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Design and optimization of a capillary electrophoretic mobility shift assay involving *trp* repressor–DNA complexes

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Abstract

An investigation of DNA–protein interactions by capillary electrophoresis (CE) with laser fluorometric detection is performed that combines the rapid and minimal sample consumption methods of CE with the selective separation influence of mobility shift assays. An inspection of the well characterized interaction between the *trp* repressor of *Escherichia coli* and the *trp* operator (DNA) is the basis of the assay. The use of fluorescently tagged operator not only lends itself to laser-induced fluorescence detection but also precludes the use of radiolabeled detection. It is demonstrated that composition and pH of the running buffer are critical for maximized efficiency and resolution of operator from the repressor–operator complex. Quantitative studies showed reaction of repressor with operator resulted in the diminishing of free operator signal and the simultaneous creation of the repressor–operator peak that is well resolved from the free operator. Also examined was the ability to perform qualitative studies involving non-specific interactions between the operator and a complex protein sample. It is shown that the specificity of operator for repressor can be used to selectively separate the repressor from a complex sample that includes non-specific proteins.

Keywords: Proteins; DNA

1. Introduction

Analytical methodologies involving DNA and proteins are showing widespread success throughout the life sciences. The analysis of these complex biopolymers pervades such disciplines as biochemistry, molecular biology, genetics and others. Capillary electrophoresis (CE) has had its share of success as a viable technique for the analysis of DNA and other large biopolymers (i.e., proteins). Typical oligonucleotide samples for CE analysis include enzyme restriction fragments of single and double stranded DNA chains [1,2], selected sections of DNA obtained by the polymerase chain reaction (PCR) [3–6] and sequence analysis [7–9]. Typical analysis of proteins by CE includes molecular mass determinations [10,11], enzyme micro assays [12–14], separation of antigen–antibody complexes [15–17] and peptide mapping [18]. The superiority of CE with regard to efficiency, speed, small sample quantities and the possibility of automation makes it well suited for the study of protein–DNA interactions.

Studies of protein–DNA interactions permeate most areas of biochemical research. Currently the established method for these studies is the electrophoretic mobility shift assay (EMSA), also known as gel retardation assays [19,20]. However, this method suffers from some significant limitations, most importantly, long assay time, difficulty with accurate quantitation and difficulty in determining dissocia-

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tion constants. CE is well suited as a replacement for traditional methods. The current research, termed capillary electrophoretic mobility shift assay (CEMSA), will incorporate the advantages of both EMSA and CE, while overcoming some of the major limitations of the former. This cursory look at CEMSA will provide both a quantitative and qualitative basis for the development of this technique.

Gel retardation assays are based on the observation that the formation of protein–DNA complexes generally reduces the electrophoretic mobility of the DNA component in the gel matrix [21]. This widely used technique can be used to study complex formation and stability [19,22,23], define binding sites on nucleic acids [22,24,25], analyze DNA conformations in complexes [22,26,27] and study dynamic processes involving transcription and splicing [22,28].

The interaction between the *trp* repressor of *Escherichia coli* and the *trp* operator (DNA) serves as a well-characterized system for the development of CEMSA. DNA binding in the *trp* repressor/*trp* operator system is regulated by allosteric control. The *trp* repressor is responsible for the regulation of L-tryptophan biosynthesis in *E. coli*. This is accomplished via a simple negative feedback loop [29]. When the concentration of L-tryptophan is low, *trp* repressor is inactive (aporepressor) and the *trp* operon is expressed, resulting in the biosynthesis of L-tryptophan. A relatively high concentration of tryptophan leads to formation of the active repressor, which in binding the operator, inhibits transcription initiation in the *trp* operon. The binding of tryptophan induces a conformational change in the *trp* repressor that alters the orientation of the recognition helices so as to fit into the two successive major grooves of the operator DNA [29].

We will use the *trp* repressor–*trp* operator interaction to explore the capabilities of CEMSA. In CEMSA, as in traditional EMSAs, the mobility of a specific protein–DNA complex differs from that of free DNA. Evaluation of running buffer conditions allows optimization of complex formation and resolution of free DNA from complex. Qualitative studies involving non-specific interactions between operator and a complex protein sample are demonstrated. These studies demonstrate the technique's ability to selectively differentiate between bioaffinity specific interactions and non-specific ones.

