phosphotyrosine to the electrophoresis buffer. One of the two antibodies that were tested exhibited peak broadening while the other showed no change in peak shape but had a decreased mobility proportional to the amount of phosphotyrosine present. The migration shifts which were of the order 0.05 to 0.15 min at 439 V/cm were a consequence of the antibody–antigen complexes having a slower mobility than the non-complexed antibody. On the basis of measurement of migration shifts at different antigen concentrations, dissociation constants were estimated and shown to be independent on the applied field strength. Thus, when certain requirements are fulfilled, immuno-capillary electrophoresis is a fast and simple method for establishing binding characteristics of unlabelled antigen and antibody molecules under non-denaturing conditions and consumes minute amounts of sample.

1. Introduction

Free solution capillary electrophoresis (CE) has been used in a number of cases to characterize receptor–ligand interactions [1–7]. These applications are analogous to traditional slab gel electrophoretic methods such as "gel mobility-shift" [8] or "affinity electrophoresis" [9,10] where changes in the migration of acceptor molecules are correlated with the concentration of ligand included in the gel matrix, the magnitude of difference between the migration of non-complexed acceptor and ligand molecules, and the value of the binding constant for the interaction. The ensuing mobility shifts are directly proportional to the amount of time the acceptor molecule spends in complexed form during electrophoresis. The versatility of CE for analysis of a very wide range of structurally different compounds including small biomolecules has made it a more general approach for the study of molecular interactions than the traditional slab gel methods. Added attractions are speed, high efficiency, low sample consumption, and on-line detection of unlabelled components. The high separating power of CE means that most cases of complex formation that leads to changes in charge/mass ratios will be detectable [11–13]. This is also true for macromolecules such as proteins. Thus, when the right conditions for their reproducible analysis can be found interactions of proteins with charged (and in some cases neutral) ligands may also be studied and quantitated by CE [4–6,11]. Within

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the field of protein–ligand affinity measurements, the determination of thermodynamic binding constants for antigen–antibody interactions is of considerable interest [14,15] and although the use of CE for analyzing immune-complex formation has been reported [16,17], no study has as yet dealt with the determination of binding constants for antigen–antibody reactions by affinity CE.

In the present study monoclonal antibodies against a negatively charged ligand, phosphotyrosine, are used as a model system to test the use of affinity CE for the study of antigen–antibody reactions. The screening of binding behavior by CE to define optimum binding assay conditions, the estimation of dissociation constants, and the investigation of the influence of field strength on binding parameters are demonstrated.

2. Experimental

2.1. Chemicals

O-Phospho-L-serine (P0878) and O-phospho-L-tyrosine (P9405) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade water and analysis-grade chemicals (sodium chloride, sodium tetraborate, and boric acid) were from Merck (Darmstadt, Germany). The non-interacting molecule used as a marker in the CE analyses was the peptide Asp–Ala–Glu–Phe–Arg–NH_2 which was synthesized as described [5].

2.2. Antibodies

Monoclonal anti-phosphotyrosine antibodies were from Upstate Biochemicals (UBI; Lake Placid, NY, USA) (catalog No. 05-321) and from Boehringer Mannheim (Mannheim, Germany) (catalog No. 1083 147). The former antibody was supplied as a 1 mg/ml solution in phosphate-buffered saline, 0.01% sodium azide. Because this preparation gave multiple salt-associated peaks upon CE analysis with detection at 200 nm, the buffer was diluted 1:10 by centrifugal filtration through an M_r 10 000 cut-off cellulose filter from Millipore (catalog No. UFC3 LGC 25) (Malvern, MA, USA) using approximately 1 ml of electrophoresis buffer (isotonic borate, pH 9.1) to a 100-μl sample. Some antibody was lost during this procedure and the resulting exact concentration was unknown. The antibody from Boehringer Mannheim was supplied as lyophilized protein and buffer salts and appeared in CE as a well-defined peak after reconstitution in HPLC-grade water.

2.3. Capillary electrophoresis

Electrophoresis buffers were isotonic borate buffers of pH from 7.4 to 9.1 prepared according to [18]. Before use all buffers were filtered through 0.22-μm pore size sterile filters from Millipore (Malvern, MA, USA) (catalog No. SLGV 025 BS). The CE instrument was a P/ACE System 5010 from Beckman Instruments (Fullerton, CA, USA). Electrophoresis was performed in uncoated, 57 cm (50 cm effective length) × 50 μm fused-silica capillaries from Beckman Instruments. Detection was at 200 nm and the thermostat for the cooling fluid was set at 18°C. Between analyses, the capillary was rinsed for 2 min with 0.1 *M* NaOH, 2 min with water and for 1 min with the electrophoresis buffer. Data collection, storage and analysis were performed using the System Gold Software (Beckman Instruments).

