

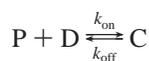
Sweeping Capillary Electrophoresis: A Non-Stopped-Flow Method for Measuring Bimolecular Rate Constant of Complex Formation between Protein and DNA

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Noncovalent protein–DNA complexes play an important role in gene expression, DNA replication, DNA integrity control, and DNA damage repair.¹ To understand the dynamics of these biological processes it is essential to know the kinetic parameters of the formation and dissociation of relevant complexes (C) between the protein (P) and DNA (D):



The knowledge of these parameters is also required for the development and optimization of analytical and molecular biology methods based on protein–DNA interactions. The monomolecular rate constant of complex dissociation, k_{off} , can be determined by either surface plasmon resonance (SPR)² or by nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).³ As for the bimolecular rate constant of complex formation, k_{on} , until now, the only technique available for its direct measurements was stopped-flow spectroscopy.⁴ Stopped-flow spectroscopy relies on the change of spectral properties of either protein or DNA during complex formation. Such changes are often insignificant, which limits the applicability of stopped-flow methods to studies of protein–DNA interactions. Here we introduce sweeping capillary electrophoresis (SweepCE), a new method for directly measuring k_{on} , and demonstrate its use for studying protein–DNA interactions. In contrast to stopped-flow spectroscopy, SweepCE does not rely on spectral changes of the protein or DNA upon complex formation. It requires only that electrophoretic mobilities of the protein and DNA be different, which is always achievable. Moreover, SweepCE is complementary to NECEEM; the two methods constitute a powerful capillary electrophoresis platform for comprehensive studies of protein–DNA interactions.

The concept of SweepCE is based on the sweeping of slowly migrating DNA by fast migrating protein during electrophoresis. The capillary is prefilled with a solution of DNA, and electrophoresis is then carried out from a solution of the protein in a continuous mode. Because the electrophoretic mobility of the protein is greater than that of DNA, the protein continuously mixes with DNA and forms the protein–DNA complex. The complex migrates with a velocity higher than that of DNA and causes sweeping of DNA. The value of k_{on} for complex formation can be determined from the time profile of DNA concentration using a simple mathematical model of the sweeping process. Mathematical analysis is an essential part of SweepCE.

First, we demonstrated the phenomenon of DNA sweeping by a DNA-binding protein in capillary electrophoresis (CE). The capillary (bare silica) was prefilled with 10 nM fluorescently labeled DNA (5'-fluorescein-GCGGAGCGTGGCAGG) in 25 mM sodium tetraborate buffer at pH 9.0. The injection end of the capillary was then immersed into a solution of 100 nM SSB protein in the same buffer and a positive voltage of 20 kV was applied to the protein solution. SSB is a single-stranded DNA-binding protein,⁵ which

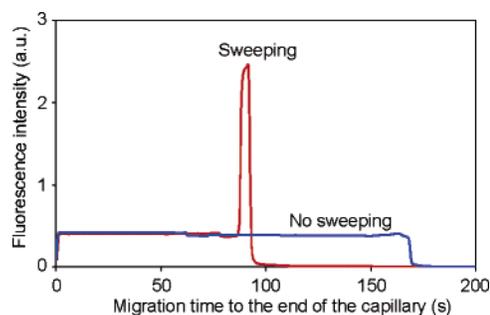


Figure 1. Sweeping of DNA by a DNA-binding protein in capillary electrophoresis. Experimental conditions are described in the text. The “no-sweeping” control experiment was performed in the absence of the DNA binding.

migrates faster than DNA and does not change the electroosmotic flow significantly.⁶ Fluorescence detection was used in these experiments so that only DNA and its complex with the protein were detectable. In the absence of the protein, DNA gradually eluted from the outlet end of the capillary while the capillary was gradually filled with a bare run buffer from its inlet end. This DNA replacement process generated an electropherogram with a characteristic plateau, which ended at 170 s, when the tail of DNA exited the capillary (Figure 1, blue line). In the presence of the protein, the tail of DNA was swept by the faster moving protein, which resulted in a “sweeping region” with an increased overall concentration of DNA (Figure 1, red line). It is the shape of the “sweeping region” that can be used for the determination of the bimolecular rate constant, k_{on} , of protein–DNA interaction.

