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ELECTROPHORETIC MOBILITY SHIFT ASSAY OF ZINC FINGER PROTEINS: COMPETITION FOR Zn^{2+} BOUND TO SP1 IN PROTOCOLS INCLUDING EDTA

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Abstract

The electrophoretic mobility shift assay (EMSA) offers a principal method to detect specific DNA-protein interactions. As commonly conducted, the reaction and electrophoresis running buffers contain large concentrations of EDTA. EDTA has large affinity for Zn^{2+} and readily competes with zinc-finger peptides for Zn^{2+} resulting in protein unfolding. Nevertheless, EMSA is routinely used to detect zinc-finger protein-DNA adducts. This paper examines the chemistry that permits the detection of zinc-finger-DNA complexes in the presence of EDTA, using Zn_3 -Sp1 and a cognate DNA binding site, GC1. Twice as much adduct was detected when the reaction was conducted in the absence than in the presence of EDTA. The observation of Zn -Sp1-GC1 was shown to depend on three properties: the inertness of Zn -Sp1-GC1 to reaction with EDTA and the comparatively similar rates of reaction of EDTA and GC1 with Zn_3 -Sp1 under the conditions of the assay that permit some Zn_3 -Sp1-GC1 to form. Inquiring about the mechanism of stabilization of Zn_3 -Sp1 by GC1, EDTA readily reacted with Zn_3 -Sp1 bound to a non-specific DNA, poly(dI-dC). Two structurally similar but oppositely charged chelators, nitrilotriacetate (NTA) and tris-(2-ethylaminoethyl) amine (TREN), that react with free Zn_3 -Sp1 failed to compete for zinc bound in the Zn_3 -Sp1-GC1 adduct. On the basis of these and other results indicated that the stability of Zn_3 -Sp1-GC1 has a thermodynamic not a kinetic origin. It is concluded that the observation of zinc finger proteins in the EMSA rests on a fortuitous set of chemical properties that may vary depending on the structures involved.

Keywords

zinc-finger; protein; EDTA; EMSA

1. Introduction

The electrophoretic mobility shift assay (EMSA) is a powerful, widely used method that tests the capacity of purified proteins or proteins in cell extracts to bind selectively to

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specific double stranded DNA sequences. In general, the method compares the electrophoretic mobility of a free DNA sequence that has been tagged for visualization purposes with the DNA sequence incubated with a protein or mixture of proteins [1]. If specific protein-DNA adducts form, they are detected by much reduced rates of migration of the labeled protein-DNA complex in the electric field of the electrophoresis apparatus.

Zinc finger transcription factors comprise the largest class of DNA binding proteins as well as the largest group of Zn-metalloproteins according to recent bioinformatic studies [2,3]. Of these, by far the most common zinc finger motif is that based on $\beta\beta\alpha$ sequential secondary structure and Zn^{2+} coordination by 2 cysteinyl sulfhydryl groups in the β strands and 2 histidyl imidazole nitrogens in the α helix ($\text{C}_2\text{H}_2 \text{Zn}^{2+}$ coordination).

The EMSA method is routinely used to detect the presence of a Zn-finger transcription factor and to examine its DNA binding properties [4–9]. Recent examples from the authors' laboratories include Zn-transcription factor IIIA and Zn-Sp1 [10–13]. Another involving C_2H_2 transcription factor is MTF-1, the metal ion responsive transcription factor that is responsible for up-regulation of metallothionein synthesis in the presence of elevated Zn^{2+} or Cd^{2+} [14,15].

The reaction buffer and/or the electrophoretic running buffer for the EMSA commonly contain EDTA [4–9]. EDTA is a potent metal ion chelating agent that exhibits high affinity for a variety of biologically essential as well as non-essential metal ions, including Zn^{2+} and Cd^{2+} . At pH 7.4 and 25° the log formation constant for Zn-EDTA is 13.3 [16] Zn-finger transcription factors that utilize the C_2H_2 ligand set bind Zn^{2+} with apparent stability constants for Zn^{2+} that range from about 10^7 to 10^{11} [11,17]. Therefore, it is evident that even under stoichiometric conditions, EDTA should almost completely sequester Zn^{2+} from the most robust of these structures if the kinetics of reaction are favorable.

At the lower end of Zn^{2+} stability among such zinc finger proteins is, for example, transcription factor IIIA; its purification and storage procedures call for the presence of Zn^{2+} in isolation buffers to help to stabilize the association of Zn^{2+} with the finger mini-domains [10,11]. Similarly, with MTF-1, changing the ambient Zn^{2+} concentration *in vitro* in the μM range alters its DNA binding capacity, presumably through changing the occupancy of some of its zinc fingers [15]. Sp1 appears to bind Zn^{2+} more robustly and does not require storage in the presence of excess Zn^{2+} [18].

With each of these transcription factors, incubation with EDTA inactivates it toward specific association with cognate DNA [10,13,15]. Thus, contact with EDTA during the EMSA as described above raises the practical question whether at least some Zn-finger proteins react with EDTA and become inactivated toward DNA adduct formation during routine use of this method [19]. The fact that some Zn-finger transcription factors can be assayed for DNA binding in the presence of EDTA brings forth the related issue of how, mechanistically, this might occur. These two questions are the subject of this report. They are addressed by considering the properties of reaction of the Zn-finger transcription factor $\text{Zn}_3\text{-Sp1}$ with EDTA and related metal chelating agents in the absence and presence of cognate DNA binding sequences [20–22].