Optimum conditions for the recovery of the monoclonal antibodies (mAbs) were first established by a pH scan (electrophoresis at different pH values). A satisfactory recovery was defined as the reproducible appearance of antibody peaks of similar migration times and areas upon repeated injections. Both antibodies in this study were recoverable in CE at pH values between 8.2 and 9.1 (not shown).

Binding experiments were performed by adding aliquots of the antigen (3.83 m*M* phosphotyrosine stock solution in electrophoresis buffer) or a control (3.83 m*M* phosphoserine stock solution in electrophoresis buffer) to the electrophoresis buffer inlet vials in final concentrations from 1.9 to 479 μM. For the Boehringer Mannheim antibody, the sample consisted of final concentrations of 0.3 μM antibody and

162 μM of a non-interacting peptide marker in a volume of 80 μl electrophoresis buffer. The buffer for most experiments was isotonic borate, pH 8.9 (40 mM sodium tetraborate, 40 mM boric acid, 19 mM NaCl) or pH 8.4 (22.5 mM sodium tetraborate, 110 mM boric acid, 36 mM NaCl). The sample was injected for 1 s corresponding to a volume of 0.9 nl according to the specifications of the manufacturer. Electrophoresis with the anode at the sample injection end of the capillary was performed at voltages between 15 and 25 kV (corresponding to currents of 35 to 75 μA). For the migration shift experiments, samples were analyzed three times using inlet electrophoresis buffer from one vial. Outlet buffers were electrophoresis buffer without addition of ligand. The electrophoresis buffer in the outlet vial was replaced each time a new inlet vial was used.

2.4. Data handling

To compensate for small differences in electrophoretic velocity in consecutive analyses, the migration times (t) of the antibody peak was adjusted to a fixed value of the migration time of the internal marker (the peptide added to the mAb sample); e.g. in the case of the pH 8.9, 25 kV experiments, the migration time values were all adjusted to a marker migration time of 6.15 min. Differences (Δt) of the adjusted migration times in the absence and in the presence of the concentration c of the ligand were then plotted as a function of c to give a direct binding curve. For estimation of dissociation constants, the data were linearized in a double reciprocal plot of $1/\Delta t$ as a function of $1/c$ using data points between the maxima and minima of the direct binding curve. The dissociation constant K_D was then determined from the equation of the best-fit straight line as the value of x for $y = 0$ where x equals $-1/K_D$ [19–22].

3. Results and discussion

Fig. 1 illustrates schematically some of the typical effects when receptor–ligand interactions characterized by different reaction rates are analyzed by affinity CE. The interaction patterns correspond to binding of a receptor to a ligand of a lower electrophoretic mobility which is distributed uniformly in the electrophoresis buffer. The binding is assumed to be homogeneous. Interactions which are characterized by identical binding constants may exhibit considerable differences in behavior in affinity CE because of the sensitivity of the technique to the rate constants. Analysis of peak shape changes may in fact in certain cases be used to calculate kinetic constants [6,23]. If the on-and-off rates are sufficiently fast compared with the time of the electrophoresis, all receptor 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 non-complexed species which have different electrophoretic velocities. The resulting migration time will therefore change without change in peak shape (Fig. 1, second panel). Estimation of binding constants on the basis of migration shifts is then feasible [2,5,6,11].

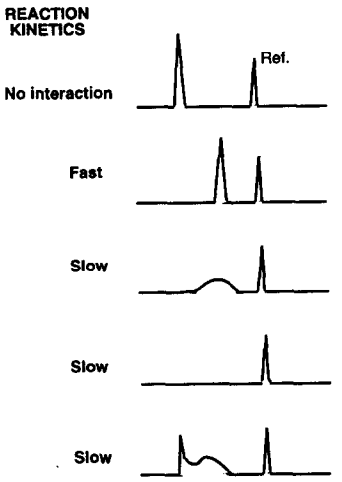

Fig. 1. Schematic drawing of some of the possible interaction patterns in affinity CE with hypothetical, homogeneous receptor–ligand systems characteriized by different reaction kinetics. Ref. is a non-interacting component while the other peak represents a molecule interacting quickly (second panel) or more slowly (lower panels) with ligands of lesser electrophoretic mobility which are present in the electrophoresis buffer.

In the event of slower kinetics, however, the

time that each receptor molecule spends in complexed form during the electrophoresis differs between the individual molecules, and peak broadening will occur (Fig. 1, third panel). This may in some cases occur to the extent that no peak is detectable or, in situations where the receptor–ligand ratio is high, the sample may be fractionated into non-interacting and interacting molecules (Fig. 1, bottom panel).