2. Experimental

2.1. Materials

Tris(hydroxymethyl)aminomethane, boric acid, ethylenediaminetetraacetic acid (EDTA), methyl cellulose (MC) polymer (M_r 100 000, 4000 mPa s viscosity at 2.0% w/v), and L-tryptophan were obtained from Sigma (St. Louis, MO, USA) and were used to prepare running buffers. Acrylamide, γ-methacryloxypropyltrimethoxysilane, N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate were obtained from Sigma and used to prepare column coatings. Myoglobin and lysozyme were also obtained from Sigma. Fused-silica capillaries (50 μm I.D.×365 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were coated with linear polyacrylamide using a modified version [2] of Hjerten's method [30].

2.2. Running buffer and sample preparation

Running buffers containing methyl cellulose were prepared in a manner previously described [2]. Purified *trp* repressor was prepared as described in Czernik et al. [25] and stored at −20°C in small aliquots at concentrations greater than 1 mg/ml in 10 m*M* sodium phosphate buffer, pH 7.6, containing 150 m*M* NaCl and 0.1 m*M* EDTA. E12 purification will be described elsewhere [31]. The concentrations of *trp* repressor (60 μM) and E12 (12.3 μM) were determined at 280 nm using molar absorptivities of 13 940 $cm^{-1}\ M^{-1}$ and 17 900 $cm^{-1}\ M^{-1}$, respectively. The symmetrical 26-nucleotide DNA was synthesized incorporating a fluorescein-conjugated base at the 3′ end using a Persceptive Biosystems (Framingham, MA, USA) Expedite synthesizer by the Molecular Biology Core facility at U.A.M.S. The oligonucleotide was annealed by heating to 100°C for 5 min and slowly cooling to room temperature over a period of 1 h. After self-annealing, the duplex sequence is as follows:

5′–GAGCGTACTAGTTAACTAGTACGCTC–Fl

Fl–CTCGCATGATCAATTGATCATGCGAG–5′

where Fl designates a fluorescein molecule. The underlined portions represent the base pairs that have

been shown to be most important for repressor binding in genetic and biochemical studies. DNA concentrations were based on spectral measurements using a Hewlett-Packard (Palo Alto, CA, USA) Model 8452A diode-array spectrophotometer and the conversion of 1.0 absorbance units at 260 nm = 50 μg ml^{-1}. Working solutions of DNA were diluted with 45 m*M* TBE, 0.5 m*M* L-tryptophan. Preparation of *trpR*–DNA complexes involved the addition of the appropriate amounts of each analyte (depending on the desired ratio) and diluting to the desired volume with running buffer. A 1:1 *trpR*–DNA complex represents an approximate concentration of $2.6 \cdot 10^{-6}$ *M* for each analyte. All other ratios are based on this concentration. Myoglobin and lysozyme stock solutions were prepared by weighing out the appropriate amount of protein and diluting to the desired concentration with running buffer. DNA–protein samples involving myoglobin, lysozyme and E12 were prepared in the same fashion as the *trpR*–DNA complexes.

2.3. Apparatus and electrophoresis

The electrophoresis and fluorescence apparatus was assembled in-house [32]. A Spectra-Physics (Mountain View, CA, USA) Model 162A air-cooled argon ion laser, operating at 488 nm was employed as the excitation source. A 25 mm diameter, f/1, quartz lens was used to focus the laser radiation through the capillary. The collection optics consisted of a 600 μm (core) diameter fiber optic which was positioned within 200 μm of the capillary, at the point through which the beam was focused. The other end of the fiber was carefully butted against a narrow bandpass filter (20 nm bandpass centered at 520 nm), to isolate the fluorescence emission. The filter was placed against a shutter assembly on the face of the photodetector, a Hamamatsu (Bridgewater, NJ, USA) Model HC120-01 PMT assembly. The PMT power supply was assembled in-house, as were a noise filter and offset circuit for the signal output [32]. Electropherograms were recorded using a strip chart recorder.

A Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply was used to apply electrophoretic fields. Injections were performed electrokinetically (for running buffers incorporating MC) by placing the inlet of the capillary into the sample vial and applying −2 kV for 20 s. Following injection, the capillary inlet was replaced into the mobile phase vial and a running potential of −20 kV was applied across the 50 cm (40 cm to the detector) capillary. Hydrostatic injections were performed by placing the inlet of the capillary in the sample vial and raising it 10 cm for 10 s.