2. Experimental

2.1 Materials

EDTA (reagent grade) and other chemicals (molecular biology grade) were purchased from Sigma-Aldrich Chemical Co. Recombinant human Sp1 (rh Sp1) was obtained from Promega Corp. Its buffer includes 5 μM Zn^{2+} to insure complete saturation of the Zn-finger binding

sites. Electrophoretic mobility shift assays were performed using reagents in the DIG Gel Shift Kit, 2nd Generation (Roche). DIG labeled oligonucleotides for DNA binding reactions were synthesized by Integrated DNA Technologies. Oligonucleotides labeled with an infrared probe were purchased from LI-COR Biosciences.

2.2 Electrophoretic Mobility Shift Assay

In EMSA experiments, the DNA (3.6 nM) labeled with a fluorescent or infrared absorbing molecule, was reacted with protein (45 nM) in a reaction buffer that also contained (ca. 85 μ M base pairs) of nonspecific competing DNA (polydI-dC) and (400 μ M) of nonspecific competing peptide (poly-lysine) and then electrophoresed through a polyacrylamide gel with 6% cross-linking.

Commercially available EMSA kits use EDTA as a common quench agent in the DNA labeling reaction and as a prominent component of the reaction mixture. It is also part of the electrophoresis running buffer. Other metallic and metal binding ligand species are also present in the protein-DNA reaction medium. These components and their respective concentrations are listed in Table 1. The electrophoresis running buffer contained 0.2 mM Tris-borate-EDTA [26].

In the present study, recombinant human Sp1 and DIG- or IR-labeled oligomer from the promoter of mouse *sglt1* (GC1) were subjected to EMSA [12]. DNA migration was then detected by chemiluminescence or infrared methods. Besides protein and DNA, the reaction solution also contained either the Promega reaction buffer or the same buffer without EDTA and DTT, which included 20 mM Hepes, pH 7.6, 10 mM $(\text{NH}_4)_2\text{SO}_4$, Tween 20, 0.2% (w/v), 30 mM KCl and contributions from the Zn_3 -Sp1 stock solution and the reaction mixture used to synthesize DIG or IR labeled DNA [23–25]. The removal of DTT did not affect the formation of Zn_3 -Sp1·GC1.

In the present experiments, Zn_3 -Sp1 (45 nM) was incubated for 15 min at room temperature with increasing concentrations of EDTA or other metal chelators. Then, 3.6 nM labeled DNA sequence was added and the binding reaction mixture incubated for an additional 15 min at 25° C. Reaction mixtures were separated on a 6% non-denaturing acrylamide gel and then blotted onto a nylon membrane. After incubating the blot with anti-DIG-alkaline phosphatase conjugated antibody, CSPD substrate was added and the chemiluminescent bands were detected and quantified with a Kodak Image Station 2000. Alternatively, when IR-labeled DNA was employed, the bands were detected directly in the gel on a glass slide using an Odyssey IR imager. All summary data reported here were averages $\pm$ standard deviation for 3 or more runs.

2.3 Kinetic Analysis of the Reactions of Zn_3 -Sp1 with EDTA

The rate of reaction of Zn_3 -Sp1 with EDTA was observed with the EMSA method by monitoring the presence of Zn_3 -Sp1·GC1 after the time dependent interaction of Zn_3 -Sp1 with EDTA at 25° C. The reaction kinetics were treated as a pseudo-first order process, so that $\ln([\text{Zn}_3\text{-Sp1}]_t)$ was plotted vs. time (t). The negative slope of the resultant straight line was the observed rate constant (k_{obs}) for the reaction [23,27–29].

2.4 Zn_3 -Sp1·GC1 Association Rate Constant

The association rate constant (k_{on}) for the Zn_3 -Sp1·GC1 was calculated after measuring the fraction of Zn_3 -Sp1·GC1 complex formed as a function of time during the reaction of Zn_3 -Sp1 with GC1 at 25° C. The reactions were conducted using 2.5 nM Zn_3 -Sp1 and 15 nM GC1. The latter concentration was 100 times the K_d for the reaction as determined below. Thus, the reaction kinetics could be treated as proceeding to completion. The formation of

Zn₃-Sp1-GC1 complex was quenched at different time points by the addition of 50 fold excess of unlabeled GC1 and lowering the temperature to 4° C. Then, treating the results as a second order rate process and plotting $\ln([Zn_3-Sp1]_t/[GC1]_t)$ vs time yielded a slope proportional to the negative of the rate constant of the reaction.

2.5 Dissociation Constant (K_d) of the Zn₃-Sp1-GC1 DNA Complex

The dissociation constant, K , of Zn₃-Sp1 bound to the GC rich promoter sequence of Na⁺-dependent glucose transporter (GC1) was measured by Scatchard analysis as follows. Zn₃-Sp1 (10 nM) was titrated with increasing concentrations of specific infrared labeled GC1 DNA in a concentration range of 0.9 to 25nM at 25° C. Then standard EMSA was carried out as described above. A Scatchard plot of $[Zn_3-Sp1-GC1]/[GC1]_{free}$ vs $[Zn_3-Sp1-GC1]$ has a slope of $-1/K_d$ and x- intercept of $[Zn_3-Sp1]_{TOTAL}$.

