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Studies of Protein−DNA Interactions by Capillary Electrophoresis/Laser-Induced Fluorescence Polarization

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Protein−DNA interactions were studied on the basis of capillary electrophoretic separation of bound from free fluorescent probe followed by on-line detection with laser-induced fluorescence polarization. Changes in electrophoretic mobility and fluorescence anisotropy upon complex formation were monitored for the determination of binding affinity and stoichiometry. The method was applied to study the interactions of single-stranded DNA binding protein (SSB) with synthetic oligonucleotides and single-stranded DNA. Increases in fluorescence anisotropy and decreases in electrophoretic mobility upon their binding to SSB were observed for the fluorescently labeled 11-mer and 37-mer oligonucleotide probes. Fluorescence anisotropy and electrophoretic mobility were used to determine the binding constants of the SSB with the 11-mer (5×10^6 M^{-1}) and the 37-mer (23×10^6 M^{-1}). Alternatively, a fluorescently labeled SSB was used as a probe, and the formation of multiple protein−DNA complexes that differ in stoichiometry was observed. The results demonstrate the applicability of the method to study complex interactions between protein and DNA.

The binding of proteins to DNA is an integral component of many cellular activities including the control of gene expression, site-specific recombination, replication, and repair of DNA damage.[1,2] Quantitative measurements of these interactions are essential not only to understanding the molecular basis for these cellular functions but also to the development of bioanalytical methods that derive their specificity from these interactions. Several techniques have been used for binding studies, including fluorescence spectroscopy, nuclear magnetic resonance, mass spectrometry, and gel electrophoresis. The most commonly used method is the gel electrophoresis mobility shift assay (EMSA),[2−5] which is based on the observation that binding of a protein to DNA fragments generally leads to a reduction in the electrophoretic mobility of the DNA fragment in nondenaturing polyacrylamide or agarose gels. The assay typically involves the addition of a binding protein to a DNA sample, separation of bound and free DNA by gel electrophoresis, and visualization of the separated DNA either by autoradiography or fluorescence imaging. While used extensively, EMSA requires relatively large amounts of sample and lengthy analysis time. Moreover, the assay is not suitable when dissociation of protein−DNA complex occurs during gel electrophoresis.

Efforts to alleviate these problems have led to the development of other rapid and sensitive approaches. Fluorescence polarization (FP) provides a nonradioactive approach for measuring protein−DNA interactions.[6−8] FP is directly related to the molecular volume of the fluorescent molecule. An increase in molecular volume due to binding of a small fluorescent molecule to a protein impedes the fluorescent molecule's rotational motion in solution and results in an increase in polarization. Thus, the binding of a fluorescently labeled DNA fragment to a recognition protein can be studied by monitoring the changes in fluorescence polarization. Thus, FP measurement is well-suited for optimizing binding conditions and for screening binding ligands. However, its use in quantitative analysis is limited to simple binding systems because it is unable to distinguish multiple protein−DNA complexes. Expansion of its quantitative applications necessitates its coupling with a separation technique such as capillary electrophoresis (CE). With a high-sensitivity laser-induced fluorescence (LIF) detector, CE offers the benefit of rapid analysis of complex binding systems. Separation and detection of protein-bound and free fluorescent DNA probes can be complete within a few minutes, and sample volumes required are reduced by at least 100-fold from the amount that is needed for EMSA.[9−11] CE/LIF has been used to study DNA-binding affinities of sea urchin embryo transcription factor, SpP3A2,[9] a yeast transcription factor, GCNK 58,[11] and a bacterial protein, *trp* repressor.[10] One difficulty is, however, associated with the identification of protein−DNA complexes since LIF gives no information about the characteristics of these complexes.

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Recently, we developed a new approach incorporating the advantages of FP with those of CE and applied it to qualitative measurements of noncovalent interactions involving proteins with peptides, proteins, and DNA.[12] A fluorescently labeled probe molecule, which can be a peptide, protein, or DNA fragment, was mixed with an affinity recognition agent (e.g., an antibody) in solution, and the resulting mixture was then analyzed by on-line coupled capillary electrophoresis and laser-induced fluorescence polarization (CE/LIFP). Changes in the electrophoretic mobility and fluorescence polarization of the fluorescent probe upon complex formation with the binding partner were measured simultaneously, thereby providing complementary information on the binding interaction. This information could not be obtained with either CE or LIFP used alone.