3. Results and discussion

In an attempt to develop a capillary-based assay, evidence of complex formation was a primary concern. Two scenarios present themselves when dealing with the creation of protein–DNA complexes. The first scenario is whether free DNA and complex are resolved. The second scenario is the degree to which the complex remains intact throughout the separation. In both cases, however, it is paramount that the free DNA peak is reduced in response to repressor addition, to be sure that a complex is being formed. Fig. 1 depicts experimental evidence of the formation of a complex that remains intact, at least somewhat, during the separation. It is shown that as

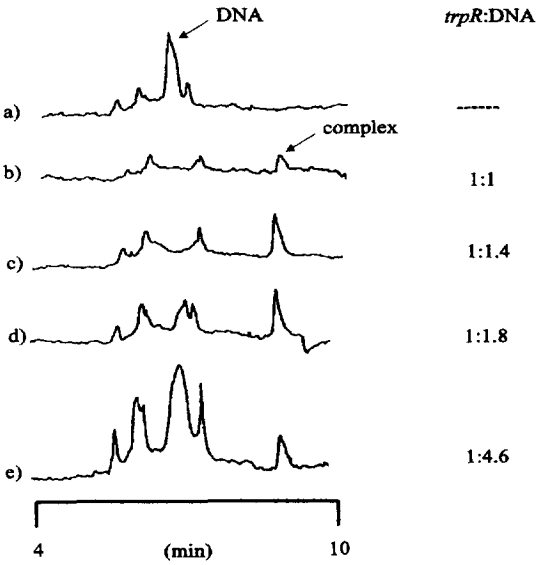

Fig. 1. Electropherogram depicting evidence of complex formation. (a, b) Free DNA and an approximate 1:1 (*trpR*–DNA) complex, respectively with [DNA] $\cong 2.6 \cdot 10^{-6}$; (c–e) sequential addition of DNA to the complex. The *trpR*–DNA ratio is noted beside each electropherogram. Conditions: 45 m*M* TBE, 0.5 m*M* L-tryptophan, 0.5% methyl cellulose, pH 8.

more DNA is added to a 1:1 (*trpR*–DNA) complex, the complex peak grows until the *trpR* exists only in the complexed form. At this point, addition of more DNA results in an increase in the free DNA signal only. The fact that the complex peak grows upon addition of DNA above an apparent 1:1 amount may suggest that the complex dissociates under electrophoretic conditions, or simply that the concentration of active, full length strands of DNA is relatively low in the non-gel-purified DNA sample. It can be assumed that wall adsorption is negligible due to coating of the capillary walls with linear polyacrylamide. Fig. 1 further shows that resolution of complex and free DNA exists. At this point attention was turned to developing running buffer conditions that maximized efficiency and resolution of operator from complex.

For site-specific binding of repressor to operator, a saturating amount of L-tryptophan is required [23]. *Trp* repressor binds two molecules of L-tryptophan to become competent for high affinity operator binding [33,34]. Fig. 2 shows the dependence of complex formation on L-tryptophan. It is demonstrated that without L-tryptophan in the running buffer (45 m*M* TBE, pH 8), a complex is created (evidenced by a reduction of the free DNA peak), but is not maintained, i.e. it dissociates since no complex peak is observed. However, upon addition of L-tryptophan (0.5 m*M*) to the running buffer, a complex peak is observed.

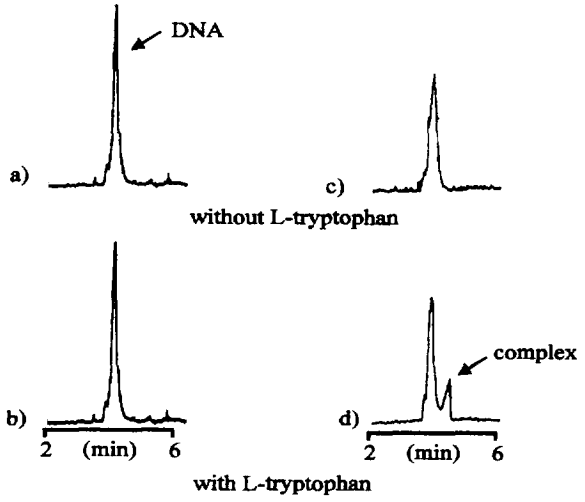

Fig. 2. Dependence of complex formation on L-tryptophan. Running buffer conditions are 45 m*M* TBE, pH 8. (a, b) DNA without and with 0.5 m*M* L-tryptophan in the running buffer, respectively; (c, d) an approximate 1:1 (*trpR*–DNA) complex without and with 0.5 m*M* L-tryptophan, respectively.

