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Short communication

DNA-binding affinities of MyoD and E47 homo- and hetero-dimers by capillary electrophoresis mobility shift assay

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Abstract

A simple capillary electrophoresis mobility shift assay (CEMSA), with no gel and uncoated capillaries, for the accurate determination of protein–DNA affinities free in solution was applied to constructs of the MyoD/E47 DNA-binding proteins. The determined affinities are compared to those obtained by EMSA. MyoD-E47 covalent heterodimer binds DNA more tightly (K_d=1.8 n*M*) than MyoD (K_d=14.2 n*M*) or E47 (K_d=11.5 n*M*) covalent homodimers. The effect of non-specific DNA on binding affinities was more important than salt concentration in the MyoD/E47 series. Application of this method to the MyoD/E47 system demonstrates the generality of our CEMSA.

Keywords: Capillary electrophoresis mobility shift assay; DNA; MyoD-E47 protein

1. Introduction

Capillary electrophoresis has been applied to the determination of small molecule binding affinities and called affinity capillary electrophoresis (ACE) [1–3]. Protein/DNA binding has been detected by several capillary electrophoresis mobility shift assays (CEMSA) that used coated capillaries with gel packing [4–7]. CEMSA has also been used to measure DNA-binding of the helix-turn-helix protein, c-Myb, in the micromolar range using UV detection [8]. Recently, we described a fast and simple CEMSA without gels in uncoated capillaries to measure protein/DNA dissociation constants in the nanomolar regime [9,10]. To demonstrate its utility we have applied the method to MyoD and E47 DNA complexes [11].

MyoD and E47 belong to the family of basic region helix-loop-helix (bHLH) transcription factors, which regulate key steps in early development [12]. MyoD is specifically involved in skeletal muscle development, while the E proteins are general transcription factors [13]. MyoD activity is contingent upon heterodimerization with E47 or E12 and DNA-binding [14]. Transcriptional activation is regulated by many balancing protein–protein and protein–DNA interactions. Determining the binding affinities involved gives valuable insight into the mechanisms of transcriptional activation.

In previous EMSA studies on the bHLH constructs, it was found that both MyoD and E47 form homodimeric complexes that bind DNA with similar equilibrium dissociation constants, despite having very different protein dimer stabilities [11]. Also, the MyoD-E47 heterodimer DNA complex was found to

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form preferentially over homodimer DNA complexes. This suggested that more favorable DNA contacts are made by one or both subunits of the heterodimer, in comparison with either homodimer complex, and that protein–protein interactions are less significant. We determined DNA binding affinities by CEMSA for the bHLH constructs of E47 homodimer, MyoD homodimer, and E47-MyoD heterodimer under varying conditions, and compared them to those determined by the traditional gel EMSA to demonstrate the utility and generality of the assay.

2. Experimental

2.1. Apparatus

CE was performed on a Beckman P/ACE 2100 series instrument equipped with the Beckman Laser Module 488 and LIF detector. The instrument was interfaced with an IBM PC running Beckman System Gold software (version 8.1). Separation was performed in uncoated fused-silica capillaries (Polymicro Technologies) of 75 μm I.D., 375 μm O.D., 27 cm total length and 20 cm effective length from the inlet to the detector.

2.2. Materials

The fluorescently labeled and unlabeled DNA strands were synthesized at the Biomolecular Research Facility of the University of Virginia. The fluorescent dye 5′-carboxyfluorescein phosphoramidite was attached to the 5′ end of one strand. After column purification the single stranded oligonucleotides were stored as stock solutions in 10 m*M* Tris·HCl (pH 7.4), 1 m*M* EDTA buffer at −20°C. Concentrations were measured by UV at λ_{260} (30 μg/ml=1 AU). Sense and antisense DNA, 1.1 equiv. of 5′-GTACGCACACCTGCTGCCTGA-3′ and 1 equiv. of 5′-X-TCAGGCAG-CAGGTGTGCGTAC-3′ where X is fluorescein, were annealed in 10 m*M* 3-(*N*-morpholino)-propane sulfonic acid (MOPS)–triethylamine (Et_3N) (pH 7.5), 100 m*M* KCl at 90°C for 5 min and slowly cooled to room temperature over 2 h. The E47 (60 residue), E47GGC (63 residue), MyoDGGC (65 residue) and disulfide crosslinked E47-MyoD proteins [11] were generously supplied by Professor Tom Ellenberger (Harvard Medical School) and stored as 50–300 μ*M* stock solutions in 50 m*M* ammonium acetate (pH 7.0), 2 m*M* dithiothreitol (DTT) at −60°C.