For binding systems that have low affinity, dissociation may take place during separation. We overcame this problem by including the binding reagents in the CE separation buffer to stabilize the complex. In this paper, we report a detailed study on the use of CE/LIFP in the analysis of protein−DNA interactions. Single-stranded DNA binding protein (SSB) from *Escherichia coli*, synthetic oligonucleotides, and single-stranded DNA (ssDNA) were chosen as the study systems. A fluorescently labeled oligonucleotide or SSB protein was used as a probe and the binding interactions with its partner were studied in either of two formats, depending on the stability of the complexes formed. For weak binding interactions, the binding partner was included in the running buffer to stabilize the complexes during CE separation. The electrophoretic mobility and fluorescence anisotropy of the fluorescent probe were measured as a function of the concentration of its binding partner in the running buffer. Both the electrophoretic mobility and fluorescence anisotropy were used to determine the binding constants and cooperativity. For high-affinity interactions, mixtures containing the fluorescent probe and its binding partner at varying ratios were incubated prior to separation. The complexes formed off-column were then separated by CE with a running buffer free of the binding components. The electrophoretic mobility and fluorescence anisotropy measurements were used for the identification of the complexes and for the study of binding stoichiometry.

The SSB protein plays an important role in the DNA replication, recombination, and repair process[13−16] although mechanisms for its functions in these processes have not yet been elucidated. The SSB protein exists as a tetramer in solution with a subunit weight of ~20 000. It binds cooperatively to single-stranded DNA (approximately 32−60 nucleotides per protein), keeping the DNA in an extended configuration and protecting it from nuclease digestion. One of the reasons for choosing the binding of SSB with DNA as a study system is its rich stoichiometry. The resolving power and identification capabilities make the CE/LIFP approach especially attractive for studying the formation of protein−DNA complexes that differ in stoichiometry.

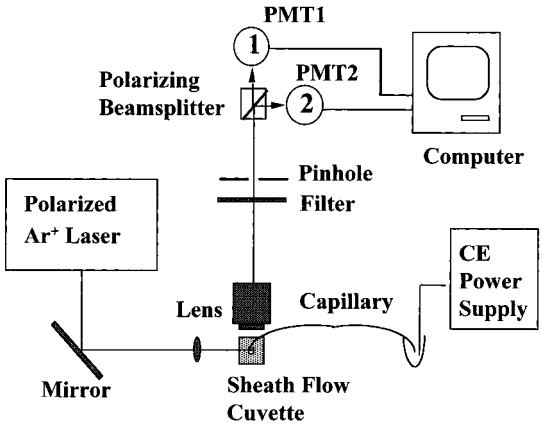

Figure 1. Schematic diagram showing capillary electrophoresis separation with laser-induced fluorescence polarization detection.

EXPERIMENTAL SECTION

Materials and Reagents. Disodium fluorescein of purified grade was obtained from Fisher Scientific (Fair Lawn, NJ). Fluorescein isothiocyanate (FITC) isomer I, SSB protein, $d(pT)_{18}$, single-stranded M13mp8 phage DNA, and fluorescence polarization immunoassay (FPIA) dilution buffer (pH 7.4), containing phosphate, bovine protein, and sodium azide, were obtained from Sigma (St. Louis, MO). Fluorescein-dUTP was obtained from Molecular Probes (Eugene, OR). A 5′-oligolabeling kit containing T4 polynucleotide kinase, ATPS, and 5-iodoacetamidofluorescein (IAF) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, England). Fluorescein-labeled oligonucleotides 11-mer (5′-F-CGCGATACGCC-3′) and 37-mer (5′-F-CCTTAAGCTTCCTCAACCACTTACCATACTCGAGATT-3′) were provided by J. Lee of Cross Cancer Institute and T. Carnelley of Department of Public Health Sciences, University of Alberta.

Apparatus. The experimental setup used to perform CE/LIFP analysis is schematically shown in Figure 1. Briefly, the home-built system consisted of a CE power supply (model CZE 1000R, Spellman, Plainview, NY), a fused-silica capillary (Polymicro Technologies, Phoenix, AZ), and a laser-induced fluorescence polarization detector. The detector consisted of a polarized argon ion laser (model 2014-65ML, Uniphase, San Jose, CA), laser focusing and fluorescence collection optics, a broad-band polarizing beam splitter cube (Melles Griot, Irvine, CA), and two photomultiplier tubes (PMT1 and PMT2) (R1477, Hamamatsu, Shizuoka, Japan). A sheath flow cuvette (NSG Precision Cells, Farmingdale, NY) was used as a postcolumn fluorescence detection cell.[17] The operation of the power supply and the acquisition of data were controlled by a Power Macintosh computer with the application software written in LabView (National Instruments, Austin, TX).

There is no fundamental difference between the apparatus we use to measure fluorescence anisotropy and the conventional fluorescence detectors that are capable of anisotropy measurement. The only difference is that our cuvette (flow cell) is much smaller (0.2 × 0.2 mm square) and that our detector is capable of handling the small volumes suitable for CE separation.