Once complex formation and resolution were observed, a cursory study of the effects of running buffer constituents on CEMSA performance was conducted. TBE has been used as the buffer in traditional gel-based assays of *trpR* binding to DNA and was thus chosen as a starting point for this CEMSA work [23]. It has been shown that the pH of a gel-based assay for *trpR* plays an important role in the ability of *trpR* to retard the electrophoretic mobility of the DNA fragment. As the pH of the buffer approaches the isoelectric point (p*I*) of *trpR* (5.9), retardation is enhanced [23]. In the capillary-based assay, this was not observed. A comparison of buffers with pHs of 5, 6, 8 and 10 (45 m*M* TBE, 0.5 m*M* L-tryptophan) resulted in no discernible difference in resolution; however, an increase in signal was observed as pH increased. Since pH 8 buffer resulted in adequate signal for free DNA and optimal signal and reproducibility for the complex, it was used for further studies.

It has been demonstrated that running buffer additives play an important role in the optimization of protein separations. Various protein separations and enzyme micro assays, studied by CE, have employed such running buffer additives as polyethylene glycol, dextran, cross-linked and linear polyacrylamide gels [7,11,12,35,36]. In each case, the additives served as polymer networks imparting sieving. The addition of soluble polymers to running buffers for the analysis of DNA restriction fragments serves the same size-selective sieving purpose [2].

Moreover, in gel-based assays, the cross-linked acrylamide matrix imparts a caging effect [22]. This serves to effectively raise the local concentration of protein and DNA. Therefore, when a protein–DNA complex dissociates under electrophoresis conditions, they usually re-associate rapidly to reform the complex. In this CEMSA work, the linear polyacrylamide is chemically bonded to the column wall and thus serves only to inhibit wall adsorption of analytes. Therefore, a similar caging phenomenon may be desired for the capillary-based assay. It should be noted that because of the speed of these CEMSA separations, this may not be needed.

Since the presence of MC imparts size selectivity in DNA restriction digest assays, the effect of MC in the running buffer was investigated. The molecular mass and percentage of MC used in these studies was not expected to yield a mesh that would be small enough to impart size selection. However, MC conditions have also been shown to influence efficiency in prior work [2]. This increase in efficiency, combined with the adequate resolution inherent to shift assays, led to the use of MC as a running buffer additive. It is likely that cross-linked polyacrylamide and other polymer additives will exhibit similar effects. The addition of MC not only increased resolution, but it also led to an increase in efficiency of both the free DNA and the complex, as seen in Fig. 3. What appears to be multiple peaks from the free DNA response can be attributed to the fact that non-gel-purified DNA was used. The increase in efficiency afforded to the complex peak may be attributed to the same phenomenon seen in gel-based assays. The MC is a non-rigid soluble polymer network that may impart the caging effect alluded to above. Not only does it serve to keep any dissociated protein or DNA in proximity, it may also slow down the diffusion of any wall-adsorbing species. In effect, it acts not only as a sieving medium, resulting in increased resolution, but it also helps prevent wall adsorption. The final result is increased resolution and efficiency.

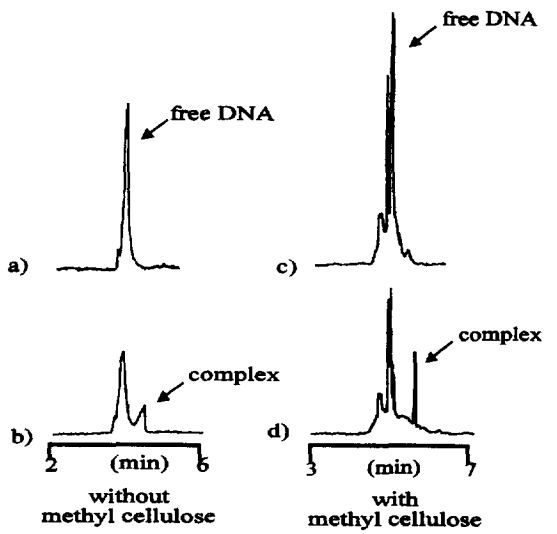

Fig. 3. Electropherograms depicting the effect of methyl cellulose in the running buffer. (a, b) Free DNA and an approximate 1:1 (*trpR*–DNA) complex, respectively. Running buffer conditions were 45 m*M* TBE, 0.5 m*M* L-tryptophan, pH 8; (c, d) the same but with 0.75% methyl cellulose also in the running buffer.

