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COMMUNICATIONS

Published on Web 10/26/2002

Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures − A Single Experiment Reveals Equilibrium and Kinetic Parameters of Protein−DNA Interactions

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Received August 20, 2002

Protein−DNA interactions play an important role in gene expression, DNA replication, DNA integrity control, and DNA damage repair.[1] Electrophoresis has been the workhorse method for in vitro studies of such interactions. Classically, electrophoresis mobility shift assay (EMSA) on slab gels was used in these kind of studies.[2] Lately, EMSA in capillaries and affinity capillary electrophoresis have been developed for studies of protein−DNA interactions.[3] The assumption of equilibrium in all of these methods allows for the determination of the equilibrium binding constant, K_b, but makes it difficult or impossible to find the monomolecular rate constant of complex decay, k_{-1}. Here we introduce for the first time a unique electrophoretic method, nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), and describe its use in studies of protein−DNA interactions. In NECEEM, a short plug of the equilibrium mixture is introduced into the capillary and subjected to electrophoresis under nonequilibrium conditions. The new method allows for the determination of two parameters, K_b and k_{-1}, in a single experiment that requires only a minute amount of the protein. NECEEM has the potential for significantly augmenting the arsenal of methods available for studies of protein−DNA interactions. Moreover, we foresee the potential role of NEECEM in the development of extremely sensitive protein assays based on aptamers (synthetic affinity probes).

In this study, we used the following protein−DNA pair: an *E. coli* single-stranded DNA binding (SSB) protein and a fluorescently labeled 15-mer oligonucleotide, 5′-fluorescein-GCGGAGCGT-GGCAGG. We prepared the equilibrium mixture of the protein and DNA by mixing the solutions of 16 μM protein and 205 nM DNA at a desired volume ratio and by incubating at room temperature for 1 h prior to the analysis. The equilibrium mixtures were made in the run buffer used in NECEEM. The equilibrium mixtures contained three components: free protein (P), free DNA (DNA), and the protein−DNA complex (P·DNA). We used laser-induced fluorescence detection; therefore, only DNA and P·DNA were detectable due to the fluorescent label on DNA.

Eighty picoliters of the equilibrium mixture was injected into the capillary and analyzed by NECEEM. In general, the observed electropherograms exhibited three essential features: peaks 1 and 2 and curve 3 (Figure 1). Sole peak 1 was observed when P was not present in the equilibrium mixture, indicating that peak 1 represents DNA (the upper panel in Figure 1). When the concentration of P was intermediate, all three features were observed (the middle panel in Figure 1). The height of peak 1 decreased with increasing concentration of P. When the concentration of P was high enough, peak 1 completely disappeared (the bottom panel in Figure 1). Curve 3 was shown to be perfectly fitted by a single-exponential curve and therefore will be called the "exponential part" of the electropherogram.

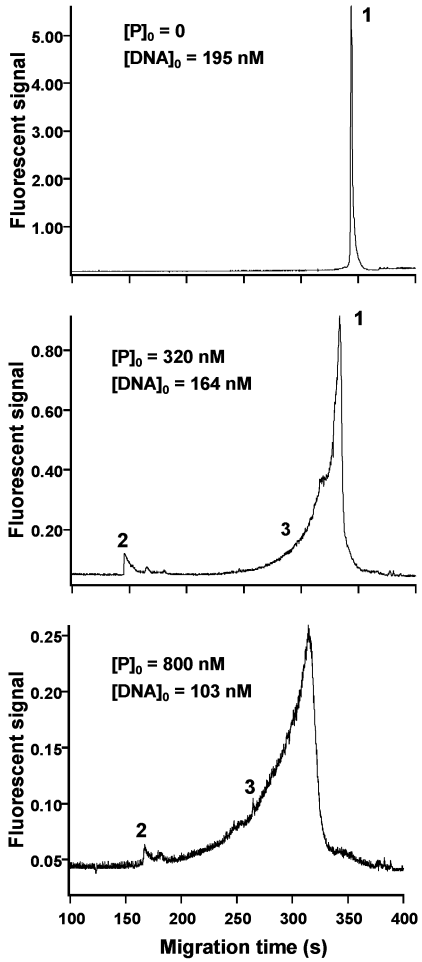

Figure 1. Nonequilibrium capillary electrophoresis of the equilibrium mixtures of the SSB protein and a fluorescently labeled 15-mer oligonucleotide, 5′-fluorescein-GCGGAGCGTGGCAGG (DNA). The total concentrations of the protein and DNA in the mixtures are shown in the panels.

To reveal the identities of the three features in the electropherograms, we need to consider the fate of the three components, P, DNA, and P·DNA, while being separated in NECEEM. Because of the significant difference in the charge-to-mass ratio, the electrophoretic mobilities of P and DNA differed considerably; P·DNA had an intermediate value of electrophoretic mobility. Because of these differences in the three electrophoretic mobilities, the equilibrium fractions of DNA and P were immediately removed from the electrophoretic zone of P·DNA as soon as electrophoresis

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10.1021/ja028212e CCC: $22.00

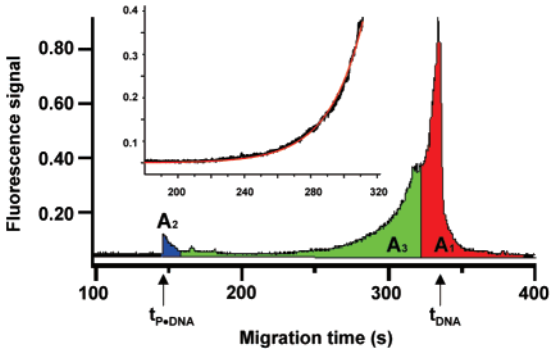

Figure 2. The determination of K_b and k_{-1} requires a number of parameters that can be obtained from a single NECEEM experiment. The mainframe illustrates the determination of areas A_1 and $A_2 + A_3$ as well as migration times t_{DNA} and $t_{P\cdot DNA}$. The inset illustrates fitting of experimental data (black line) with the single-exponential function (red line).