3. Results

3.1 EDTA and EMSA Buffers

Table 1 reveals that the reaction buffer for a standard EMSA protocol contained 200 μ M EDTA, a powerful, broad specificity, metal chelating agent as well as substantial concentrations of DTT, a reductant that also has significant affinity for metal ions such as Zn²⁺ [19]. EDTA and perhaps DTT can compete for Zn²⁺ bound to Sp1, which binds 3 Zn²⁺ ions per molecule [30]. EDTA's conditional stability constant for Zn²⁺ binding at pH 7.4 is $10^{13.3}$, whereas, that for Zn²⁺ ion associated with one of the Sp1 fingers is 1.7×10^9 at pH 7.0 [16,18]. Thus, on an equilibrium basis, nanomolar concentrations of native Zn₃-Sp1 cannot exist in the presence of even much smaller concentrations of EDTA. The same conclusion applies to all other Zn-finger proteins of the classical C₂H₂ type [17]. The experiments below probe the underlying chemistry that permits the EMSA to work in the presence of EDTA.

3.2 Electrophoretic Mobility Shift Assay with Zn-Sp1 and GC1 DNA Binding Site: Effect of EDTA

The EMSA for Zn₃-Sp1 binding to the GC1 DNA sequence in the mouse Na⁺-glucose co-transporter 1 gene promoter was conducted using a standard commercial protocol that included EDTA in the reaction buffer and a standard electrophoresis running buffer (Table 1). The migration of GC1 oligonucleotide (lane 1) was retarded by the formation of Zn₃-Sp1 GC1 in lane 3 of Fig. 1A. The same reaction was carried out in the standard reaction buffer system minus EDTA. As seen in lane 2, a more intense band of the protein-DNA adduct was observed, indicative of the presence of a 2-fold larger concentration of Zn₃-Sp1 that was able to bind to GC1 in the absence of EDTA (Fig. 1B). In lanes 4 and 5, comparison of the extent of DNA binding by a non-Zn-finger transcription factor, Oct-2A, in the presence and absence of EDTA in the reaction buffer failed to demonstrate an effect of EDTA. Thus, it was concluded that EDTA partially reacted with the Zn-binding site in Sp1 under the normal conditions of the EMSA assay carried out in the presence of EDTA.

The experiment was repeated using the standard reaction buffer and after removing EDTA (200 μ M) from the running buffer. In data not shown, neither mobility shift of Zn₃-Sp1-GC1 nor OCT2A-OT1 was altered by the presence of EDTA in the running buffer. Thus, in all other experiments the standard running buffer that contained EDTA was used. Understanding the chemical basis of these results was the focus of the next series of experiments.

3.3 Reaction of Zn-Sp1 with EDTA

Initially, using the standard protocol buffers containing EDTA, Zn-Sp1 was reacted with additional increasing concentrations of EDTA for 15 min followed by the EMSA analysis that probed for the presence of residual DNA binding Sp1. Fig. 2 shows that EDTA inhibited the capacity of Zn-Sp1 to bind to GC1 in a concentration-dependent manner during the initial incubation and that much of the reaction was complete at 100 μ M EDTA. We interpreted these results in terms of the following, a substitution reaction, in which EDTA competes with the Sp1 protein for binding Zn^{2+} :

Once $\text{Zn}_3\text{-Sp1}$ lost its complement of Zn^{2+} , it unfolded and was unable to interact productively with GC1.

The results in the EDTA free reaction buffer were quantitatively different (Fig. 3). An attenuated multi-phasic response to EDTA was observed after 15 min reaction time. Between 0–5 μ M EDTA, 35% of the EMSA band intensity was lost. Increasing the EDTA concentration to 230 μ M gradually depressed $\text{Zn}_3\text{-Sp1}$'s capacity to bind to GC1 to about 45% of control. At larger concentrations, $\text{Zn}_3\text{-Sp1-GC1}$ complexation further declined and was almost absent at 1 mM EDTA. Recognizing that the standard reaction buffer contains 200 μ M EDTA, one can see that even in the absence of added EDTA the commercial reaction buffer would reduce the concentration of $\text{Zn}_3\text{-Sp1-GC1}$ by about 50%, consistent with the results in Fig. 1.

3.4 EMSA of $\text{Zn}_3\text{-Sp1-GC1}$: Effect of EDTA

The conditions above were modified so that EDTA was reacted with $\text{Zn}_3\text{-Sp1-GC1}$, preformed in the absence of EDTA. According to Figs. 2 and 3, concentrations up to 1 mM EDTA did not reduce the shifted band intensity detected in the EMSA with either the commercial reaction buffer or the buffer minus EDTA over a 15 minute time period. Extending the time to 60 min did not alter the results (data not shown). Thus, $\text{Zn}_3\text{-Sp1-GC1}$ was unreactive even with a concentration of EDTA that was more than 2 fold larger than one that nearly completely inactivated free $\text{Zn}_3\text{-Sp1}$ in Fig. 3. It was noted that similar lack of $\text{Zn}_3\text{-Sp1}$ reactivity was observed when a consensus Zn-Sp1 binding sequence (TCGGGGCGGGGCGAGC) was substituted for GC1 (data not shown). Thus, once the complex between $\text{Zn}_3\text{-Sp1}$ and GC1 had formed, the Zn^{2+} sites in Sp1 became either thermodynamically or kinetically available for reaction with EDTA.

