

Video Article

Analysis of AtHIRD11 Intrinsic Disorder and Binding Towards Metal Ions by Capillary Gel Electrophoresis and Affinity Capillary Electrophoresis

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Abstract

Plants are strongly dependent on their environment. In order to adjust to stressful changes (e.g., drought and high salinity), higher plants evolve classes of intrinsically disordered proteins (IDPs) to reduce oxidative and osmotic stress. This article uses a combination of capillary gel electrophoresis (CGE) and mobility shift affinity electrophoresis (ACE) in order to describe the binding behavior of different conformers of the IDP AtHIRD11 from *Arabidopsis thaliana*. CGE is used to confirm the purity of AtHIRD11 and to exclude fragments, posttranslational modifications, and other impurities as reasons for complex peak patterns. In this part of the experiment, the different sample components are separated by a viscous gel inside a capillary by their different masses and detected with a diode array detector. Afterward, the binding behavior of the sample towards various metal ions is investigated by ACE. In this case, the ligand is added to the buffer solution and the shift in migration time is measured in order to determine whether a binding event has occurred or not. One of the advantages of using the combination of CGE and ACE to determine the binding behavior of an IDP is the possibility to automate the gel electrophoresis and the binding assay. Furthermore, CGE shows a lower limit of detection than the classical gel electrophoresis and ACE is able to determine the manner of binding a ligand in a fast manner. In addition, ACE can also be applied to other charged species than metal ions. However, the use of this method for binding experiments is limited in its ability to determine the number of binding sites. Nevertheless, the combination of CGE and ACE can be adapted for characterizing the binding behavior of any protein sample towards numerous charged ligands.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57749/>

Introduction

Plants are more dependent on their environment than many other life forms. Since plants cannot move to other places, they have to adjust to changes in their surroundings (e.g., drought, cold and high salt concentrations). Consequently, higher plants developed specialized stress proteins like dehydrins, which fulfill manifold tasks to reduce cell stress related to high salinity. These proteins bind water and ions inside cells, reduce oxidative stress by binding Cu^{2+} -ions, and interact with phospholipids as well as the cytoskeletons. Moreover, binding Zn^{2+} -ions allows these proteins to act as transcription factors. Their ability to bind Ca^{2+} -ions after phosphorylation has also been reported¹.

The multifunctional behavior of these proteins is related to the absence of hydrophobic amino acid residues. Consequently, they lack any hydrophobic interactions inside the peptide chain and also a constrained structure. However, because these proteins lack a restrictive structure, they can occupy different conformers under the same conditions. Therefore, they can be described best as an ensemble of structures rather than as a single conformation. Proteins with these properties are known as intrinsically disordered proteins (IDPs) and are a widely used concept for stress proteins and crosstalk between different pathways in eukaryotic cells².

One of these stress-related IDPs is AtHIRD11. It is one of *Arabidopsis thaliana*'s most highly drought-expressed IDPs. Hence, the different conformers can be separated by their effective radius to charge ratio, and capillary electrophoresis (CE) has been used for further investigations. Previous ACE experiments demonstrated the interactions between AtHIRD11 and transition metal ions such as Cu^{2+} -, Zn^{2+} -, Co^{2+} -, and Ni^{2+} -ions. The detailed results can be found in Hara *et al.*³ and Nachbar *et al.*⁴.

The ACE method that will be used here is based on our earlier published works⁶. However, the addition of the EOF marker acetanilide to the protein sample is not suitable. AtHIRD11 shows broad peak patterns, and adding the EOF marker to the sample would disguise two peaks. Therefore, the marker is used in a separate run. Before the binding behavior is examined, it is confirmed that the peaks found during previous experiments are from different conformers. Thus, CGE is used to distinguish between the protein conformers, the post-translational modified protein, and impurities, such as fragments of AtHIRD11, by their different masses. Subsequently, the characterized AtHIRD11 sample's binding behavior towards various different metal ions is investigated.

The purpose of this article is to describe an experimental setup to distinguish between an IDP and other components of a sample in order to evaluate the differences in the binding behavior of different conformers.