Fluorescent Labeling of SSB Protein and $d(pT)_{18}$. The SSB protein was labeled with FITC, and the extent of modification was

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estimated according to the methods described by Brinkley.[18] A 10-fold molar excess of the dye was added to a solution of the SSB protein (1.4 μg/mL) in 25 mM $Na_2B_4O_7$ (pH 9.1). The reaction was allowed to proceed for 1 h at room temperature and then stopped by adding excess of hydroxylamine. The FITC-labeled protein was purified using a prepacked Bio-spin column (Bio-Gel P-6, Bio-Rad, Hercules, CA). The absorbance of FITC (280 nm) and FITC-SSB (490 nm) in 25 mM $Na_2B_4O_7$ (pH 9.1) was measured using a Hewlett-Packard (Pala Alto, CA) model 1040A diode array detector equipped with a Gilson (Villies le Bel, France) model 307 HPLC pump. The molar ratio of the fluorophor to protein was calculated from the absorbance measurements using the following extinction coefficients: FITC,[19] $\epsilon_{490} = 73\,000$ cm^{-1} M^{-1}; SSB,[20] $\epsilon_{280} = 120\,000$ cm^{-1} M^{-1}. Absorbance of FITC at 280 nm was corrected for in the calculation of the molar ratio of FITC to protein. The labeling of d(pT)$_{18}$ at the 5′-end with 5-iodoacetamidofluorescein was accomplished by following a protocol provided by Amersham.

Complex Formation. For weak interactions, protein−DNA complexes were formed on-column with excess amounts of the protein. Buffer solutions containing various concentrations of the SSB protein or oligonucleotide were used as CE running buffers. The fluorescently labeled DNA was injected into the capillary for CE/LIFP analysis. For strong interactions, protein−DNA complexes were formed off-column. Various volumes (0, 2.5, 5.0, 10, 15 μL) of the M13 phage DNA solution (43 nM) were mixed with 1.0-μL aliquots of FITC-SSB protein solution (12.5 μM) in 0.5-mL microcentrifuge tubes. FPIA dilution buffer was added to each tube to a final volume of 50 μL. The tubes were vortexed for 30 s and the mixtures incubated at room temperature for at least 15 min prior to CE/LIFP analysis.

CE/LIFP Analysis. A capillary of 20 μm i.d, 148 μm o.d., and 35 cm in length was used in conjunction with 25 mM $Na_2B_4O_7$ (pH 9.1) as a typical running buffer, unless stated otherwise. Prior to sample analysis, the capillary was preconditioned periodically by successive rinsing with 0.1 M NaOH, deionized water, and the running buffer to ensure the reproducibility of the separation. Samples were electrokinetically injected into the capillary by applying an electric field of 143 V/cm for 5 s. Separation was carried out under an applied field strength of 714 V/cm. The electrophoretic mobility (μ) of a solute was calculated using the following equation:[21,22]

$$\mu = L(1/t_{eo} - 1/t)/E \qquad (1)$$

where L and E are the capillary length and applied electric field strength and t_{eo} and t are the migration times of the solvent and solute, respectively.

Horizontally and vertically polarized fluorescence intensities measured by the LIFP detector were optimized by aligning a tightly focused laser beam with a small-diameter sample stream and by balancing signals from the two PMTs. An aqueous solution of disodium fluorescein (10^{-9} M) was passed through a capillary inserted in a sheath flow cuvette. The sheath fluid, identical to the CE run buffer, was introduced into the cuvette hydrodynamically by keeping the inlet reservoir of the sheath buffer 1 cm higher than the outlet reservoir. The vertically polarized laser beam was focused onto a spot ~20 μm below the tip of the capillary. The angle and position of the cuvette relative to the detection optical path were adjusted so that roughly equal signals with maximum outputs from both PMTs were achieved. The values of fluorescence anisotropy (A) were calculated according to[6−8]

$$A = (I_v - I_h)/(I_v + 2I_h) \qquad (2)$$

where I_v and I_h are the fluorescence intensities of vertically and horizontally polarized components, respectively.

There is possible unequal transmission of the two orthogonal polarizations by the emission optical trains and unequal sensitivity of the PMT detectors. To correct for the potential bias, we adjusted the PMT voltage until the fluorescence intensities from the two PMTs (the vertically and horizontally polarized fluorescence) were identical for dilute fluorescein (10^{-9} M), assuming its anisotropy negligible.

