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Thermochemistry of Protein−DNA Interaction Studied with Temperature-Controlled Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures

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We introduce temperature-controlled nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) and demonstrate its use to study thermochemistry of protein−DNA interactions. Being a homogeneous kinetic method, temperature-controlled NECEEM uniquely allows finding temperature dependencies of equilibrium and kinetic parameters of complex formation without the immobilization of the interacting molecules on the surface of a solid substrate. In this work, we applied temperature-controlled NECEEM to study the thermochemistry of two protein−DNA pairs: (i) *Taq* DNA polymerase with its DNA aptamer and (ii) *E. coli* single-stranded DNA binding protein with a 20-base-long single-stranded DNA. We determined temperature dependencies of three parameters: the equilibrium binding constant (K_b), the rate constant of complex dissociation (k_{off}), and the rate constant of complex formation (k_{on}). The $K_b(T)$ functions for both protein−DNA pairs had phase-transition-like points suggesting temperature-dependent conformational changes in structures of the interacting macromolecules. Temperature dependencies of k_{on} and k_{off} provided insights into how the conformational changes affected two opposite processes: binding and dissociation. Finally, thermodynamic parameters, ΔH and ΔS, for complex formation were found for different conformations. With its unique features and potential applicability to other macromolecular interactions, temperature-controlled NECEEM establishes a valuable addition to the arsenal of analytical methods used to study dynamic molecular complexes.

Two kinetic capillary electrophoresis methods have been recently introduced for quantitative studies of protein−DNA interactions: nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[1] and sweeping capillary electrophoresis (SweepCE).[2] In NECEEM, the protein and DNA are mixed and allowed to reach the dynamic equilibrium:

$$\text{protein} + \text{DNA} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{protein−DNA}$$

The equilibrium mixture contains three components: free protein, free DNA, and a protein−DNA complex. A short plug of the equilibrium mixture is injected into the capillary, and the three components are separated under nonequilibrium conditions, in a run buffer that does not contain the protein or DNA. The equilibrium fractions of free protein and free DNA migrate as unchanging electrophoretic zones, while the complex does not. As the result of electrophoretic separation of the three components, the complex is no longer at equilibrium with free protein and free DNA. When out of equilibrium, the complex dissociates and its concentration decreases exponentially. The protein and the ligand dissociated from the complex broad zones with exponential concentration profiles. As a general concept, NECEEM can be used in a variety of applications. The proved applications of NECEEM include the following: (i) determination of the rate constant, k_{off}, of complex dissociation and the equilibrium constant, K_b, of complex formation,[3,4] (ii) quantitative analyses of proteins using aptamers as affinity probes,[5] and (iii) determination of temperature inside the capillary during capillary electrophoresis (CE).[6] NECEEM requires that the complex be well separated from at least one of free components, free protein or free DNA. Finding optimal buffer conditions for acceptable separation quality may be difficult for some protein−DNA pairs. Affinity-mediated NECEEM was suggested as a generic way of achieving good separation of the protein−DNA complex from free DNA.[7] In affinity-mediated NECEEM, the run buffer is supplemented with a background affinity agent, which can bind free DNA but cannot bind the protein−DNA complex. Binding to the background affinity agent changes the mobility of free DNA, which, in turn, mediates NECEEM. Sequence-nonspecific DNA-binding proteins were suggested as suitable background affinity agents for changing the mobility of free DNA.[7] We believe that affinity-mediated NECEEM can be also realized with a protein-binding affinity agent, such as an antibody, provided that it binds free protein but does not bind the protein−DNA complex.

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(1) Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2002**, *124*, 13674−13675.
(2) Okhonin, V.; Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2004**, *126*, 7166−7167.
(3) Krylov, S. N., Berezovski, M. *Analyst* **2003**, *128*, 571−575.
(4) Okhonin, V.; Krylova, S. M.; Krylov, S. N. *Anal. Chem.* **2004**, *76*, 1507−1512.
(5) Berezovski, M.; Nutiu, R.; Li, Y.; Krylov, S. N. *Anal. Chem.* **2003**, *75*, 1382−1386.
(6) Berezovski, M.; Krylov, S. N. *Anal. Chem.* **2004**, *76*, 7114−7117.
(7) Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2003**, *125*, 13451−13454.