When the half-time of the complexes is long compared with the time for the electrophoresis, assessment of binding constants by CE should be performed by the type of experiment in which samples of different ratios of receptor and ligands are preincubated followed by electrophoretic separation of free from complexed molecules and quantitation by peak area integration [24,25]. However, in cases with fast interaction kinetics the lifetime of the receptor–ligand complex is shorter than the time required for non-complexed receptor molecules to leave an injected sample plug. Thus, no changes in peak areas of receptor molecules is to be expected when preincubation is applied to this type of interaction.

It is therefore recommended to initiate CE-based binding studies with a set of experiments where putative ligands are added to the electrophoresis buffer. As shown in Figs. 2 and 3, the anti-phosphotyrosine antibodies of this study were initially evaluated in such a way for their interactions with phosphotyrosine and with phosphoserine.

All molecules in these analyses are negatively charged at the chosen buffer pH but move towards the cathode because the electroosmotic flow overcomes the electromigration in the opposite direction [26]. Both antibodies had a migration time of approximately 5.5 min at pH 8.9, 25 kV without any additions to the electrophoresis buffer. Phosphotyrosine when injected as a sample had a migration time under the same conditions of approximately 15.6 min. The molecule used as a non-interacting ligand control should preferably have about the same electrophoretic velocity as the ligand itself, but the migration of phosphoserine was not measured directly in this study because the molecule is not detectable at

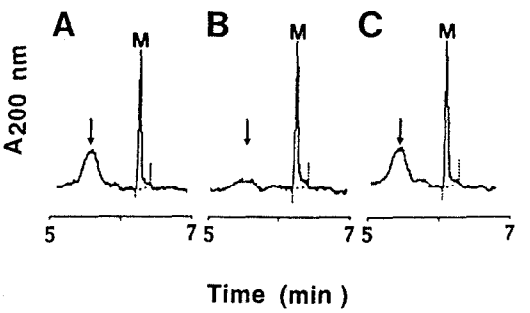

Fig. 2. Interaction of monoclonal anti-phosphotyrosine antibodies with phosphotyrosine in CE. Anti-phosphotyrosine antibody from Upstate Biotechnology. Electrophoresis at 25 kV in isotonic borate, pH 8.9 of a mixture of 20 μl antibody (arrows) (approximately 0.1 mg/ml in isotonic borate, pH 9.1) and 1 μl of a synthetic peptide (M) (Asp–Ala–Glu–Phe–Arg–NH_2) (1 mg/ml in water) pressure injected for 5 s. Detection was at 200 nm and electrophoresis took place in electrophoresis buffer with no additions (A), or with the addition of 3.8 μM phosphotyrosine (B) or 3.8 μM phosphoserine (C).

200 nm. However, as mobility in CE is related to the charge/mass ratio and phosphoserine is smaller than phosphotyrosine, its migration is most likely to be even slower than that of phosphotyrosine assuming a similar ionisation of the two molecules.

Specific interactions of the mAb from UBI could be demonstrated, i.e., binding to phosphotyrosine (Fig. 2B) but not to phosphoserine (Fig. 2C) affects the mAb peak (arrows). However, the experiment also showed that the interaction kinetics of this mAb was slow compared with the time of the experiment so that a flattened peak appeared in the presence of phosphotyrosine (Fig. 2B). Therefore, for this antibody, binding constants might be estimated through preincubation experiments as outlined above. Alternatively, a mobility shift type of experiment might have been feasible if a more complete equilibration between complexed and non-complexed species were ensured, e.g. by increasing the time of the experiment by using a lower voltage. Work is in progress to evaluate the use of the relationship between peak shape and different field strengths in affinity CE as a possible means of estimating rate constants.

For the other anti-phosphotyrosine mAb used

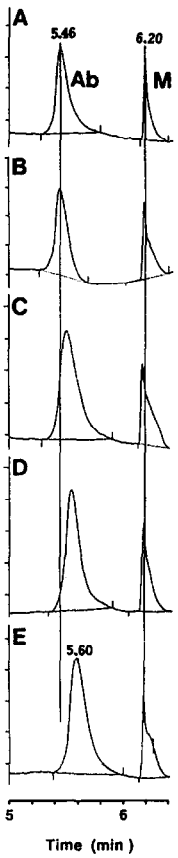

Fig. 3. Interaction of monoclonal anti-phosphotyrosine antibodies with phosphotyrosine in CE. Anti-phosphotyrosine antibody from Boehringer Mannheim. Electrophoresis under the same conditions as given above for Fig. 2 of a mixture of 80 μl 0.05 mg/ml antibody (Ab) in isotonic borate, pH 8.9 and 8 μl of the peptide marker (M) (1 mg/ml in water). Pressure injection for 1 s followed by electrophoresis in the presence of 57 μM phosphoserine (B) or 2 μM (C), 14 μM (D) and 57 μM (E) phosphotyrosine. Vertical lines indicate the time points for the mAb (Ab) and the reference marker (M) in the control experiment (A).