RESULTS AND DISCUSSION

CE/LIFP may be used in binding studies through the measurement of changes of either electrophoretic mobility or fluorescence anisotropy of a binding component upon affinity interactions. Usually, the binding component of lower molecular weight is used as a probe to induce greater mobility or anisotropy changes upon binding. In the present study, both fluorescently labeled DNA fragments and binding protein were examined as probes for the study of protein−DNA interactions.

Fluorescein-Labeled Oligonucleotides as Probes. In the first series of experiments, fluorescein-labeled 11-mer (F-11-mer) was evaluated as a probe for examining its binding with the SSB protein. Figure 2 shows electropherograms of F-11-mer in the absence (Figure 2A) and presence (Figure 2B) of the SSB protein in the running buffer. The vertical (I_v) and horizontal (I_h) components of fluorescence were measured simultaneously. Fluorescein-labeled dUTP (F-dUTP) was used as a reference compound to correct for possible fluctuations in electroosmotic flow and unequal detection sensitivity between the two detection channels of the instrument. In the absence of the binding protein, the fluorescent probe has an electrophoretic mobility of $\mu = 3.99 \times 10^{-4}$ cm^2 V^{-1} s^{-1} and a fluorescence anisotropy of $A = 0.05$ (Figure 2A). In the presence of 0.7 μM SSB protein in the running buffer, the electrophoretic mobility of the oligonucleotide probe is reduced to 1.93×10^{-4} cm^2 V^{-1} s^{-1} whereas the anisotropy is increased to 0.25 (Figure 2B). The decrease in electrophoretic mobility and increase in anisotropy are due to the binding of the F-11-mer with the SSB protein. In contrast, the mobility and anisotropy of F-dUTP are essentially unchanged, consistent with the fact that the SSB protein has very low binding affinity for the mononucleotide.

The electrophoretic mobility and anisotropy changes observed in the experiments arise from the effects of the binding protein

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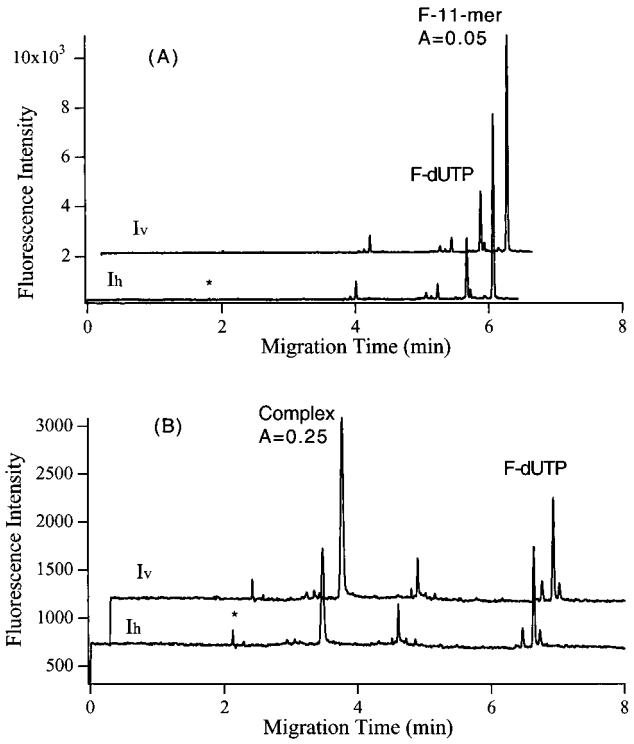

Figure 2. Electropherograms showing 3 nM fluorescently labeled 11-mer in the absence (A) and in the presence (B) of 0.7 μM SSB protein in the running buffer. The conditions used were as follows: separation capillary, 35 cm × 20 μm i.d.; running buffer, 25 mM disodium tetraborate (pH 9.1); separation voltage, 25 kV; excitation wavelength, 488 nm; emission wavelength, 515 nm; and temperature, 25 ± 1 °C. Approximately 1 nL of sample solution was injected electrokinetically. The asterisk indicates the migration time of the solvent, t_{eo}. The traces I_v and I_h, corresponding to vertically and horizontally polarized fluorescence intensities, are offset for clarity.