Protocol

1. Preparation of the Capillary Electrophoresis Instruments

1. Prepare the capillaries

1. Use a glass cutter to cut a bare-fused silica capillary with a polyimide external coating and an inner diameter of 50 μm into 33 cm (for the CGE experiment) and 30 cm (for the ACE experiment) long capillaries. Perform the cutting on a glass plate.
Note: The 2 different experimental setups were developed for 2 different instruments. In order to use them on other instruments, a proper method transfer has to be performed and the capillary length has to be adjusted to the allowed length of the instrument.
2. Use a pen to mark the middle of a 1-cm wide detection window at a distance of 24.5 cm from one end of the capillary for the CGE experiment and at 21.5 cm from one end of the capillary for the ACE experiment (effective length).
3. Remove the polyimide external coating from the capillaries by burning it off with a blowtorch, 0.5 cm before and after the mark. Similarly, use the blowtorch to remove 1 cm of the coating at both ends of the capillaries.
Note: The effective length may vary slightly since it relies on the capillary electrophoresis instrument used.
4. Clean the ends of the capillaries and the detection windows with ethanol and a soft tissue.
Note: The uncoated capillaries are less flexible and likely to break.

2. Install the capillaries

1. Install a capillary into the CE system with the detection window near the outlet.
NOTE: The various CE instrument models have different holding systems for the capillaries. Refer to the manual of the used instrument for the exact instructions.

2. Prepare the Solutions

1. Prepare the solutions for the CGE analysis

1. Prepare the tris(hydroxymethyl)aminomethane (Tris)-SDS buffer (100 mM/1% w/v) at pH 8.0.
 1. Use a respiratory mask and a booth for weighing 1.00 g of sodium dodecyl sulfate (SDS) in a 100-mL beaker.
 2. Add 1.21 g of Tris into the 100 mL beaker and dissolve it using 50 mL of deionized water and a magnetic stir bar on a magnetic stirrer.
 3. Put a pH electrode in the solution and adjust the pH to 8.0 using 1.0 M HCl.
 4. Fill the solution into a 100 mL volumetric flask and add deionized water up to the mark. Shake it gently to avoid any foam forming.
2. Prepare the 0.1 M NaOH solution.
 1. Weigh 4.0 g of NaOH in a 100 mL volumetric flask. Fill it up to the mark with deionized water to make a 1 M stock.
 2. Use a 10 mL bulb pipette to transfer 10 mL of this solution into another 100 mL volumetric flask and fill it up to the mark with deionized water.
3. Prepare the 0.1 M HCl solution.
 1. Put 10 mL of 10 M HCl into a 100 mL volumetric flask using a 10 mL volumetric pipette and fill it up to the mark with deionized water. Repeat this step to obtain the 0.1 M HCl.
4. Prepare the sample solution.
 1. Weigh 0.5 mg of the protein into a 1 mL tube. Add 0.5 mL of the Tris-SDS gel buffer from step 2.1.1 with a micropipette.
 2. Dissolve the protein by shaking gently to avoid any foam forming and degradation of the protein.
NOTE: Use ultrasonication if the protein cannot be dissolved as described; avoid any heating of the solution.
5. Fill the solutions into the vials.
 1. Filter each solution through a 0.22 μm polyvinylidene fluoride (PVDF) filter using an adequate syringe directly into the vial.
NOTE: For the gel-containing solutions, the back pressure can be high due to its high viscosity.
 2. Prepare 15 vials in total and mark each to avoid any mix-ups.
 1. Fill 3 vials with a commercially available sodium dodecyl sulfate (SDS) gel buffer at pH 8.
NOTE: The use of a commercially available SDS gel buffer provides more precise results.
 2. Fill 1 vial with 0.1 M NaOH and 1 vial with 0.1 M HCl for flushing.
 3. Fill 1 vial with the sample solution.
 4. Fill 5 vials with deionized water and 4 vials with 0.1 mL of deionized water (dipping vials).
NOTE: The dipping vials are used as waste vials during the flushing of the capillary before each run. The capillary ends are dipped in water in order to rinse off any gel residues.