2.3. Procedure

The capillary was washed daily for 30 min with 0.1 *M* NaOH, 15 min with water and 15 min with run buffer (10 m*M* MOPS–Et_3N pH 7.5). Prior to each run the capillary was rinsed for 2 min with 0.1 *M* NaOH and 2 min with run buffer. Serial dilutions of freshly thawed bHLH protein were prepared in 10 m*M* MOPS–Et_3N (pH 7.5), 10% glycerol, 2 m*M* DTT and 200 μg/ml bovine serum albumin (BSA) and then mixed with an equal volume (typically 10 μl) of DNA in 10 m*M* MOPS–Et_3N (pH 7.5), 100 m*M* KCl to give the appropriate final concentrations. The sample mixture was preincubated at 4°C for 10–30 min prior to injection and maintained at 4°C between injections. Samples were injected onto the capillary under pressure for 2 s. Electrophoresis was performed at 25 kV in run buffer at 21°C. Electrophoresis was at reversed polarity to conventional EMSA, with the cathode at the detector end. Binding constants were calculated by non-linear least squares fit of the data to the binding equation using KaleidaGraph (version 3.0.5), Fig. 1.

3. Results and discussion

3.1. CEMSA

Our CEMSA method [9] was developed with the DNA-binding protein GCNK58 [15], a bZIP construct of the yeast transcription factor GCN4. Experiments investigating column length, protein adsorption and stability of protein–DNA complexes to increasing field strength were performed to give an efficient and fast method [10]. The use of the zwitterionic [16] run buffer MOPS gives lower currents and less joule heating than other buffers. The established method was successfully applied to the bHLH DNA-binding proteins, with little alteration. Electrophoresis is performed free in solution with no gel, and in uncoated fused-silica capillaries.