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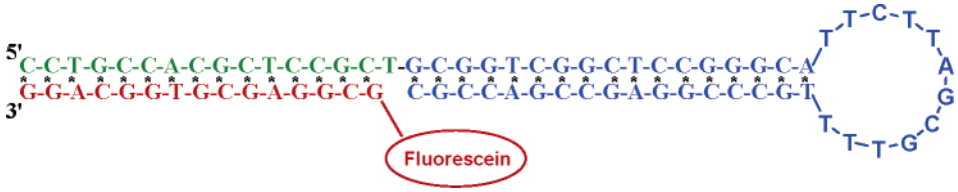


Figure 1. DNA aptamer for *Taq* polymerase. The aptamer is shown in blue, the extension in green, and the fluorescently labeled hybridization probe in red.

SweepCE was introduced as the only non-stopped-flow method for directly measuring the bimolecular rate constant, k_{on}, of protein−DNA complex formation.[2] Conceptually, in SweepCE, the capillary is prefilled with DNA and the inlet of the capillary is inserted into the solution of the protein, the interaction studied. When electrophoresis starts, the fast-moving protein is mixed with a slowly moving DNA in a continuous mode and "sweeps" DNA upon protein−DNA complex formation. The shape of the concentration profile of swept DNA is very sensitive to k_{on}, and thus, its analysis provides accurate information on the value of k_{on}.

In this work, we introduce temperature-controlled NECEEM and demonstrate its application to study the thermochemistry of protein−DNA interactions. Knowing how temperature influences kinetic and equilibrium binding parameters of noncovalent protein−DNA interactions is important for understanding fundamental biological processes, such as gene expression and DNA replication.[8−12] It is also essential for developing analytical applications of DNA aptamers and DNA-binding proteins in affinity and hybridization analyses and in optimizing the polymerase chain reaction (PCR).[7,13−15] Conventional methods for thermochemical studies of protein−DNA interactions have limitations. Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) are not applicable to finding kinetic parameters.[16−18] Surface plasmon resonance (SPR) can serve to determine equilibrium and kinetic parameters, but it is a heterogeneous method, which requires the immobilization of either DNA or protein on the surface of a sensor.[19,20] Being a homogeneous kinetic method, temperature-controlled NECEEM uniquely allows finding temperature dependencies of equilibrium and kinetic parameters of complex formations without the immobilization of reagents on the surface.

Moreover, it requires much lower quantities of the protein than DSC, ITC, and SPR. In this work, we explain the concept of temperature-controlled NECEEM and apply the method to study the thermochemistry of protein−DNA interactions.

(8) Ma, H.; Zou, Y. *Biochemistry* **2004**, *43*, 4206−4211.
(9) Schubert, F.; Zettl, H.; Hafner, W.; Krauss, G.; Krausch, G. *Biochemistry* **2003**, *42*, 10288−10294.
(10) Galletto, R.; Bujalowski, W. *Biochemistry* **2002**, *41*, 8921−8934.
(11) Mosig, G.; Gewin, J.; Luder, A.; Colowick, N.; Vo, D. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8306−8311.
(12) Sutani, T.; Yanagida, M. *Nature* **1997**, *388*, 798−801.
(13) Savran, C. A.; Knudsen, S. M.; Ellington, A. D.; Manalis, S. R. *Anal. Chem.* **2004**, *76*, 3194−3198.
(14) Dang, C.; Jayasena, S. D. *J. Mol. Biol.* **1996**, *264*, 268−278.
(15) Yakimovich, O. Y.; Alekseev, Y. I.; Maksimenko, A. V.; Voronina, O. L.; Lunin, V. G. *Biochemistry (Moscow)* **2003**, *68*, 228−235.
(16) Carra, J. H.; Privalov, P. L. *Biochemistry* **1997**, *36*, 526−535.
(17) Knapp, S.; Karshikoff, A.; Berndt, K. D.; Christova, P.; Atanasov, B.; Ladenstein, R. *J. Mol. Biol.* **1996**, *264*, 1132−1144.
(18) Davis, K. G.; Plyte, S. E.; Robertson, S. R.; Cooper, A.; Kneale, G. G. *Biochemistry* **1995**, *34*, 148−154.
(19) Tsoi, P. Y.; Yang, M. *Biosens. Bioelectron.* **2004**, *19*, 1209−1218.
(20) Shumaker-Parry, J. S.; Aebersold, R.; Campbell, C. T. *Anal. Chem.* **2004**, *76*, 2071−2082.