2. Prepare the solutions for the ACE analysis

1. Prepare the 1 M NaOH solution.
 1. Weigh 4.0 g of NaOH in a 100 mL volumetric flask.

2. Fill it up to the mark with deionized water to make a 1 M stock.
2. Prepare the 0.1 M ethylenediaminetetraacetic acid (EDTA) in a 0.1 M NaOH solution.
 1. Transfer 10 mL of 0.1 M NaOH in a 100 mL volumetric flask using a 10 mL bulb pipette. Fill it up to the mark with deionized water.
 2. Weigh 2.42 g of EDTA on a weighing boat and dissolve the solid compound in the 100 mL of 0.1 M NaOH.
3. Prepare the Tris buffer (30 mM) solution.
 1. Add 3.63 g of Tris, 200 mL of deionized water, and a stir bar in a 500 mL beaker. Place the beaker on a stir plate and switch it on.
 2. Put a pH meter electrode in the beaker and adjust the pH to 7.4 using 0.1 M HCl.
 3. Fill the solution in a 1 L volumetric flask and fill up to the mark with deionized water.

NOTE: This procedure has to be repeated or a bigger volumetric flask is needed since more than 1 L is needed for the whole analysis.
4. Prepare the acetanilide electroosmotic flow (EOF)-marker (60 μ M) solution.
 1. Add 6 mg of acetanilide and 100 mL of Tris buffer in a 100 mL volumetric flask.
 2. Put the volumetric flask in an ultrasonic bath and switch it on for 30 min.
5. Prepare the sample (1 mg/mL) solution.
 1. Put a 0.5 mL microcentrifuge tube on a precision balance and add 0.3 mg of freeze-dried AtHIRD11 powder into the tube.
 2. Use a micropipette to add 0.3 mL of a 30 mM Tris buffer (pH 7.4) in the tube. Shake the tube carefully to dissolve the protein.
6. Prepare the ligand solutions.
 1. Prepare the CaCl_2 (5 mM) stock solution.
 1. Take a weighing boat, put it on an analytical balance and weigh 36.76 mg of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$.
 2. Using a micropipette, flush the $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ from the weighing boat into a 50 mL volumetric flask with the previously prepared Tris buffer.
 3. Fill the volumetric flask with the Tris buffer up to the mark.
 2. Prepare the remaining stock solutions (5 mM).
 1. Repeat step 2.2.6.1 using the other metal salts instead of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ [MgCl_2 : 23.80 mg, BaCl_2 : 26.02 mg, $\text{Sr}(\text{NO}_3)_2$: 52.91 mg, MnCl_2 : 31.46 mg, SeCl_4 : 52.91 mg, $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$: 41.47 mg, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$: 42.62 mg, ZnCl_2 : 3.41 mg, and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$: 59.43 mg].

NOTE: The ZnCl_2 solution has a concentration of 0.5 mM. Since strong interactions between the ions and the inner capillary wall were observed, the concentration was reduced by a factor of 10^4 .
 3. Prepare the 500 μ M CaCl_2 solution.
 1. Fill 10 mL of the CaCl_2 stock solution and put it in a 100 mL volumetric flask.
 2. Fill the volumetric flask with a Tris buffer to the mark and shake the flask.
 4. Prepare the 250 μ M CaCl_2 solution.
 1. Fill 5 mL of the CaCl_2 stock solution and put it in a 100 mL volumetric flask.
 2. Fill the volumetric flask with a Tris buffer to the mark and shake the flask.
 5. Repeat steps 2.2.6.3 and 2.2.6.4 for the other stock solutions.
7. Fill the solutions in the vials.

NOTE: Every repeated run needs a set of inlet and outlet vials. The use of fresh ligand solutions at the inlet and outlet reduces the shifts in migration time after each run.

 1. Fill the 250 μ M CaCl_2 solution in a 10 mL syringe.
 2. Put a 0.22 μ m PVDF filter on the syringe and push 2 mL of the solution through the filter using the syringe in order to discard this filtered solution.
 3. Fill 10 vials up to the maximum allowed volume with the remaining 250 μ M CaCl_2 solution of the syringe. Mark each as **250 μ M CaCl_2 solution inlet vial**.
 4. Fill 10 vials until they are half-filled with the 250 μ M CaCl_2 solution. Mark each as **250 μ M CaCl_2 solution outlet vial**.
 5. Repeat steps 2.2.7.1 - 2.2.7.4 for the other metal salt-containing solutions.
 6. Repeat the steps for every pair of inlet and outlet vials using the 30 mM Tris buffer (pH 7.4) solution instead.