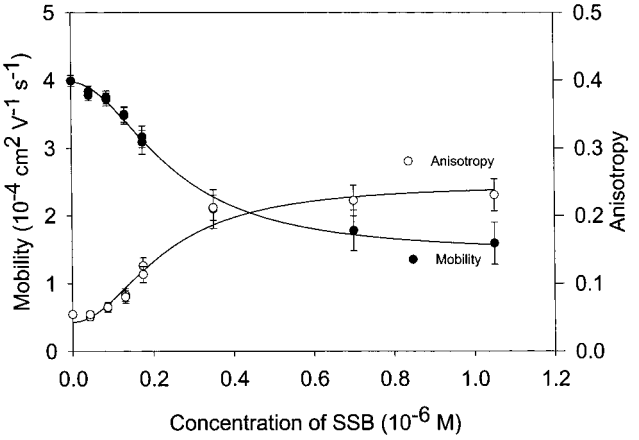

Figure 3. Binding curves obtained by measuring the electrophoretic mobility (●) and fluorescence anisotropy (○) of F-11-mer at varying concentrations of SSB protein in the running buffer. Conditions were the same as for Figure 2.

on the molecular motion of the probe. In the absence of the binding protein, the fluorescently labeled oligonucleotide probe migrates with a mobility similar to that of F-dUTP in the free zone electrophoresis mode. It has a low fluorescence anisotropy because of its small molecular size (MW 4000) and random motion in solution. When bound to the SSB protein (MW 80 000), the size of the fluorescent molecule is markedly increased, resulting in a slower molecular motion in the solution. Therefore, it is not surprising that binding of the SSB protein to the oligonucleotide probe gives rise to a marked increase in anisotropy. The mobility and anisotropy of the complex approach to those of the binding protein ($\mu = 1.07 \times 10^{-4}$ cm^2 V^{-1} s^{-1} and $A = 0.23$ for FITC-SSB; see Figure 6). While the electrophoretic mobility of a compound is proportional to its charge-to-mass ratio,[21,22] the fluorescence anisotropy is mainly determined by its molecular size, shape, and fluorescence lifetime.[6]

The mobility and anisotropy of the F-11-mer were measured with the running buffer containing various concentrations of the SSB protein, resulting in the binding profiles as shown in Figure 3. Nanoliter amounts of the F-11-mer (10^{-9} M) were injected into the capillary that was filled with the running buffer containing 10^{-7}−10^{-6} M SSB protein. Thus, the concentration of the binding protein in the running buffer was in large excess and was not affected significantly by its binding with the F-11-mer. Under this condition, the quantitative interpretation of the binding profiles can be carried out using a standard four-parameter logistic equation:[23]

$$y = (a + bK^n x^n)/(1 + K^n x^n) \qquad (3)$$

where y is the observed response such as electrophoretic mobility or fluorescence anisotropy of the oligonucleotide probe at a given concentration of the binding protein, x; K is the apparent binding constant; a and b are the responses of the free and bound probe, respectively; and superscript n is the Hill coefficient describing the steepness of the curve. The experimental data in Figure 3 were fitted to the above equation using nonlinear regression analysis (SigmaPlot, version 4, SPSS Inc.). Binding constant (K) measurements based on mobility and anisotropy of the F-11-mer were similar, which were approximately 4.4 × 10^6 M^{-1} and 5 × 10^6 M^{-1}, respectively. These values are comparable to previous measurements by other methods. For example, Molineux et al.[24] reported that SSB has an affinity of ∼2 × 10^6 M^{-1} for d(pT)$_8$ and Krauss et al.[14] reported an affinity of 1.4 × 10^6 M^{-1} for d(pT)$_{16}$ using fluorescence quenching methods. Binding constants and fitting parameters from nonlinear regression analysis are summarized in Table 1.

Having demonstrated the feasibility of the approach for quantitative binding studies, we further explored its application to the analysis of binding interactions involving protein−DNA complexes that differ in stoichiometry. Fluorescein-labeled 37-mer (F-37-mer) was chosen as a DNA probe since its complexes with the SSB protein are of higher stability, allowing us to examine distribution of different species in the binding interactions. Figure 4 shows electropherograms of F-37-mer with the absence and presence of the SSB protein in the CE running buffer. Changes in electrophoretic mobility and fluorescence anisotropy upon formation of complexes between F-37-mer and SSB protein are observed as expected. It is noted that the initial single peak of F-37-mer was split into two when bound to the SSB protein. Both

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Table 1. Binding Interactions between Fluorescently Labeled Oligonucleotides and SSB Analyzed by Nonlinear Regression of Mobility and Anisotropy Data According to $Y = (a + bK^n x^n)/(1 + K^n x^n)$

y	n	a	b	K (10^6 M^{-1})	R^2
F-11-mer					
mobility	3.0 ± 0.2	3.84 ± 0.03	1.63 ± 0.04	4.4 ± 0.1	1.00
anisotropy	3.9 ± 0.4	0.05 ± 0.002	0.23 ± 0.01	5.0 ± 0.1	1.00
F-37-mer					
mobility	5.1 ± 1.7	3.56 ± 0.09	1.80 ± 0.07	24 ± 1	0.96
anisotropy	5.7 ± 1.3	0.02 ± 0.01	0.24 ± 0.01	22 ± 1	0.98