started. The equilibrium fraction of DNA migrated as a single electrophoretic zone and resulted in peak 1 with the longest migration time. The equilibrium fraction of P also migrated as a single zone, but due to the lack of a fluorescent label, P did not contribute to the electropherograms. The equilibrium fraction of P·DNA could not migrate as a single zone and generate a single peak, because the equilibrium of the complex was not maintained in NECEEM. P·DNA continuously decayed during the separation, resulting in the nonequilibrium production of P and DNA. The rate of DNA production reduced exponentially following the monomolecular decay of the complex during NECEEM. The exponential part of the electropherograms reflects the decay of P·DNA and release of DNA. Peak 2 corresponds to P·DNA that remained intact at the time of its elution from the capillary.

The proposed interpretation was reinforced by measuring the fluorescence anisotropy of the fluorescently labeled DNA during NECEEM.[3e,f] A relatively small DNA molecule rotates fast and has low fluorescence anisotropy, while a relatively large P·DNA complex rotates slowly and has high fluorescence anisotropy. Both peak 1 and the exponential part had low anisotropy, corresponding to DNA, while peak 2 had high anisotropy, corresponding to P·DNA. In a separate experiment, we confirmed that the quantum yield of DNA-bound fluorescein did not change upon DNA binding to P.

The assignment of peak 1 to the equilibrium fraction of DNA and both peak 2 and exponential part 3 to the equilibrium fraction of P·DNA allows us to calculate the equilibrium binding constant:

$$K_b = \frac{1 + (A_2 + A_3)/A_1}{[P]_0\{1 + A_1/(A_2 + A_3)\} - [DNA]_0}$$

$[P]_0$ and $[DNA]_0$ are the total concentrations of P and DNA in the equilibrium mixture. A_1, A_2, and A_3 are areas of peak 1, peak 2, and exponential part 3, respectively. The area A_1 and the sum area $A_2 + A_3$ were determined as illustrated in Figure 2. Both A_1 and A_3 were generated by unbound DNA; therefore, the diffusion-caused front of A_1 and tail of A_3 had identical shapes but opposite directions. The front and the tail canceled each other, allowing us to use the vertical boundary line between A_1 and A_3. Using the above formula, we calculated the value of K_b to be $(3.6 \pm 0.7) \times 10^6$ M^{-1} on the basis of six experiments with different $[P]_0$ to $[DNA]_0$ ratios. This value is in agreement with those obtained for the SSB−DNA complex by other methods: 4.4×10^6 and 5.0×10^6 M^{-1} for binding of the SSB protein with an 11-mer oligonucleotide measured by affinity capillary electrophoresis and fluorescence anisotropy, respectively,[3e] and 1.4×10^6 M^{-1} for binding of the SSB protein with a 16-mer oligonucleotide measured by the fluorescence quenching method.[4]

The monomolecular rate constant of complex decay, k_{-1}, can be determined from the exponential part by fitting the experimental data in the time window $t_{P\cdot DNA}$ to t_{DNA} (see the inset to Figure 2) with a single-exponential function:

$$I_t = I_{t_{DNA}} \exp\left\{k_{-1}\frac{t_{P\cdot DNA}}{t_{DNA} - t_{P\cdot DNA}}(t - t_{DNA})\right\}$$

where I_t and $I_{t_{DNA}}$ are the fluorescence intensities at time t and t_{DNA}, respectively, and t_{DNA} and $t_{P\cdot DNA}$ are migration times of DNA and P·DNA, respectively. The $t_{P\cdot DNA}/(t_{DNA} - t_{P\cdot DNA})$ coefficient reflects the apparent change of the time window in which the complex decay is monitored: $t_{P\cdot DNA}$ to t_{DNA} instead of 0 to $t_{P\cdot DNA}$. The value of k_{-1} for our protein−DNA complex was determined to be $(3.3 \pm 1.6) \times 10^{-2}$ s^{-1} in six experiments with a varied $[P]_0$ to $[DNA]_0$ ratio. In a number of previous works, this constant was estimated to be less than 1 s^{-1}.[5] NECEEM made it possible for the first time to measure this constant directly. The found values of K_b and k_{-1} allowed us to calculate the bimolecular rate constant of complex formation, $k_1 = (7.2 \pm 3.6) \times 10^4$ M^{-1} s^{-1}.

In conclusion, we present here the first application of NECEEM, a novel method for studies on noncovalent macromolecular interactions. While classical affinity analyses often fail to work with low-affinity complexes, NECEEM takes advantage of a rapidly decaying complex to calculate all equilibrium and kinetic parameters of the reaction in a single experiment. Knowledge of these parameters is essential for understanding the dynamics of regulatory biological processes.[6] Another unique feature of NECEEM is its extremely high sensitivity. We were able to determine the parameters of the protein−DNA interaction with the amount of protein as low as 10^{-18} mol. The next exciting applications of NECEEM will include extremely sensitive analyses of proteins using aptamers, synthetic nucleotide affinity probes.

Acknowledgment. The authors thank Dr. Yingfu Li (Mc Master University) for providing the fluorescently labeled oligonucleotide and Dr. X. Chris Le (University of Alberta) for valuable advice on performing the fluorescence anisotropy experiments. This work was supported in part by grants from the Natural Sciences and Engineering Council of Canada (238990) and the Research Corp. (RIO575).

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JA028212E