Note: The 30 mmol/L Tris buffer pH 7.4 is used in between the runs in order to obtain migration time data for the protein in absence of metal ions. It is necessary in order to neglect changes in the EOF.
 7. Repeat the above steps for one vial using the acetanilide EOF-marker (60 μ M) solution instead. Also, repeat these steps using the sample (1 mg/mL) solution instead.

3. Capillary Gel Electrophoretic Separation

Note: Prepare the separation according to the method described in previous work by Nachbar *et al.*⁴.

1. **Prepare the analysis**
 1. **Condition the capillary**
 1. Set the thermostat to 23 $^{\circ}\text{C}$.

2. Flush the capillary for 10 min at 2.5 bar with 0.1 M NaOH.
3. Flush the capillary for 5 min at 2.0 bar with 0.1 M HCl.
4. Flush the capillary for 2 min at 2.0 bar with deionized water.

2. **Fill in the SDS gel buffer**

1. Fill a capillary for 10 min at 2.0 bar with the commercially available SDS gel buffer at pH 8.0.

3. **Flush the capillary before each separation run**

1. Flush the capillary for 3 min at 4.0 bar with 0.1 M NaOH.
2. Flush the capillary for 1 min at 4.0 bar with 0.1 M HCl.
3. Flush the capillary for 1 min at 4.0 bar with deionized water.
4. Flush the capillary for 10 min at 4.0 bar with the SDS gel buffer.

2. **Run the separation**

1. Inject the sample solution for the CGE experiments (step 2.1.4) hydrodynamically by applying 0.1 bar for 4 min at the inlet.
2. Apply -16.5 kV and a pressure of 2.0 bar at both ends of the capillary for 25 min.

4. Affinity Capillary Electrophoretic Analysis

Note: Prepare the separation according to the method described in previous works by Nachbar *et al.*⁴ and Alhazmi *et al.*⁵.

1. **Condition the capillary**

1. Set the thermostat to 23 °C.
NOTE: The temperature is used according to published protocols and can be changed to other temperatures^{4,5}. However, the interactions are dependent on the temperature and will change accordingly.
2. Flush a capillary for 40 min at 2.5 bar with 0.1 M NaOH.
3. Flush the capillary for 10 min at 1.0 bar with deionized water.
4. Flush the capillary for 30 min at 1.0 bar with a 30 mM Tris buffer (pH 7.4).

2. **Preparation for the ACE methods**

1. Prepare the method for the measurements without ligands.
Note: Acetanilide was not added to the sample solution, as it disguises 2 protein peaks.
 1. Rinse the capillary for 1 min at 2.5 bar with a 0.1 M EDTA solution.
 2. Rinse the capillary for 1 min at 2.5 bar with deionized water.
 3. Rinse the capillary for 1.5 min at 2.5 bar with a Tris buffer for equilibration.
 4. Inject the acetanilide solution for 6 s at 0.05 bar and change the inlet and outlet vials to the Tris buffer vials.
 5. Apply 0.05 bar for 2.4 s in order to push the acetanilide solution from the tip of the capillary further inside.
 6. Apply 10.0 kV for 6 min and detect the acetanilide peak at a wavelength of 200 nm.
 7. Repeat steps 4.2.1.1 - 4.2.1.6. using the protein sample instead of the acetanilide solution and detect all the protein peaks.
2. Prepare the method for the measurements with ligands.
 1. Rinse the capillary for 1 min at 2.5 bar with a 0.1 M EDTA solution.
 2. Rinse the capillary for 1 min at 2.5 bar with deionized water.
 3. Rinse the capillary for 1.5 min at 2.5 bar with the ligand solution for equilibration.
 4. Inject the acetanilide solution for 6 s at 0.05 bar and change the inlet and outlet vials to the ligand-containing buffer vials.
 5. Apply 0.05 bar for 2.4 s in order to push the acetanilide solution from the tip of the capillary further inside.
 6. Apply 10.0 kV for 6 min and detect the acetanilide peak at a wavelength of 200 nm.
 7. Repeat steps 4.2.2.1 - 4.2.2.6 using the protein sample instead of the acetanilide solution and detect all the protein peaks.
3. Alternately repeat steps 4.2.1 and 4.2.2 in order to calculate the change in the charge-size ratios for the various protein-metal ion interactions (described under **Representative Results**).
 1. Change the protein solution after every 60 h to a fresh one.
NOTE: At this point, the experiment can be paused. This procedure is at least repeated 10x for each concentration of the ligands since the peak pattern varies slightly from run to run. If the solution is used longer than 60 h, the variations become too large.
4. **Run the methods**
 1. Run the method described under step 4.2.2, using the alkaline earth ligand solutions (CaCl₂, MgCl₂, BaCl₂, and SrCl₂ solutions).
 2. Continue using the method described under step 4.2.2, using the MnCl₂, SeCl₄, CoCl₂, CuCl₂, ZnCl₂, and NiCl₂ solutions instead of the alkaline earth ligand solutions.
NOTE: The latter transition metal chlorides showed stronger interactions with the capillary and cannot be removed completely. These interactions result in shifts in the migration time from run to run. Consequently, they were investigated after the other metal ions.