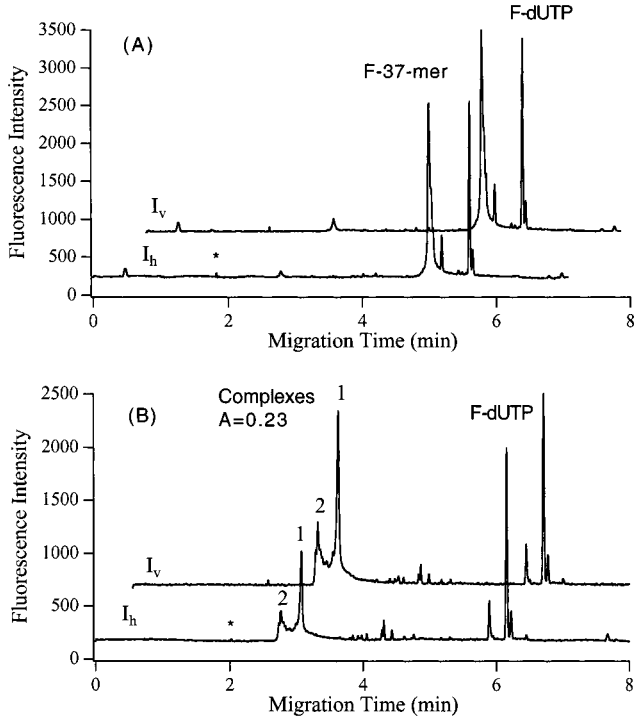

Figure 4. Electropherograms showing 3 nM fluorescently labeled 37-mer in the absence (A) and in the presence (B) of 0.35 μM SSB protein in the running buffer. Conditions were the same as for Figure 2.

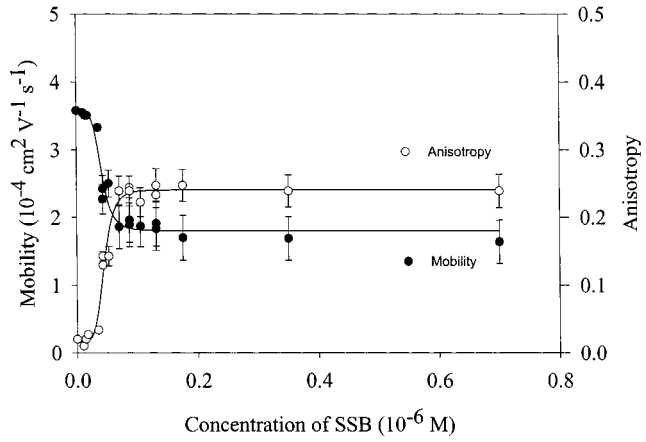

Figure 5. Binding curves obtained by measuring the electrophoretic mobility (●) and fluorescence anisotropy (○) of F-37-mer at varying concentrations of SSB protein in the running buffer. Conditions were the same as for Figure 2.

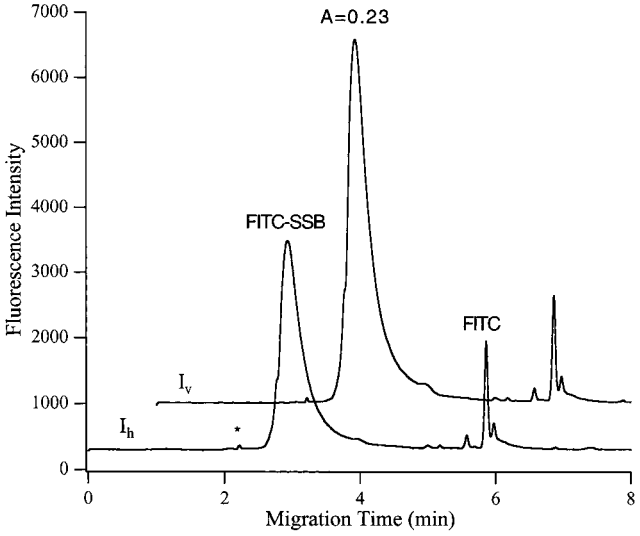

Figure 6. Electropherograms showing FITC-labeled SSB protein in 25 mM disodium tetraborate, pH 9.1. Conditions were the same as for Figure 2.