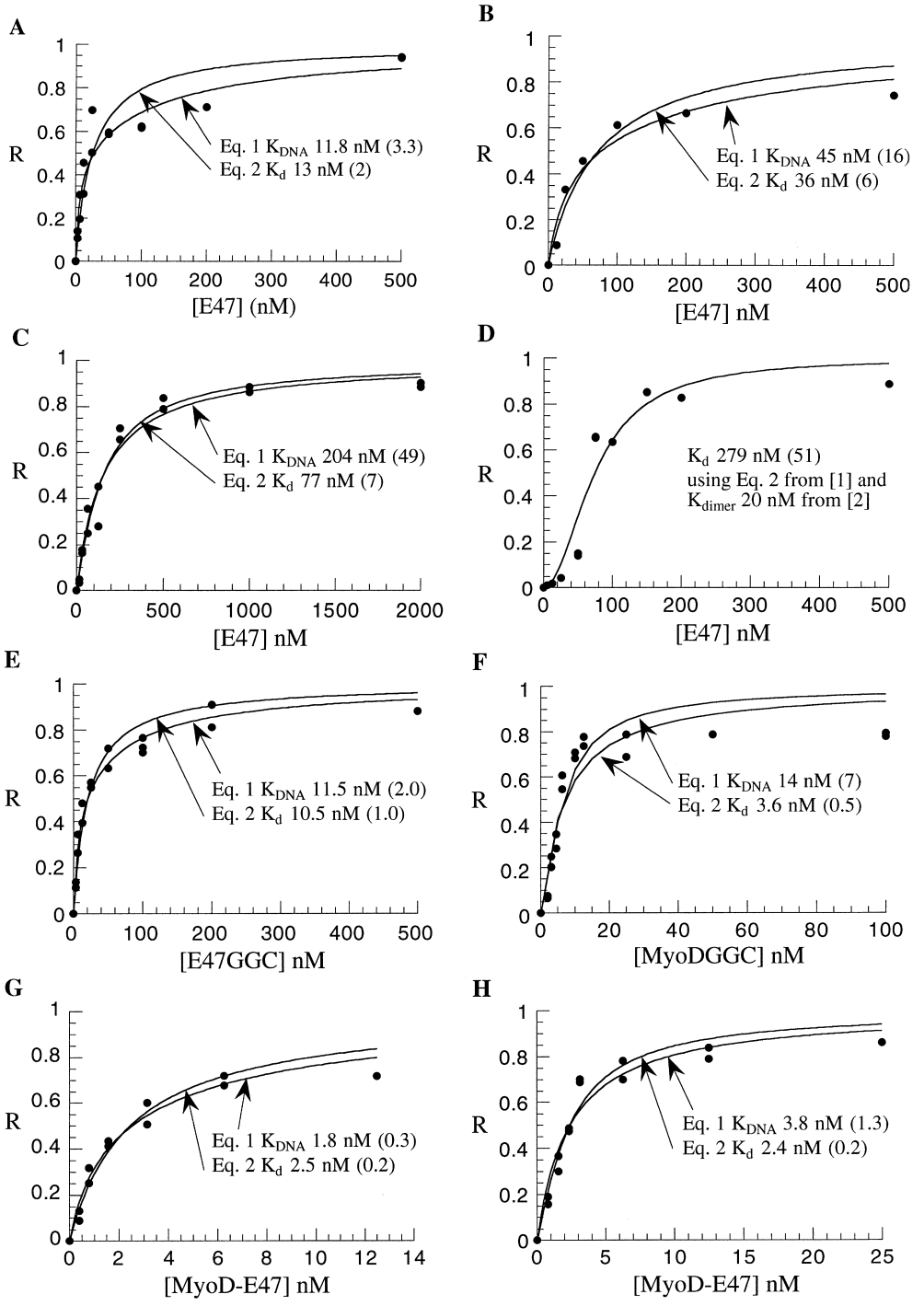

Fig. 1. DNA binding data for bHLH constructs fit to Eqs. 1 and 2 (± error in n*M*). (A), E47 with standard buffer including 50 m*M* KCl; (B), E47 with 150 m*M* KCl; (C), E47 with 50 μg/ml poly(dI-dC)·(dI-dC); (D), E47 with 10 n*M* DNA; (E), E47GGC; (F), MyoDGGC; (G), MyoD-E47 with 50 m*M* KCl; (H), MyoD-E47 with 150 m*M* KCl.

Uncoated capillaries are more robust, simpler and give large electroosmotic flow for fast separations. Resolution of free DNA from protein–DNA complex is achieved in a 2 min. separation, with an interim 4 min wash cycle. Typically, an assay of eight concentrations in duplicate can be run in about 100 min with the automatic sample tray.

3.2. Binding affinities

The CEMSA data for each protein was fit by a non-linear least squares analysis to two binding equations (Fig. 1). Eq. (1), also used in the EMSA, describes the dissociation constant of a monomeric protein (P) interacting with DNA (K_{DNA}) and a cooperativity factor (α) that accounts for the higher affinity of dimeric protein (P_2) for DNA [11]. The simpler Eq. (2) describes the equilibrium dissociation constant (K_d) including both dimerization and DNA-binding [9]

$$R = \frac{\frac{[P]}{K_{DNA}} + \frac{[P]^2}{\alpha K_{DNA}^2}}{1 + \frac{2[P]}{K_{DNA}} + \frac{[P]^2}{\alpha K_{DNA}^2}} \quad (1)$$

$$R = \frac{0.5[P]}{K_d + 0.5[P]} \quad (2)$$

where

$$R = \frac{[P_2 \cdot DNA]}{[P_2 \cdot DNA] + [DNA]}.$$

Sample buffer composition can affect binding affinities. Our sample mixtures were prepared in with 50 m*M* KCl to keep current low and Joule heating low, instead of 20 m*M* Tris pH 7.5 with 100 m*M* KCl used in the EMSA. Our sample buffer did not contain DTT for the disulfide-bound dimers because it was not found to be necessary in the EMSA study [11]. The higher ionic strength and saturation of non-specific DNA interactions in the EMSA samples account for the slightly weaker binding affinities obtained in that study for the bHLH constructs.

EMSA gave a higher K_{DNA} (160 n*M*) [11] than CEMSA (12 n*M*) for E47, Fig. 1A. This reflects the higher ionic strength of the sample buffer and the presence of non-specific DNA in the EMSA. Increasing the amount of salt from 50 m*M* to 150 m*M* KCl in the sample buffer also increased the K_{DNA} value for E47 slightly from 12 to 45 n*M* in the CEMSA (Table 1, Fig. 1A and B). E47 homodimer is more stable than MyoD homodimer by approximately 10 kJ/mol, thus E47 is a relatively stable dimer even at low concentrations [11], and presumably at higher salt concentration. Including 50 μg/ml poly(dI-dC)·(dI-dC) in the sample buffer gave a K_{DNA} value of

Table 1
MyoD and E47 DNA-binding affinities measured by EMSA and CEMSA

Fig. 1	Protein dimer	EMSA [11] K_{DNA} (n*M*)[a]	K_{DNA} (n*M*) see Eq. (1)[b]	α (Cooperativity factor) Eq. (1)	K_d (n*M*) see Eq. (2)[b]
A	E47	–	11.8±3.3	5.8±2.7	13±2
B	E47(high salt)[c]	–	45±16	2.9±1.9	36±6
C	E47 (nsDNA)[d]	160±97	204±49	0.57±0.25	77±7
D	E47 (10 n*M* DNA)	–			279±51[e]
E	E47GGC	–	11.5±2.0	3.3±0.9	10.5±1.0
F	MyoDGGC	16±5.3	14.2±7.4	0.23±0.23	3.6±0.5
G	MyoD-E47	–	1.8±0.3	2.0±0.7	2.5±0.2
H	MyoD-E47(high salt)[c]	14.0[f]	3.8±1.3	0.4±0.26	2.4±0.2