Representative Results

Figure 1 shows the electropherogram for the AtHIRD11 sample obtained during the CGE experiments. The peptide size increases from left to right. Peak number 4 has the largest mass and indicates the intact protein. The smaller peaks 2 and 3 represent smaller impurities (e.g., degradation products). The first peak and the inconsistency of the baseline before could also be reproduced without the protein sample. Therefore, it is related to the SDS gel itself and does not represent any sample-associated impurities.

Figure 2 represents the electropherogram for acetanilide during the ACE experiments. The acetanilide solution shows only 1 high peak since it should have no impurities. The detection time for the peak maximum indicates the migration time of the electroosmotic flow (t_{EOF}) and is used for the calculation of the interaction.

Figure 3 is the electropherogram of the AtHIRD11 sample during the ACE experiment in the absence of SDS and metal ions. It shows, besides the 2 impurities from the CGE electropherogram, at least 5 peaks which are related to the protein itself. Peak 1 and 2 can be assigned to impurities since their migration times indicate that they are small or highly positively charged. This assumption is supported by the increase of the peak height and the area over time. Since AtHIRD11 peaks 3 and 4 are close to the EOF, they are hardly charged and cannot be separated in every run. They almost show no interactions and represent conformations without accessible residues essential for the interactions with the metal ions. AtHIRD11 shows several different charge-to-size-ratios due to different effective radii of different conformers. These conformers can merge continuously. Subsequently, peaks 5 - 7 are broader than the usual protein peaks. The broad peaks give a hint on the kinetics of the interactions. Since the peaks are not narrow, a fast and a very slow conversion between the different conformers can be excluded. In both cases, the peaks would be baseline-separated⁴. In this case, a pre-equilibration step can change the peak pattern and would give a better insight into the interactions with slower kinetics.

Figure 4 shows the graphical evaluation of the measured migration time shifts in the presence of the various metal ions for peak 6. It is represented by the calculated value $\Delta R/R_f$. This value indicates how strong a shift is (by its value) and the overall change of the protein-metal ion complexes charge (by its sign). In order to differentiate between a significant interaction and a coincidental shift, the confidence intervals for $\alpha = 0.05$ are calculated as well. In cases where the confidence interval is not intersecting with the zero line, the interaction is considered as significant. Results are taken into account if the peak was present in at least 6 out of 10 electropherograms. Peak 6 was not present in any run with 500 μM Co^{2+} . The complete list of the interactions for each AtHIRD11 peak is published in an earlier work by Nachbar *et al.*⁴.