The observation that MC improved efficiency led to the optimization of MC conditions. Three different percentages of MC (0.25, 0.5 and 0.75%) were compared regarding their influence on signal, resolution and efficiency. All running buffers consisted of 45 m*M* TBE, 0.5 m*M* L-tryptophan, pH 8, and the soluble polymer. Running buffers containing 0.25% MC yielded broad, highly inefficient responses for free DNA, as well as decreased resolution and efficiency of the complex peak as compared to 0.75% MC. A comparison between 0.5% and 0.75% MC resulted in slightly greater efficiency for free DNA from the 0.5% buffer, but significantly greater efficiency for the complex peak from the 0.75% buffer. While the effects of soluble polymer percentages and size (M_r) have been investigated for DNA fragments [2], to our knowledge they have not been studied for DNA–protein separations.

Upon optimization of running buffer conditions, simple quantitative studies were performed to show the changes in peak height as a function of increasing repressor concentration. In this work, high concentrations of operator DNA were required to offset poor fluorescent labeling efficiency (hence poor detectability). At concentrations greater than 100-fold above the K_D [37], binding of repressor to operator is stoichiometric [38]. This allows easy manipulation of the amounts of either analyte in solution. Fig. 4 shows a plot depicting change in peak height as a function of increasing [*trpR*]. Each data point represents a ratio of *trpR*–DNA, beginning with a ratio of 0:1 and increasing in increments of 0.25 units. In the plot, both the free DNA peak height and the complex peak height are monitored as a function of [*trpR*]. As the concentration of *trpR* approaches that of the DNA in solution, a subsequent decrease in the free DNA peak height is observed. This expected behavior can be attributed to the consumption of free DNA by the increasing amounts of repressor. Up to, and slightly beyond, the apparent ratio of 1:1, this effect is relatively linear. This slight deviation can be attributed to the low concentration of active DNA in the non-gel-purified DNA sample, as alluded to earlier. Once the ratio approaches and

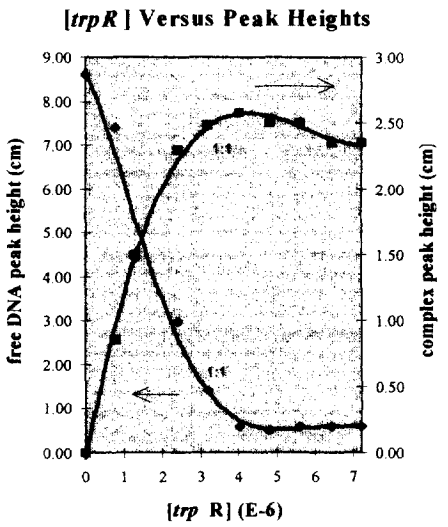

Fig. 4. Plot depicting change in peak height as a function of [*trpR*]. Labels representing the 1:1 data points for each curve are listed. Conditions: 45 m*M* TBE, 0.5 m*M* L-tryptophan, 0.75% methyl cellulose, pH 8.

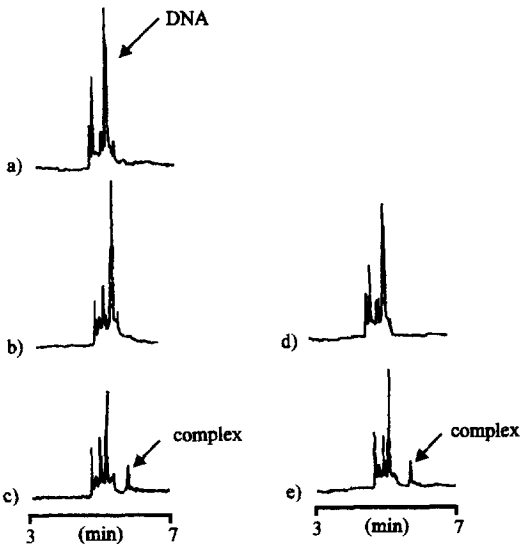

Fig. 5. Electropherograms depicting the effect of non-DNA-binding proteins on the selectivity of the *trp* repressor–*trp* operator interaction. (a) Free DNA; (b, c) 1:1 complexes of myoglobin–DNA without and with *trpR*, respectively; (d, e) 1:1 complexes of lysozyme–DNA without and with *trpR*, respectively. Conditions: 45 m*M* TBE, 0.5 m*M* L-tryptophan, 0.75% methyl cellulose, pH 8.

exceeds an apparent ratio of 1:1 (i.e., as [*trpR*]> [DNA]), a leveling effect is seen in the peak height of the free DNA. The same effect is also seen in the plot of complex peak height as a function of increasing [*trpR*]. The minimum detectable injected concentration, based on a complex peak at twice the baseline noise, is 5×10^{-8} *M trpR*. A relative standard deviation of complex peak height, based on five sequential injections of the 0.75:1 *trpR*–DNA complex, is 4.9%.