3.5 Kinetic Analysis of the Reaction of $\text{Zn}_3\text{-Sp1}$ with EDTA

According to information in the Introduction, on an equilibrium basis EDTA should completely extract Zn^{2+} from free $\text{Zn}_3\text{-Sp1}$ over the entire range of ligand concentrations utilized in Fig. 3. Thus, we hypothesized that the multiphasic behavior revealed above must have a kinetic origin. In order to investigate its basis, the rate of reaction 1 was probed by incubating Zn-Sp1 with 10 to 400 μ M EDTA and varying the reaction time before initiating the GC1 binding step in the EDTA free buffer, which should preserve remaining $\text{Zn}_n\text{-Sp1}$ that can still bind to DNA.

As seen in Fig. 4, the rate of reaction 1 was readily measurable at each EDTA concentration. Each ligand substitution process, treated as a pseudo-first order reaction ($[\text{EDTA}] \gg [\text{Zn-Sp1}]$), exhibited, predominantly, a single first order rate constant (k_{EDTA}). Over the 10–400 μ M EDTA range, this rate process was linear in EDTA concentration with a second order rate constant of $2.7 \text{ s}^{-1}\text{M}^{-1}$ and a first order component (k_{EDTA}') indicated by the non- zero

intercept of $2.1 \times 10^{-4} \text{ s}^{-1}$ (Fig. 5). Between 0 and 250 μM EDTA, an induction phase was also noted. Its contribution to the overall reaction kinetics decreased as the EDTA concentration increased (Fig. 5). Apart from the induction phase the overall rate law was $k = k_{\text{EDTA}'} + k_{\text{EDTA}}[\text{EDTA}]$.

For comparison with the study described above, primary data at 15 min were converted to percent of control concentrations of $\text{Zn}_3\text{-Sp1-GC1}$ and plotted on Fig. 3 as a function of EDTA concentration. Other percent of control points for 500, 750, and 1000 μM EDTA were obtained by extrapolating the results in Fig. 5 to obtain the corresponding k_{obs} values and from those, the concentrations of remaining DNA binding protein. The plateau in the extent of reaction between 0 and 250 μM EDTA was evidently due to the induction period of the reaction over these concentrations.

3.6 Kinetics of Binding $\text{Zn}_3\text{-Sp1}$ to GC1

The observed rate constant for reaction 1 at 200 μM EDTA, the concentration of EDTA in the commercial reaction buffer, was $7 \times 10^{-4} \text{ s}^{-1}$ with a corresponding half time of reaction of 1000 s or 17 min (Fig. 5). Properties of the reaction of $\text{Zn}_3\text{-Sp1}$ with GC1 (reaction 2) were measured in order to understand the kinetic basis for GC1's inhibition of the reaction of EDTA with Zn-Sp1 .

The rate of association of Zn-Sp1 with GC1 was measured by EMSA in order to determine whether this reaction could compete kinetically with reaction 1. Results in Fig. 6 demonstrate that under the conditions of the EMSA reaction, $\text{Zn}_3\text{-Sp1}$ binds to GC1 with a second order rate constant (k_1) of $4.6 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$. For comparison, using initial rate data from the left graph in Fig. 6, the second order rate constant was $6.9 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$.

Coupled with the data in Figs. 3 and 5, the k_1 data provide the basis for a hypothesis to explain the observation of positive electrophoretic mobility shifts in the standard buffer mixture containing EDTA. EDTA and $\text{Zn}_3\text{-Sp1}$ react at a modest rate ($\text{velocity}_{\text{EDTA}} = k_{\text{EDTA}}[\text{Zn}_3\text{-Sp1}][\text{EDTA}]$) at the same time that $\text{Zn}_3\text{-Sp1}$ starts to associate with GC1 ($\text{velocity}_{\text{GC1}} = k_{\text{GC1}}[\text{Zn}_3\text{-Sp1}][\text{GC1}]$). In effect, EDTA and DNA compete for $\text{Zn}_3\text{-Sp1}$. At any time, the relative extent of the two reactions is given by the ratio, $(k_{\text{EDTA}}[\text{EDTA}] + k_{\text{EDTA}'})/(k_{\text{GC1}}[\text{GC1}])$. For example, at the beginning of the reaction when $[\text{GC1}] = 3.6 \times 10^{-9} \text{ M}$, $[\text{EDTA}] = 2 \times 10^{-4} \text{ M}$, and $[\text{GC1}] = 3.6 \times 10^{-9} \text{ M}$, the ratio is 3–4.5, depending on the value for k_{GC1} , indicative of qualitatively similar extents of product formation for the competitive reactions 1 and 2. As the reaction proceeds and $[\text{GC1}]$ declines, the ratio gets larger and increasingly favors the formation of Zn-EDTA . Because the two competing reactions occur with the same order of magnitude rate under the conditions of the assay, at least some $\text{Zn}_3\text{-Sp1-GC1}$ forms (ca. 50% in the monitored reaction), becomes inert to reaction with EDTA, and displays the shifted EMSA band.