in this study (Fig. 3), the binding rates were fast enough within the 5–6 min of the experiment to result in migration shifts of otherwise unchanged peaks. The shifts were proportional to the amount of phosphotyrosine in the electrophoresis buffer but did not occur with phosphoserine added at comparable concentrations (Fig. 3B). Neither addition affected the internal migration marker (M) (Figs. 2 and 3). Affinity CE in the presence of a 3–800 molar excess of antigen over antibody-binding sites were conducted with this antibody (calculation based on the assumption that the mAb concentration of the sample is preserved throughout the analysis, i.e. disregarding band sharpening or diffusion during electrophoresis [27]). After compensating for small differences in the migration times of the internal marker as detailed in the Experimental section above, migration shift data were plotted (Fig. 4A). Phosphoserine had no influence on the migration time at any concentration and phos-

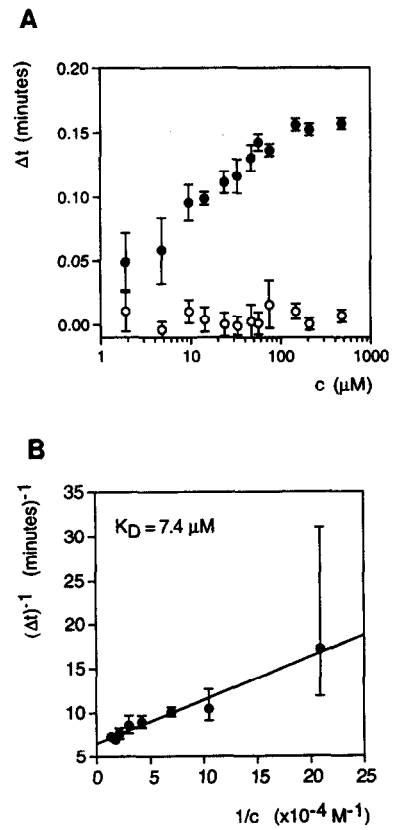

Fig. 4. CE-based binding curves for the interaction of anti-phosphotyrosine mAb with phosphotyrosine (●) and phosphoserine (○). (A) Shifts in migration time of the antibody as a function of concentration c, of ligands added to the electrophoresis buffer. Data are the means ± the standard deviations of triplicate experiments. (B) Double reciprocal plot of the data points between the maxima and minima from the experiments of A (the three upper and the lowest data point of A are excluded). The dissociation constant, K_D is calculated from the equation of the best-fit straight line ($r^2 = 0.97$).

photyrosine slowed down the resulting migration of the antibody peak until a plateau of maximum migration change of 0.15 min was reached at approximately 100 μM phosphotyrosine. Upon linearization of the data (Fig. 4B) a K_D of 7.4 μM was calculated from the equation of the best-fit straight line (correlation coefficient $r^2 = 0.97$). This value may also be estimated from Fig. 4A as the value of c that corresponds to half-maximum Δt. In the linear plot (Fig. 4B) the error bars which derive from the standard deviations of the results in Fig. 4A indicate that the relative errors are getting considerable at migration time differences below approximately 0.06 min. This reflects the limits of precision of the analytical setup. Thus, for K_D measurements using affinity CE in this system, conditions where binding shifts of more than approximately 0.07 min are measured should be used. This may be achieved through the proper combination of capillary length, field strength and buffer systems and is of course ultimatively dependent on a suitable difference in the electrophoretic velocity between complexed and non-complexed molecules.

The flexibility of the approach is illustrated by Fig. 5 where migration change data obtained at different voltages and pH values are shown. Lower field strengths result in slower migration velocities and the values of the migration shifts are therefore increased. It appears that the dissociation constants obtained at pH 8.4 within experimental error are indistinguishable from those obtained at pH 8.9.

This is also the case for the results obtained using different field strengths. With globular molecules such as immunoglobulins, high field strengths are not expected to induce conformational or orientation effects such as those affecting the electromigration of rod-shaped and large polymers such as DNA and small virus [28,29] which might influence binding interactions. Other hypothetical effects of electrical fields on binding interactions are also ignored and within the limits of these experiments these assumptions seem to be valid because the calculated binding constants were independent of field strength changes (Fig. 5).