EXPERIMENTAL SECTION

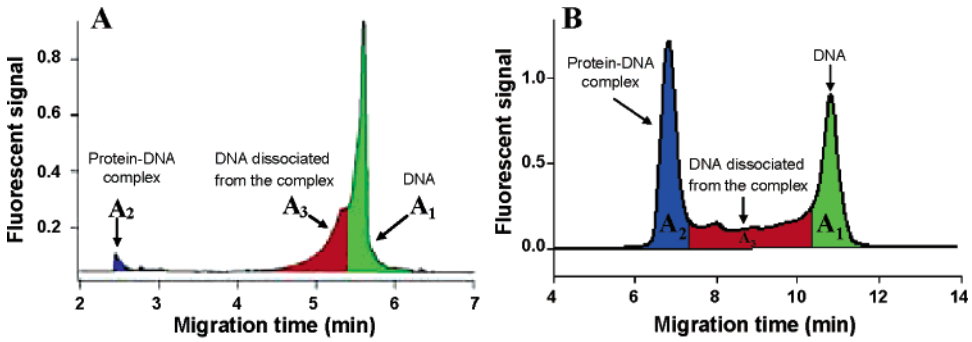
Chemicals and Materials. Single-stranded DNA-binding protein (SSB) from *Escherichia coli* and buffer components were obtained from Sigma-Aldrich (Oakville, ON, Canada). An extended 62-mer DNA aptamer for *Taq* DNA polymerase and a fluorescently labeled 20-mer ssDNA, 5′-FAM-AACGAGAAGCGCGATCACAT-3′, were custom-synthesized by IDT (Coralville, IA). The 15-base extension in the aptamer sequence was made to fluorescently label the aptamer by making its hybrid with a fluorescently labeled 15-mer DNA oligonucleotide, 5′-FAM-GCGGAGCGTGGCAGG, which was kindly donated by Dr. Yingfu Li (McMaster University, Hamilton, ON, Canada). Fluorescein was attached to oligonucleotides through the six-carbon spacer arm to eliminate interference with aptamer binding to the protein. The structure of the aptamer is shown in Figure 1. Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). All solutions were made using Milli-Q quality deionized water and filtered through a 0.22-μm filter (Millipore, Nepean, ON).

NECEEM. Temperature-controlled NECEEM was performed with a CE instrument (P/ACE MDQ, Beckman-Coulter) with thermostabilization of the capillary (the outer walls of the capillary were washed with a liquid heat exchanger maintained at a fixed temperature) and sample vials. The instrument employed laser-induced fluorescence detection with a 488-nm line of an argon ion laser for fluorescence excitation.

An uncoated fused-silica capillary was used with the following dimensions: 50 cm × 75 μm i.d. × 350 μm o.d. The length from the injection end to the detection window was 40 cm. Electrophoresis was run with a positive electrode at the injection end and an electric field of 400 V/cm. The run buffer for all NECEEM experiments was 25.0 mM sodium tetraborate at pH 9.3. The quality of thermostabilization was shown to be acceptable under these separation conditions.[6] The samples were injected into the capillary by a pressure pulse of 5 s × 0.5 psi (3.5 kPa); the length of corresponding sample plug was 6 mm. The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

Temperature-controlled NECEEM experiments were performed in three repeats for every temperature point.

Equilibrium Mixtures. Equilibrium mixtures were prepared by mixing the protein and DNA in the incubation buffer. Prior to the analysis, they were preincubated for 1 h at room temperature (20 ± 2 °C) followed by a 10-min incubation at the required temperature inside the sample compartment of the CE instrument. The incubation buffer was 25 mM tetraborate at pH 9.3. For the



$$K_b = \frac{1 + A_1/(A_2 + A_3)}{[\text{Protein}]_0\{1 + A_1/(A_2 + A_3)\} - [\text{DNA}]_0} \qquad k_{off} = \frac{\ln\left((A_2 + A_3)/A_2\right)}{t_{\text{Protein•DNA}}} \qquad k_{on} = k_{off} \bullet K_b$$

Figure 2. NECEEM electropherograms allow measurement of equilibrium and kinetic binding parameters in a single run for two protein−DNA complexes. (A) *E. coli* ssDNA-binding protein (250 nM) with a 20-base-long ssDNA (200 nM) and (B) *Taq* DNA polymerase (70 nM) with its DNA aptamer (75 nM). The incubation buffer and the running buffer were 25 mM tetraborate at pH 9.3.