3.7 K_d of $\text{Zn}_3\text{-Sp1-GC1}$

The dissociation constant for the $\text{Zn}_3\text{-Sp1-GC1}$ complex was determined using data from EMSA reactions of Fig. 7. $\text{Zn}_3\text{-Sp1}$ was reacted with a series of concentrations of GC1 as described in the Methods (2.5). The extent of reaction was determined by EMSA and the results plotted in the left panel. In turn, these data produced the secondary Scatchard plot in the right panel. The slope is $-1/K_d$, yielding $K_d = 6.4 \times 10^{-10}$, typical of protein complexes

with specific DNA binding sites. The x-axis intercept of 10.6 μM agrees closely with the total concentration of $\text{Zn}_3\text{-Sp1}$, as expected in the Scatchard analysis.

3.8 Protection of $\text{Zn}_3\text{-Sp1}$ by GC1 Against Other Ligand Substitution Reactions

Reasons for the differential reactivity $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$ and $\text{Zn}_3\text{-Sp1}$ with EDTA was probed in a series of reactions involving other Zn-binding ligands. It was initially hypothesized that the $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$ adduct might contain a Zn^{2+} binding site sterically hindered through DNA binding that is kinetically inert to reaction with EDTA. However, available zinc-finger-DNA structures do not show that DNA association obviously buries the Zn center [26–30]. Alternatively, the similar charge of EDTA^{2-} and $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}^{n-}$ might frustrate bimolecular reaction. Thus, we used oppositely charged chelators NTA^{m-} and TREN^{n+} that were smaller than EDTA and used structurally similar tripod configuration of ligands to test whether a ligand with net positive charge and a smaller size was more reactive with $\text{Zn}_3\text{-Sp1}$ or $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$. According to Fig. 8, neither NTA nor TREN reacted with $\text{Zn}_3\text{-Sp1}$ bound to GC1, indicating that charge on the competing ligand was not a dominant feature in the reaction of competing ligands with $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$. Another Zn^{2+} chelator, EGTA, with two iminodiacetate ligand sets like EDTA but with a longer linker between them, also displayed no reactivity with $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$.

A different picture emerged in the reactions of free $\text{Zn}_3\text{-Sp1}$ with these ligands (Fig. 8). NTA and EGTA were the most effective competitors for Zn^{2+} in $\text{Zn}_3\text{-Sp1}$, successfully competing for Zn^{2+} at low μM concentration despite having a much lower affinity for Zn^{2+} at pH 7.4 (10^8 and $10^{7.9}$, respectively) than EDTA [16]. In contrast, TREN was the least effective ligand, requiring more than 500 μM concentration to lower the extent of $\text{Zn}_3\text{-Sp1}$ binding to GC1 to that achieved by 20 μM NTA and EGTA. In this case the like positive charges of TREN and the Zn-finger domain of $\text{Zn}_3\text{-Sp1}$ appear to inhibit of the reaction. The markedly different behavior of these ligands with $\text{Zn}_3\text{-Sp1}$ suggests that different pathways exist for the reaction of various ligands with this transcription factor (Figs. 3 and 8).

3.9 Reaction of $\text{Zn}_3\text{-Sp1}\cdot(\text{dI-dC})$ with EDTA

The reaction of EDTA with $\text{Zn}_3\text{-Sp1}\cdot(\text{dI-dC})$ was also investigated to model the reaction of EDTA with a non-specific $\text{Zn}_3\text{-Sp1}\cdot\text{DNA}$ adduct. Although dI-dC is a non-specific oligonucleotide when compared with the sequence of GC1, it does display a positive EMSA with $\text{Zn}_3\text{-Sp1}$ as long as the reaction buffer does not contain EDTA (Fig. 9). When EDTA was included in the reaction buffer, less protein DNA was observed. When 400 μM EDTA was added to the preformed $\text{Zn}_3\text{-Sp1}\cdot(\text{dI-dC})$ complex, no additional protection was detected. Thus, $\text{Zn}_3\text{-Sp1}$, bound to a non-specific DNA by electrostatic interactions, readily reacted with EDTA.

4.0 Discussion

The electrophoretic mobility shift assay is a powerful method to assess the *in vivo* presence of DNA binding proteins. Generally, the assay is conducted as follows. A cellular or nuclear lysate is made that contains a putative DNA binding protein. An aliquot of the lysate is mixed with a DNA probe comprised of a cognate nucleotide binding sequence of the protein of interest that is labeled for analytical purposes and the mixture is electrophoresed through a polyacrylamide gel. Retardation of the DNA probe's extent of migration indicates that a specific DNA-protein adduct has been formed.