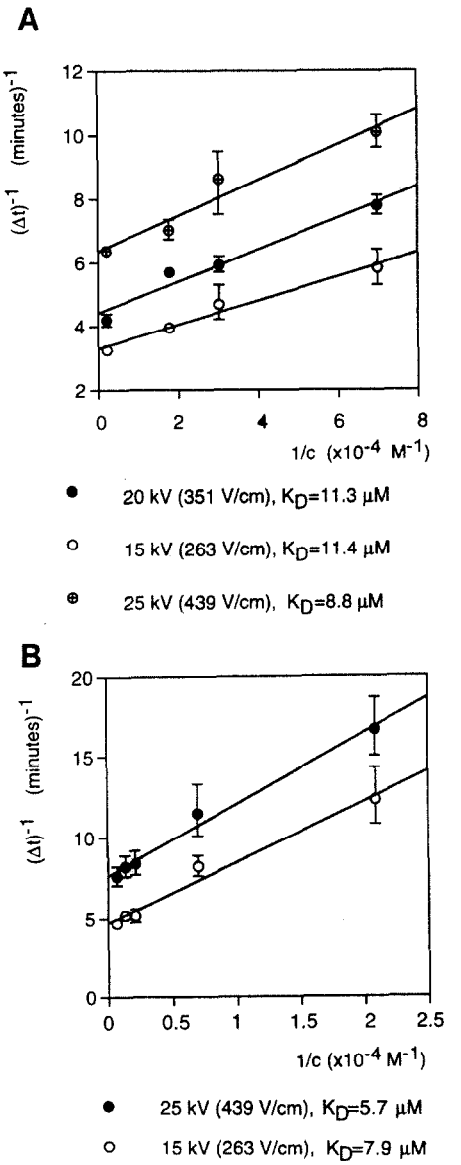

Fig. 5. CE-based binding curves for the interaction of anti-phosphotyrosine mAb with phosphotyrosine at different field strengths and pH values. Double reciprocal plots of data from immuno-CE experiments measuring migration shifts of the mAb as a function of the concentration of phosphotyrosine in the electrophoresis buffer. (A) Data obtained at different field strengths using a pH 8.9 isotonic borate buffer. Only data points where experiments have been performed at all three field strengths are included. Dissociation constants calculated on the basis of the best-fit straight line for each set of experiments. (B) Data from electrophoresis experiments performed at pH 8.4. $r^2 > 0.94$ for all experiments.

The experiments show how various separation parameters may easily be manipulated in CE. One of the main limitations for analysis of binding interactions in standard uncoated fused-silica capillaries is that the receptor molecules must be recoverable under the conditions of pH and ionic strength chosen and not participate in unwanted interactions affecting ligand binding with the walls of the capillary. Thus, in the present study it was not possible to analyze the interactions at a physiological pH because the mAb was only recoverable at pH > 8.2 even though the analyses were performed at ionic strengths corresponding to isotonic conditions.

The approach has the often repeated virtues of using unlabelled, not necessarily purified [2] receptor molecules, not requiring an exact knowledge of the concentration of receptor as long as ligand is used in excess [2,5], using small amounts of sample, and offering highly efficient and fast analyses. The determination of antibody affinity in this study required 12 triplicate analyses (discounting the control experiments with phosphoserine) each of 8 min duration. Since 0.9 nl sample was injected in each analysis, the total amount of antibody consumed during the entire analysis process corresponded to approximately 1.6 ng while a total of approximately 1.2 mg ligand (phosphotyrosine) was used.

4. Conclusions

Affinity CE is a valid method for characterization of interactions between a wide range of different molecules including proteins. It is especially well suited for the study of interactions between differently charged ligands since the method depends on differences in electromigration between complexed and non-complexed receptor molecules. In the present study, the applicability of CE is shown for the determination of binding constants for antigen–antibody binding (immuno-CE). Monoclonal anti-phosphotyrosine antibodies with sufficiently fast interaction kinetics to be amenable to binding constant determination by this method were used. It was demonstrated how antibodies initially could be screened for the suitability of the method in relation to their binding rate constants. Micromolar dissociation constants were determined and shown to be independent of the applied field strength for the separation within the range of 260–440 V/cm.

Immuno-CE as shown in this study is a convenient way to estimate affinities of mAbs with fast reaction kinetics and to compare kinetics of antibodies of similar binding constants. Further work will concentrate on the use of immuno-CE also for the characterization of polyclonal antibodies.

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