Second, we developed a mathematical model of the sweeping process that facilitates finding k_{on} from the shape of the “sweeping region”. Sweeping of DNA by the protein is governed by the rate of complex formation between them and by the velocities of the protein, DNA, and the complex in electrophoresis: v_P , v_D , v_C , respectively. The overall mass transfer process is described by the following system of nonlinear partial differential equations:

$$\begin{aligned} \frac{\partial P(t,x)}{\partial t} + v_P \frac{\partial P(t,x)}{\partial x} &= -k_{\text{on}} D(t,x) P(t,x) \\ \frac{\partial D(t,x)}{\partial t} + v_D \frac{\partial D(t,x)}{\partial x} &= -k_{\text{on}} D(t,x) P(t,x) \\ \frac{\partial C(t,x)}{\partial t} + v_C \frac{\partial C(t,x)}{\partial x} &= k_{\text{on}} D(t,x) P(t,x) \end{aligned}$$

Here P , D , and C are the concentrations of the protein, DNA, and complex, respectively; t is time passed from the beginning of separation; and x is the distance from the inlet of the capillary to the point of observation. Typically, systems of nonlinear partial differential equations can be solved only numerically. Interestingly,

the general solution for our system can be obtained analytically: the detailed step-by-step solution can be found in Supporting Information. The resulting expression for P , D , and C as functions of t and x under our initial conditions are the following:

$$P(t,x) = P_0 \frac{\exp\left(k_{\text{on}}P_0\frac{x-v_{\text{p}}t}{v_{\text{p}}-v_{\text{D}}}\right)\theta(-x+v_{\text{p}}t)}{\left\{\exp\left(-k_{\text{on}}P_0\frac{x-v_{\text{p}}t}{v_{\text{p}}-v_{\text{D}}}\right)-1\right\}\theta(-x+v_{\text{p}}t) + \left\{\exp\left(k_{\text{on}}D_0\frac{x-v_{\text{D}}t}{v_{\text{p}}-v_{\text{D}}}\right)-1\right\}\theta(x-v_{\text{D}}t) + 1}$$

$$D(t,x) = D_0 \frac{\exp\left(k_{\text{on}}D_0\frac{x-v_{\text{D}}t}{v_{\text{p}}-v_{\text{D}}}\right)\theta(x-v_{\text{D}}t)}{\left\{\exp\left(-k_{\text{on}}P_0\frac{x-v_{\text{p}}t}{v_{\text{p}}-v_{\text{D}}}\right)-1\right\}\theta(-x+v_{\text{p}}t) + \left\{\exp\left(k_{\text{on}}D_0\frac{x-v_{\text{D}}t}{v_{\text{p}}-v_{\text{D}}}\right)-1\right\}\theta(x-v_{\text{D}}t) + 1}$$

$$C(t,x) = k_{\text{on}}\int_0^t D(t-\tau,x-\tau v_{\text{C}})P(t-\tau,x-\tau v_{\text{C}})d\tau$$

Here, P_0 and D_0 are the concentrations of the protein and DNA, respectively, before the start of the sweeping process; θ is the parameter which equals 0 if $x < 0$ and equals 1 if $x \geq 0$. The three expressions can be used to build simulated electropherograms. All parameters in the three expressions, except for k_{on} , are either defined (θ) or controlled (P_0 , D_0) or can be found in independent CE experiments (v_{p} , v_{D} , and v_{C}). Therefore, fitting experimental electropherograms with the simulated ones requires the optimization of a single parameter, k_{on} , only. Standard procedures for nonlinear regression of experimental data can be used for fast and accurate determination of k_{on} .