The *trp* repressor–*trp* operator interaction is one of high specificity. Not only must the correct base pairs be present on the DNA, but tryptophan must also be present to allow the repressor to properly bind. Simple qualitative studies, involving both non-DNA-binding and DNA-binding proteins, can demonstrate the high selectivity of the operator for the repressor. An example of *trp* repressor–*trp* operator selectivity involving non-DNA-binding proteins is depicted in Fig. 5. Upon reaction of the DNA with an equimolar amount of either myoglobin or lysozyme, no complex peak is observed. This is anticipated due to the fact that neither protein recognizes the specific nucleotide sequences on the operator as binding sites. Myoglobin and lysozyme, while representing interfering species, cause relatively no change in the free DNA signal. However, upon addition of repressor, in equimolar amounts with the operator, complex creation is observed, even in the presence of the non-specific proteins. This demonstrates the selectivity of the DNA for the repressor. A greater degree of selectivity can be seen when the interfering analyte is also a DNA-binding protein.

E12 is a basic-helix-loop-helix (bHLH) protein, known to bind the enhancers of immunoglobulin genes [39]. Together with other bHLH proteins, E12 is involved in mammalian muscle development [39–41]. While E12 does not represent a protein species that specifically binds to the region on the *trp* operator, it is known to bind DNA. Fig. 6 depicts the effect of DNA-binding protein on the selectivity of the *trp* repressor–*trp* operator interaction. Upon reaction of the DNA with an equimolar amount of E12, an almost complete diminishing of the free DNA signal is observed, however no complex is observed. Non-specific interactions are probably responsible for this apparent binding to DNA. The binding constant for E12 is large enough to observe non-specific interactions but is not large enough to

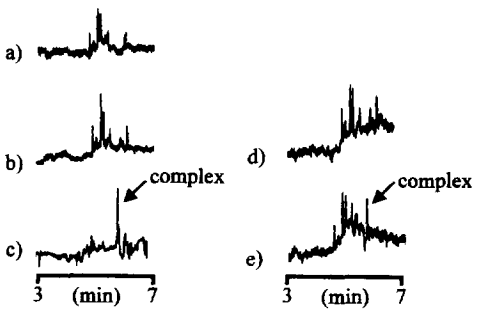

Fig. 6. Electropherograms depicting the effect of the DNA-binding protein, E12, on the selectivity of the *trp* repressor–*trp* operator interaction. (a) An approximate 1:1 complex of E12–DNA; (b, c) the 1:1 complex of (a) with *trpR* at $t = 30$ min and $t = 24$ h (after *trpR* added to E12–DNA mixture), respectively; (d, e) a sample containing three interfering proteins (myoglobin, lysozyme and E12), all in a 1:1 ratio with DNA and *trpR* at $t = 30$ min and $t = 24$ h, respectively. Conditions: 45 m*M* TBE, 0.5 m*M* L-tryptophan, 0.75% methyl cellulose, pH 8.

observe a complex peak. The addition of an equimolar amount of *trpR* yields no complex peak. However, over time (24 h), the dominant and highly specific interaction of the *trp* repressor for its operator is evident. This can be attributed to the fact that the large binding constant for *trpR* to DNA eventually overcomes the smaller binding constant of E12. Fig. 6 also depicts a similar situation when all three interfering species, (myoglobin, lysozyme and E12), are added to the DNA, followed by addition of repressor. The same highly specific behavior is apparent.

This work demonstrates methodologies for the study of protein–DNA interactions by CE. The assay, termed CEMSA, exploits the advantages of both conventional slab gel electrophoresis and CE. The use of polyacrylamide-coated columns suppressed DNA and protein adsorption to the column, while sufficiently eliminating electroosmotic flow for resolution of DNA from the complex. Assay times are generally under 6 min and, in common with most CE systems, the ability to rapidly adjust the system parameters (running buffer constituents, pH, applied voltage, etc.) is present. With capillary columns in hand, an entire series of experiments can literally be run without interruption. This is highly favorable when compared to the long turn-around times of traditional gel-based assays. The *trp* repressor–*trp* operator interaction, well characterized by other assay procedures, served as an appropriate system for the development of CEMSA.

Acknowledgments

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