$\Delta R/R_f$ is calculated using the migration time ratios R_i (in the presence of the ligand) and the migration time ratios R_f (in the absence of the ligand):

$$\frac{\Delta R}{R_f} = \frac{R_i - R_f}{R_f}$$

R_i and R_f are calculated using the peak top times $t_{protein}$ for the protein sample peaks and the corresponding peak top time for the EOF-marker t_{EOF} :

$$R = \frac{t_{EOF}}{t_{protein}}$$

For R_i , the times in the presence of the ligand are used and for R_f , the times in absence.

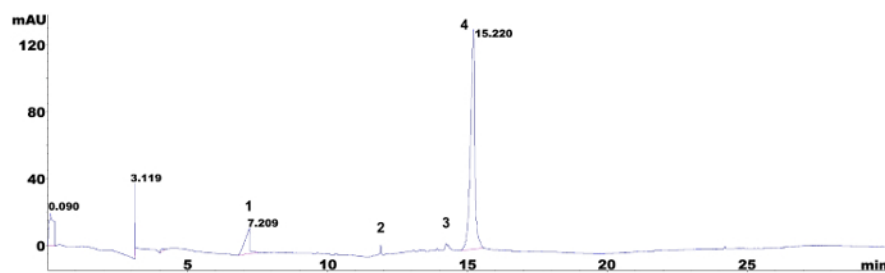


Figure 1: Electropherogram for the CGE separation of the AtHIRD11 sample in gel buffer. Peak 1 and the peaks on its left can be observed even without a sample injection, so they are artefacts. Since the masses and the overall peak areas of peak 2 and 3 are smaller than that of peak 4, they are likely impurities, such as degradation products. [Please click here to view a larger version of this figure.](#)

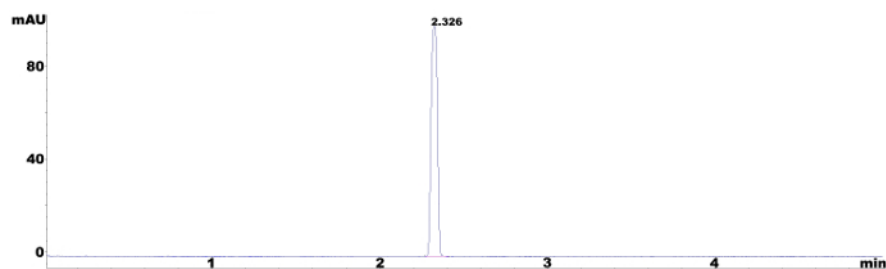


Figure 2: Electropherogram of the EOF-marker acetanilide run. The figure shows only 1 peak for the compound without any metal ions and SDS gel buffer. The time of the peak top marks the electroosmotic flow. [Please click here to view a larger version of this figure.](#)

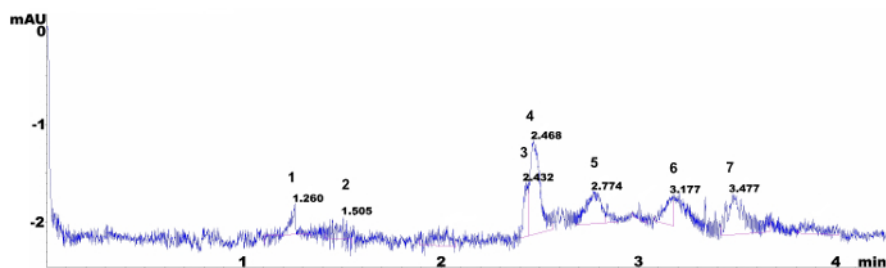


Figure 3: Example for an electropherogram of the sample separation during the ACE experiments showing a complex peak pattern in the absence of any metal ions. At least 7 peaks could be identified and related to the protein and its 2 impurities. [Please click here to view a larger version of this figure.](#)