This simplified description fails to recognize the presence of the many components of the reaction mixture and the electrophoresis buffer that might alter the outcome of the EMSA. For example, a non-specific DNA competitor, dI-dC, is present in the reaction to limit non-

specific binding to the DNA probe of other proteins that could establish favorable electrostatic interactions or partial, selective interactions with its base pair sequence. Also conspicuously present for one working with metal-containing transcription factors is a substantial concentration of EDTA. Even if the structural nature of the DNA binding protein of interest is not known, the chances are significant that it is a zinc-finger protein because such proteins comprise the largest class of eucaryotic transcription factors [2,3]. Within this group, proteins that bind Zn^{2+} with the C_2H_2 constellation of amino acid side chains are the most prevalent. There are numerous reports of EDTA inactivation of such proteins against subsequent reaction with DNA [10,13,15]. Thus, the presence of EDTA in the reaction buffer of commonly used EMSA commercial kits and the standard electrophoresis running buffer raises the question of the possible effect of EDTA on EMSA results (Table 1).

The experiments reported here not only describe the impact of EDTA on the EMSA reactions of a Zn-finger transcription factor, they also provide the first detailed analysis of the underlying ligand substitution chemistry that occurs in these reactions. The two thrusts are intertwined as the chemical results provide mechanistic insight into the role of in situ EDTA in Zn-finger protein-DNA reactions.

The experiments described in this paper reveal the following impact of EDTA on the binding of $\text{Zn}_3\text{-Sp1}$ with a cognate DNA binding site in the mouse *sglt1* promoter, GC1. When $\text{Zn}_3\text{-Sp1}$ was mixed with GC1 and electrophoresed according to a standard protocol, the anticipated mobility shift was observed (Fig. 1). However, removal of EDTA from the reaction buffer increased the formation of $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$ by a factor of two. Clearly, endogenous EDTA competed for Zn^{2+} but was only partly successful even though its affinity for Zn^{2+} is several orders of magnitude greater than that of Sp1 [16,18].

The partial formation of $\text{Zn}_n\text{-Sp1}\cdot\text{GC1}$ in the standard buffer that contains EDTA was traced to a combination of factors. First, $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$ is resistant to reaction with EDTA over a range of concentrations and times (Fig. 3). Thus, if the protein DNA complex can be formed during the competition between EDTA and GC1 for $\text{Zn}_3\text{-Sp1}$, it will be stable. Studying the transcription factor, MTF-1, Andrews et al reported this same effect without comment [15]. This behavioral has also been noted with transcription factor IIIA [10]. Second, the relative rates of reaction of EDTA and GC1 with $\text{Zn}_3\text{-Sp1}$ are comparable under the conditions of the assay as shown in Figs. 4–6 and the accompanying text. This insures that some $\text{Zn}_3\text{-Sp1}\cdot\text{GC1}$ forms in the reaction mixture before EDTA has completely removed Zn^{2+} from the protein. On that precarious basis, a positive EMSA is obtained.

Measurement of the kinetics of reaction of EDTA with $\text{Zn}_3\text{-Sp1}$ revealed that the rate includes first and second order terms. The second order component of the reaction, first order in protein and EDTA concentration, is thought to represent rate limiting bimolecular reaction of EDTA with the Zn^{2+} sites. Over a large EDTA concentration range there was no evidence of intermediate $\text{EDTA}\cdot\text{Zn}_3\text{-Sp1}$ adduct formation. The fact that the kinetics of the bulk of the reaction are simple argues that EDTA either competes equally for the three Sp1-bound Zn^{2+} ions or that removal of one is rate limiting and sufficient to prevent detectable binding to GC1.

The existence of a first order component to the rate suggested that a chemical process located at the Zn^{2+} center, such as the dissociation of one of the ligands bound to Zn^{2+} to open up the coordination sphere to EDTA, constituted a rate limiting step in another mechanistic pathway of the reaction. Once the site became available, a fast, non-rate limiting reaction occurred between the protein and EDTA (reaction 3):

Interestingly, a previous study of the reaction of an artificial Zn-finger peptide with EDTA revealed a two step reaction, the first, independent of EDTA concentration, and the second, involving a ternary adduct intermediate [31]. In contrast, the detailed kinetic analysis of the reaction of EDTA with Zn₃-Sp1 did not indicate the formation of an EDTA- Zn₃-Sp1 adduct. It was striking that the reactions in the model system occurred about three orders of magnitude faster than the reaction of Zn₃-Sp1 with EDTA. Evidently, embedding individual Zn-fingers within the larger Sp1 structure—multiple fingers and non-DNA binding region—vastly slowed the reaction. The apparent loss of kinetic reactivity of Zn₃-Sp1 is both interesting and possibly biologically significant as one considers strategies that maintain Zn²⁺ metalloproteins intact within cells that contain multiple metal binding ligands [13].

Further inquiry into the reaction of competing metal binding ligands with Zn₃-Sp1 revealed that both negatively (EDTA, NTA) and positively (TREN) charged competitors reacted with positively charged Zn₃-Sp1 zinc-finger domains but were inhibited by the preformation of the Zn₃-Sp1-GC1 complex (Fig. 8). According to Figs. 3 and 8, there were substantial differences in the rates of reaction of Zn₃-Sp1 with the various competing ligands: NTA~EGTA>EDTA>TREN. Assuming each participates in a second order reaction with Zn₃-Sp1, the differences among related polydentate aminocarboxylate ligands (NTA, EGTA, EDTA) implicate an element of steric control in the reaction, defined for EDTA. Minimizing steric differences with the use of NTA and TREN -N(CH₂-X)₃-it was evident that there was a strong electrostatic component to the reaction. Negatively charged NTA reacted much more efficiently with positively charged Zn₃-Sp1 than did TREN, which also carried a net positive charge.