[a] EMSA assay sample buffer: 20 m*M* Tris pH 7.5, 100 m*M* KCl, 1 m*M* DTT, 10% glycerol, 0.1 m*M* EDTA, 0.5% Nonidet P40 and 1.2 mg/ml sheared salmon sperm DNA [11].
[b] Standard sample buffer: 10 m*M* MOPS–Et_3N (pH 7.5), 50 m*M* KCl, 100 μg/ml BSA, 5% glycerol.
[c] Standard sample buffer except 150 m*M* KCl.
[d] Standard sample buffer plus 50 μg/ml poly(dI-dC)·(dI-dC) non-specific DNA.
[e] K_d using Eq. (2) from Ref. [9] and $K_{dimer} = 20$ n*M* from [11].
[f] K_{obs} not K_{DNA}.

203 n*M* for E47 (Fig. 1C). This is comparable within error to the EMSA value including non-specific salmon sperm DNA (160 n*M*). For MyoD-E47 heterodimer, high salt (150 m*M* KCl) sample buffer gave a K_{DNA} value of 3.8 n*M*, compared to 1.8 n*M* at 50 m*M* KCl (Fig. 1G and H). Clearly the effect of non-specific DNA on K_{DNA} is stronger than salt concentration.

Wendt et al. prepared constructs with C-terminal Gly–Gly–Cys extensions (E47GGC and MyoDGGC) that exist as covalently bound dimers even in the presence of low concentrations of the reducing agent DTT [11]. This was supported by the observation of non-cooperative hyperbolic binding curves in our CEMSA studies. These constructs were also used to form a disulfide linked MyoD-E47 heterodimer. E47 and E47GGC constructs gave nearly identical K_{DNA} values of 11.8 n*M* and 11.5 n*M*, respectively, Fig. 1A and E. Both proteins are predominantly dimeric, E47GGC as a disulfide linked dimer and E47 through strong protein–protein interactions, therefore the binding affinities represent the same protein–DNA interaction. The MyoDGGC homodimer construct gave a comparable affinity of 14.2 n*M*, Fig. 1F. This is consistent with the EMSA studies, which showed that E47 and MyoD have similar DNA-binding affinities despite having different dimer stabilities in the absence of DNA [11]. The crosslinked MyoD-E47 heterodimer gave a K_{DNA} of 1.8 n*M*, six to eight-fold stronger binding than either the E47 or MyoD homodimers, Fig. 1G. In the EMSA study, the non covalently linked MyoD-E47 heterodimer also gave a slightly stronger DNA affinity (K_{obs} 14 n*M*) than either homodimer (K_{obs} 28 n*M* and 18 n*M*) [11].

The K_d values determined from Eq. (2) were generally comparable to K_{DNA} values, except for entries C and F (Table 1), where relatively greater cooperativity was indicated by smaller α values (0.57 and 0.23). Weaker dimerization is reflected in higher K_{DNA} values relative to K_d because of cooperativity. The ternary binding constant, K_d, was relatively tighter than K_{DNA}, however, this was not reflected in obvious S-shaped binding curves. In the EMSA studies, S-shaped curves were observed when the cooperativity factor α was determined to be less than 0.03 [11]. No such binding curves were observed in the data shown for 1 n*M* DNA (Fig. 1). However, at higher concentrations of DNA (10 n*M* to 50 n*M*) cooperative S-shaped binding curves were observed for the non-crosslinked E47 construct (Fig. 1D). The cooperativity and higher K_d measured with 10 n*M* versus 1 n*M* DNA points to the importance of using concentrations of DNA lower than the K_d. Cooperativity was also observed previously by CEMSA for GCNK58 [9] and an RXR DNA-binding domain [17] (GJF and FAE, unpublished results).

4. Conclusions

CEMSA can be utilized to give quantitative DNA-binding affinities, free in solution with no gel and uncoated capillaries. The effect of non-specific DNA on binding affinities was more important than salt concentration in the MyoD-E47 series. The stronger MyoD-E47 heterodimer DNA binding supports the role of MyoD in muscle-specific transcriptional regulation [13]. The CEMSA method we have described is applicable to other DNA-binding protein systems.

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