complex peaks display strong fluorescence anisotropy ($A = 0.23$). These are likely 2:1 (peak 2) and 1:1 (peak 1) protein−DNA complexes. For the 1:1 binding interaction, variations of the μ and A values for the F-37-mer with the binding protein concentration are shown in Figure 5, from which the apparent binding constants were obtained. Again, both mobility and anisotropy measurements gave very similar results, with binding constants of $\sim 2 \times 10^7$ M^{-1} (see Table 1). This is a ~5-fold increase in binding affinity of SSB for the 37-mer compared to its binding with the 11-mer. This is consistent with the contribution of cooperativity to the binding strength. The SSB protein is a tetramer and the number of the binding sites of the SSB protein varies with the length of the oligonucleotides because each of the four subunits of the protein covers 6−8 nucleotides.[14,15] While one subunit may bind to the 11-mer, all the four subunits of the SSB tetramer could bind to the 37-mer, resulting in the corresponding increase in binding constant. Because the two complex species were not well resolved, particularly at low protein concentrations, there was a relatively large uncertainty associated with mobility and anisotropy measurements for the 2:1 complex. Consequently, we were unable to precisely determine the corresponding binding constant for the 2:1 complex.

Fluorescein-Labeled DNA Binding Protein as a Probe. Fluorescently labeled DNA oligonucleotides have exclusively been used as probes in the analysis of protein−DNA interactions by gel retardation, CE, or FP techniques. However, many bioanalytical applications require the use of a fluorescently labeled binding protein for its ability to recognize and bind to specific structures of DNA. To this end, we carried out experiments to examine the binding of FITC-labeled SSB protein with a synthetic oligonucleotide and a single-strand DNA. The FITC-labeled SSB protein was prepared as described in the Experimental Section, and the dye-to-protein molar ratio was 5.3. In a solution of 25 mM disodium tetraborate with pH 9.1, the labeled SSB protein displayed an electrophoretic mobility of 1.07×10^{-4} cm^2 V^{-1} s^{-1} and a fluorescence anisotropy of 0.23 (Figure 6).

Figure 7 shows a series of electropherograms of FITC-SSB protein obtained with running buffers containing varying amounts of oligonucleotide, $d(pT)_{18}$. Although both vertically and horizon-

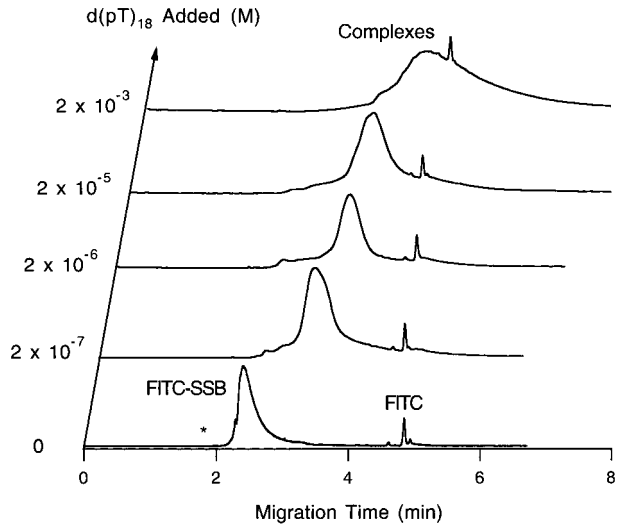

Figure 7. Effect of varying concentration of oligonucleotide, d(pT)-18, in the running buffer on the migration of FITC-labeled SSB protein. Conditions were the same as for Figure 2.

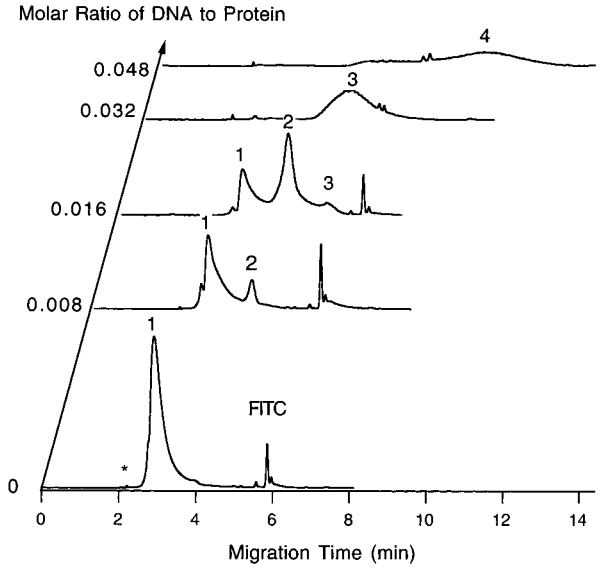

Figure 8. Separations of FITC-labeled SSB protein and its complexes with single-stranded M13 DNA formed at varying DNA-to-protein ratios. Conditions were the same as for Figure 2.