Zn₃-Sp1 contains 3 tandem zinc-finger domains that are thought to bind to cognate DNA primarily through their helical secondary structure as in other zinc-finger DNA complexes for which 3-dimensional structures are known [32–35]. An examination of published Zn-finger DNA structures, such as transcription factor IIIA and Zif268, shows that the Zn-coordination sites of most of the constituent Zn-fingers in such adducts are accessible to solvent and thus to EDTA and other competing ligands [32–35]. This analysis relied on the average not dynamic structures because the slow rate of reaction of EDTA with the Zn²⁺ sites insures the chelating agent sees the the average solvent accessible site. Thus, we hypothesize that there is a kinetic path for reaction of ligands with Zn₃-Sp1-GC1, but the reactions do not occur because they are thermodynamically unfavorable because of the stabilization of Zn₃-Sp1 by GC1.

In support of this view, among ligands examined, only the thermodynamically most powerful one, TPEN, was able to compete with Sp1 for Zn²⁺ while the protein was bound to GC1 [13]. During a 15 min incubation period, it was able to inhibit the electrophoretic mobility shift band intensity of Zn₃-Sp1-GC1 50% at 100 μM [13]. Charge did not play a controlling role in the reaction of the Zn₃-Sp1-GC1 adduct with competing ligands. TREN as well as TPEN is positively charged and like TPEN would be electrostatically favored as it approached the negatively charged protein-DNA adduct. Yet it was unreactive with Zn₃-Sp1-GC1.

We hypothesize that diminished reactivity of $\text{Zn}_3\text{-Sp1-GC1}$ results from the thermodynamic coupling of the following two reactions that together account for the overall reaction of ligands with $\text{Zn}_3\text{-Sp1-GC1}$:

The contribution of reaction 5 to the overall reaction of competing ligands with $\text{Zn}_3\text{-Sp1}$ inhibits the reaction of all but the strongest one, TPEN.

The lack of reactivity of zinc finger DNA complexes with structurally diverse metal binding ligands has potential ramifications for cellular Zn^{2+} trafficking. A previous paper demonstrated that physiological concentrations of metal binding ligands, such as apo-metallothionein (apo-MT) and glutathione (GSH) readily sequester Zn^{2+} from $\text{Zn}_3\text{-Sp1}$ [13]. However, cells containing apo-MT and GSH also include fully active $\text{Zn}_3\text{-Sp1}$. Explanations that could harmonize the reactivity results with the cellular observations include differential compartmentalization of the ligands and $\text{Zn}_3\text{-Sp1}$ and protection of $\text{Zn}_3\text{-Sp1}$ through binding interactions with other molecules, primarily, DNA. At a gross level of compartmentalization, the former may not apply because metallothionein has been detected by immunofluorescence in the nucleus of many cell types, though its metallation state is unknown [36,37]. Similarly, GSH has been observed in the nucleus through the use of a fluorescent probe [38]. Considering the latter possibility, the $\text{Zn}_3\text{-Sp1-GC1}$ adduct is unreactive with either apo-MT or GSH under conditions in which the free transcription factor undergoes facile reaction [13]. Evidently, a wide variety of metal binding ligands that can react with free $\text{Zn}_3\text{-Sp1}$ are blocked by the formation of its DNA adduct. The large range of ligand structures that display the same qualitative lack of reactivity with $\text{Zn}_3\text{-Sp1-DNA}$ strengthens the hypothesis that the explanation for its inertness to reaction lies in the unfavorable equilibrium constant for the reaction.

Lastly, there are numerous examples of zinc finger proteins detected by the EMSA under conditions that involve exposure to large concentrations of EDTA [4–9]. As with the results with $\text{Zn}_3\text{-Sp1-GC1}$, the observation of many other zinc-finger cognate DNA adducts probably depends on fortuitous comparable rates of reaction of EDTA and DNA with the zinc finger domains at the concentrations used in the assay. Whether there are instances in which the presence of EDTA prevents detection of zinc finger DNA binding proteins is unknown. Nevertheless, on the basis of the present results, EDTA competition for Zn^{2+} cannot be ignored. Since the omission of EDTA from reaction buffer had no apparent adverse effect on the EMSA results presented here, it is recommended that it be removed from these buffers in experiments that might reveal DNA binding Zn-proteins. More generally, EDTA is routinely added to a variety of kits and reagents. Its impact on related reactions and analyses involving metalloproteins needs to be investigated.