Third, we examined the proposed SweepCE approach of finding k_{on} for the interaction of SSB and the fluorescently labeled 15-mer DNA oligonucleotide described above. Due to the fluorescence label, both DNA and the protein–DNA complex were detectable. SweepCE electropherograms for three different concentrations of DNA are presented in Figure 2 (blue lines). The fluorescent signal corresponds to a sum concentration of DNA and the protein–DNA complex. To fit such a signal with our model, the fitting function should be $D(t) + C(t)$. The detector was placed in a single point at the end of the capillary; therefore, x was not a variable but a fixed parameter. We measured v_{p} , v_{D} , and v_{C} in separate capillary electrophoresis experiments and used these values along with specified P_0 and D_0 in the nonlinear regression analysis to find k_{on} . Simulated SweepCE electropherograms, which provide the best fitting of experimental data are shown in Figure 2 by red lines. The k_{on} value obtained from SweepCE analyses was $(3.4 \pm 0.6) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This value is in satisfactory agreement with that indirectly determined as $k_{\text{on}} = k_{\text{off}}/K_{\text{d}} = 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ using $k_{\text{off}} = 0.01 \text{ s}^{-1}$ and $K_{\text{d}} = 10 \text{ nM}$ obtained by NECEEM under identical to SweepCE experimental conditions. Fitting the experimental data with k_{on} , which deviated from the correct one, can be found in Supporting Information. It is remarkable that the simple model of SweepCE provides excellent quantitative description of experimental electropherograms.

Finally, we outline major characteristics of SweepCE. The simple mathematical model of SweepCE works if complex dissociation during the time of SweepCE separation is negligible. With the shortest separation times in CE being on the order of a few seconds,

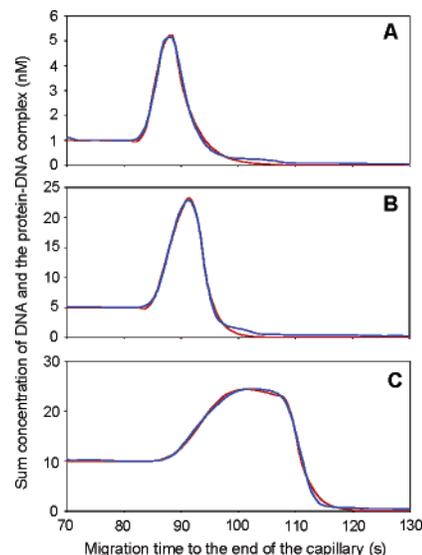


Figure 2. Fitting simulated SweepCE electropherograms with experimental SweepCE electropherograms. Experimental SweepCE electropherograms (blue lines) were obtained for the interaction between single-stranded DNA-binding protein (60 nM) and a 15-mer fluorescently labeled DNA: 1 nM (panel A), 5 nM (panel B), and 10 nM (panel C). Simulated SweepCE electropherograms (red lines) were obtained by nonlinear regression of the experimental data using the least-squares method.

the simple model can be used for finding k_{on} of complexes, whose k_{off} values are as high as 0.1 s^{-1} . For greater k_{off} , the system of partial differential equations should include the rate of complex dissociation and should be solved numerically. The numerical solution is more complex technically but can reveal both k_{on} and k_{off} . In SweepCE, very low concentrations of reacting components can be used, allowing for reliable measurements of k_{on} values as high as diffusion controlled ones ($\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$). Mixing of the reacting components in SweepCE proceeds in a continuous mode, thus excluding “dead” time, inevitable for stopped-flow methods.

To conclude, SweepCE represents the first non-stopped-flow technique for directly measuring the bimolecular rate constant of complex formation. Along with NECEEM, which measures K_{d} and k_{off} , SweepCE establishes a powerful method for comprehensive studies of protein–ligand interactions, which uses a universal instrumental platform.

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Supporting Information Available: Mathematical appendix and illustration of fitting data with incorrect k_{on} (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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