tally polarized fluorescence emissions were acquired simultaneously, only the vertical components of fluorescence emission are shown for clarity. There is a slight increase in fluorescence anisotropy ($\Delta A \approx 0.02$) of the protein probe upon binding to the oligonucleotide. This is in accordance with the fact that fluorescence anisotropy of a probe generally approaches saturation when the probe's molecular weight exceeds 20 000.[6,12,26]

It is noted that formation of the complex gives rise to some significant changes in the mobility and peak shape of the FITC-labeled SSB protein. As the concentration of $d(pT)_{18}$ in the running buffer increases, the electrophoretic mobility of the SSB protein increases with the peak becoming increasingly dispersed. The mobility increase of the low-mobility species (the FITC-SSB protein, $\mu = 1.07 \times 10^{-4}$ cm^2 V^{-1} s^{-1}) is due to its binding to a high-mobility DNA ($\mu = 2.19 \times 10^{-4}$ cm^2 V^{-1} s^{-1} for fluorescein-labeled $d(pT)_{18}$). The peak broadening suggests that the formation of multiple protein−DNA complexes is possible in the presence of increasing amount of DNA. A single protein molecule may bind several DNA molecules in the presence of excess DNA. The complexes of varying protein-to-DNA ratios comigrate in the separation capillary as a broad band. To clarify this point, we chose a DNA fragment much longer than $d(pT)_{18}$ to form stable complexes with the SSB protein. Because of increased stability, the multiple complexes formed off-column can be separated without the need for adding the binding partner to the running buffer.

Single-stranded M13mp8 phage DNA (7229 bases) was selected as a binding partner for the labeled SSB protein. Varying amounts of the phage DNA were incubated with a series of binding solutions containing a fixed amount of the FITC-labeled SSB protein. The mixtures were then analyzed by CE/LIFP with a running buffer free of the binding components. Figure 8 shows separations of FITC-SSB protein and its complexes with the DNA at various molar ratios of DNA to protein. As the amount of DNA in the reaction mixture increases, new peaks emerge and become increasingly retarded and broadened. The broad and multiple peaks with increasing migration times showed strong fluorescence anisotropy ($A = 0.25$), indicating the presence of multiple protein−DNA complexes. Three major peaks with increasing mobilities (peak 2, 2.16×10^{-4} cm^2 V^{-1} s^{-1}; peak 3, 2.66×10^{-4} cm^2 V^{-1} s^{-1}; peak 4, 3.16×10^{-4} cm^2 V^{-1} s^{-1}) were thought to correspond to the complexes with increasing DNA to protein ratios.

In the absence of the DNA, peak 1 corresponds to the SSB protein probe which has a $\mu = 1.07 \times 10^{-4}$ cm^2 V^{-1} s^{-1}. Addition of the DNA to the protein (with DNA-to-protein ratio of 0.008 and 0.016) causes a decrease in peak 1 and the appearance of peak 2 ($\mu = 2.16 \times 10^{-4}$ cm^2 V^{-1} s^{-1}), indicating the formation of DNA−protein complex. With further increase of DNA-to-protein ratio, the mobilities of the complexes (2.66×10^{-4} cm^2 V^{-1} s^{-1} for peak 3; 3.16×10^{-4} cm^2 V^{-1} s^{-1} for peak 4) shift toward that of the DNA ($\mu = 3.50 \times 10^{-4}$ cm^2 V^{-1} s^{-1}).[27] Because the amount of the protein was fixed in this series of experiments, increasing amounts of DNA in the reaction mixture favor the formation of complexes of an increasing DNA/protein ratio. This example demonstrates an application of CE/LIFP to study multiple complexes.

CONCLUSIONS

An on-line coupled CE/LIFP approach was demonstrated to study binding interactions of single-stranded DNA binding protein with oligonucleotides and DNA. Either a fluorescently labeled oligonucleotide or a binding protein can be used as a probe to facilitate the detection of protein−DNA complexation. The CE/LIFP allows for simultaneous measurements of electrophoretic mobility and fluorescence anisotropy. The binding affinity and stoichiometry can be determined by examining the changes in electrophoretic mobility and/or fluorescence anisotropy upon affinity interactions. The sample volume and time required are not only much less than those required for a gel shift assay; they also are less than required for binding determination by fluores-

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cence titration, which is advantage of the CE/LIFP system. The applications of CE/LIFP are not limited to the binding systems shown in this study. With an appropriate fluorescently labeled DNA probe, the CE/LIFP method may be used to screen for DNA binding proteins present in cell extracts or expression libraries.[28] It also has potential for the detection of DNA damage using specific recognition proteins as probes. Many proteins that are able to recognize and bind to the DNA lesions have been identified.[29]

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