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5.0 Abbreviations

CSPD	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2-(5-chloro)tricyclo [3.3.1.1 ^{3,7}]decan}-4-yl)phenyl phosphate
DIG	Digoxigenin; dI-dC, DNA duplex of poly 2'-deoxyinosinic acid and poly 2'-deoxy-cytidylic acid
DTT	Dithiothreitol
EMSA	electrophoretic mobility shift assay
GC1	Sp1 binding site in the mouse sglt1 promoter (5'-CCCCTTAGCAGGCCCTCCCTG CCACAGAACAGA CTTTACCTGCCG-3')
NTA	nitrilotriacetate
Oct 2A	DNA binding transcription factor
rhSp1	recombinant human Sp1
sglt1	sodium-glucose co-transporter 1 gene
TPEN	N,N,N',N'(2-pyridylethyl)-ethylenediammine
TREN	tris-(2-ethylaminoethyl) amine
Zn-Sp1	Sp1 containing bound Zn ²⁺ . Native protein is Zn ₃ -Sp1

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Figure 1.

EMSA of rhSp1 and GC1 in the absence and presence of EDTA. A. Lane 1, EMSA of free GC1; lane 2, Sp1 in standard reaction buffer minus EDTA; lane 3, Sp1 in standard reaction buffer containing EDTA; lanes 4 and 5, OCT2A (cognate DNA, OT1) in standard reaction mixture minus or plus EDTA. B. Summary: Bars show the average and standard deviation of 3 independent EMSA runs.

A	1	2	3	4	5
Zn₃-Sp1	+	+	+	+	+
EDTA , μM	0	5	10	100	1000
GC1	+	+	+	+	+

B	1	2	3	4	5
Zn₃-Sp1·GC1	+	+	+	+	+
EDTA, μM	0	5	10	100	1000

Figure 2.

EMSA of Zn₃-Sp1 + GC1 (A) and Sp1-GC1 (B) reacted with 0 – 1 mM EDTA in standard reaction buffer containing EDTA for 15 min.

Figure 3.

EMSA of Zn₃-Sp1 and Zn₃-Sp1 GC1 reaction with EDTA in EDTA-free reaction buffer. Data from 15 min incubation of reactants: (○) Zn₃-Sp1, (●) Zn₃-Sp1·GC1. Data from kinetic studies in Figs. 4 and 5 (▼); those for EDTA concentrations greater than 400 μM EDTA were extrapolated from Fig. 5. Results are averages $\pm$ standard deviation for 3 independent EMSA runs.

Figure 4.

Kinetics of reaction of EDTA with Zn_3-Sp1 : first order kinetic plots. Conditions: 10 – 250 μM EDTA reacted for 0 to 60 min followed by addition of GC1. Each point represents the average value for 3 independent EMSA runs.

Figure 5. Kinetics of reaction of EDTA with $\text{Zn}_3\text{-Sp1}$: secondary plots of observed rate constants (●) and fraction of total reaction assigned to induction phase (○) vs. EDTA concentration.

Figure 6.

Kinetics of association of Zn₃-Sp1 with GC1. A. EMSA band intensity of Zn₃- Sp1·GC1 is plotted against time. Initial concentrations: Zn₃-Sp1, 2.5 nM, and GC1, 15 nM. Results are averages $\pm$ standard deviation for 3 independent EMSA runs. B. Second order plot, $\ln [Zn_3 - Sp1] / [GC1]$ vs. time. The negative of the slope represents the rate constant, $2.0 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$.

Figure 7.

Scatchard plot determination of dissociation constant (K_d) for Zn_3 -Sp1·GC1. A. EMSA band intensity of Zn_3 -Sp1·GC1 is plotted against the concentration of GC1. Concentrations: Zn_3 -Sp1, 10 nM, and GC1, 0.9 – 14.4 nM. Results are averages $\pm$ standard deviation for 3 independent EMSA runs. B. Second order plot, $[Zn_3-Sp1 \cdot GC1]/[GC1]_{free}$ vs $[Zn_3-Sp1 \cdot GC1]$.

Figure 8.

Reaction of $\text{Zn}_3\text{-Sp1}$ and $\text{Zn}_3\text{-Sp1-GC1}$ with competing ligands. Conditions: ligands with $\text{Zn}_3\text{-Sp1}$ and $\text{Zn}_3\text{-Sp1-GC1}$, respectively: EGTA, 0 – 800 μM , ($\blacktriangle$, $\bullet$), TREN, 0 – 1000 μM , ($\bullet$, $\blacktriangledown$) and NTA, 0 – 800 μM , ($\blacksquare$, $\blacklozenge$); 15 min reaction. Results are averages $\pm$ standard deviation for 3 independent EMSA runs.

Figure 9. Reaction of Zn₃-Sp1 poly-dI-dC with 400 μ M EDTA. Results are averages $\pm$ standard deviation for 3 independent EMSA runs.

Table 1

Selected EMSA Reaction Mixture Components

Component	Concentration
Zn-Sp1	45 nM
(Zn ²⁺ bound to Sp1)	135 nM
ZnSO ₄ , from rhSp1 storage buffer	114 nM
CoCl ₂ , cofactor for DIG labeling of GC-1	90 μM
EDTA	200 μM
Poly-lysine	400 μM lysine equivalents
DTT, from Zn ₃ -Sp1 storage and reaction buffers	945 μM
GC-1 DNA probe	3.6 nM
(PolydI-dC)	85μM base pairs