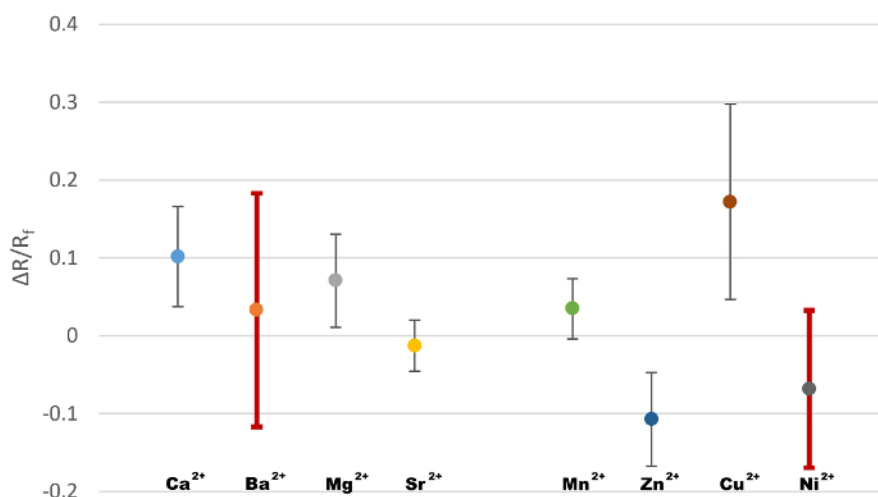


Figure 4: Graphical evaluation of the binding behavior towards the investigated metal ions using a concentration of 500 μM for peak 6. The values of $\Delta R/R_f$ give a hint on the intensity of the migration time shift and therefore on the strength of the conformational changes on metal ion binding. The sign of $\Delta R/R_f$ indicates if the complex is more positively or negatively charged compared to the unbound protein. The error bars indicate the confidence intervals for $\alpha = 0.05$. Subsequently, they show the area where the true $\Delta R/R_f$ -value can be found with a certainty of 95%. The red bars indicate the calculated results obtained by insufficient data (the peak was present in less than 6 electropherograms). [Please click here to view a larger version of this figure.](#)

Discussion

For all CE experiments, the preparation of the capillary is a critical step. Since it is a glass tube with a small diameter, it can easily break at the spots where the coating is removed when it is being handled. The installation of the capillary into the instrument should be done very carefully.

The critical steps involved in using the ACE method mainly pertain to the experimental setup. Another critical step is finding the right sample injection parameters. The injected amount of the sample has to be enough for high peaks. However, overloading it would result in a poorly resolved peak pattern with overlapping peaks. When decreasing the injected volume, it is recommended to decrease the injection pressure instead of the injection time since it takes some time for the instrument to reach the programmed injection pressure.

Unusually broad peaks can be found for different protein samples. This pattern can be influenced by increasing the voltage. Consequently, the peaks get narrower and the separation time is reduced. Nevertheless, the shorter separation time can result in not-resolved peaks and the higher

voltage increases the Joule heating and limits the effect of narrow peaks by electrophoretic dispersion^{7,8}. Therefore, the right conditions have to be determined for each new protein examined.

Combining CGE and ACE to gather information about the intrinsically disordered character of a protein and its binding behavior is a novel approach. The characterization of the sample by gel electrophoresis is a necessary step to prove that any multiple peaks observed are not related to impurities. The advantage of CGE is the possibility to automate it, the shorter preparation time, and a higher resolution compared to the classical gel electrophoresis⁹. Using ACE for IDP binding experiments shows its superiority towards other assays (e.g., immobilized metal affinity chromatography in the results) since ACE not only shows binding events. Moreover, it separates different conformers of a protein and can reveal differences in binding ligands of diverse conformers. This behavior cannot be detected by methods without a separation during the binding experiment. In addition, the analysis time is only 6 min; therefore, the results can be calculated in a short amount of time¹⁰. On the other hand, the fast analysis comes with limitations in estimating the number of binding sites¹¹.

The future of this approach lies in the possibility to screen potential binding partners for proteins, especially IDPs, in a fast manner. It gives a quick overview of the different binding behavior of the various conformers present in a sample. The use of a size-dependent separation (e.g., CGE) for the sample characterization is mandatory since ACE itself cannot distinguish between impurities and the protein itself. However, CGE gives an idea about the number of different-sized species. The application of ACE as a binding assay has been extended already from metal ion ligands to any charged ligand¹². Therefore, it can add additional information about the interactions to any characterized binding, since the combination of the separation and the binding assay in one method can give evidence of any altered binding behavior of different conformers and posttranslational modifications, respectively.

Disclosures

The authors have nothing to disclose.

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