Taq DNA polymerase−aptamer mixture, the concentrations of the protein and the aptamer were 70 and 75 nM, respectively. For the SSB−ssDNA mixture, the concentrations of the protein and DNA were 250 and 200 nM, respectively.

RESULTS AND DISCUSSION

Initially, we explain how temperature should be controlled in NECEEM for measuring temperature dependencies of protein−DNA binding parameters. The nature of the NECEEM method leads to K_b being measured under the conditions of the equilibrium mixture and k_{off} being measured under the conditions of the separation media. Thus, to determine $K_b(T)$, the temperature of the equilibrium mixture should be controlled, while to determine $k_{off}(T)$, the temperature of the separation media should be controlled.

If the conditions (the buffer and the temperature) of the equilibrium mixture and separation media are identical, $k_{on}(T)$ can be calculated as $K_b(T)k_{off}(T)$. Therefore, it is beneficial to have the two temperatures identical.

To realize the described concept of temperature-controlled NECEEM in this work, we used a CE instrument with separate temperature controls for the sample vial (which contains the equilibrium mixture) and the separation capillary (which contains the separation media); the two temperatures were set to be identical so that the k_{on} value could be calculated. Moreover, the incubation buffer for the equilibrium mixture and the electrophoresis run buffer were also identical: 25 mM sodium tetraborate at pH 9.3. DNA was labeled with fluorescein to facilitate fluorescence detection of free DNA and protein−DNA complexes. NECEEM was driven by an electric field at which thermostabilization was efficient.[6] The values of K_b and k_{off} were determined from NECEEM electropherograms as depicted in Figure 2.[1,3−5]

We first applied temperature-controlled NECEEM to study the *Taq* DNA polymerase/aptamer system. This model was chosen for the proof-of-principle work because some aspects of its temperature-dependent behavior are known.[14,15] The aptamer for *Taq* DNA polymerase was selected for a specific temperature behavior to promote hot-start PCR. Accordingly, the aptamer inhibits the *Taq* DNA polymerase activity at low temperatures but its inhibition efficiency decreases rapidly at temperatures exceeding 35 °C.[14] Temperature dependencies of K_b, k_{off}, and k_{on} for this

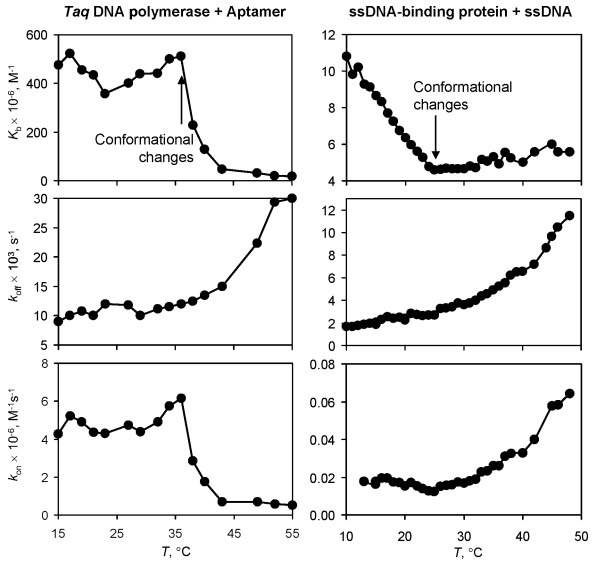


Figure 3. NECEEM-measured temperature dependencies of equilibrium and kinetic binding parameters for two protein−DNA complexes: (i) *Taq* DNA polymerase (70 nM) with its DNA aptamer (75 nM) and (ii) *E. coli* ssDNA-binding protein (250 nM) with a 20-base-long ssDNA (200 nM). Every point represents the mean of data obtained in three experiments.

protein−DNA pair are shown in the left three panels of Figure 3. The $K_b(T)$ data indicate that the equilibrium stability of the complex does not change significantly between 15 and 36 °C but decreases rapidly with temperatures exceeding the threshold level of 36 °C. This sharp suppression of complex stability at $T > 36$ °C is in perfect agreement with the data on temperature-dependent inhibition of *Taq* DNA polymerase.[14] The phase-transition-like behavior of $K_b(T)$ suggests temperature-dependent conformational changes in the structure of either the protein or the aptamer or both of them. *Taq* DNA polymerase is extremely temperature-stable, while the aptamer's hairpin structure is, in contrast, known to be temperature-sensitive.[15] Hence, the abrupt temperature-dependent transition in the stability of the protein−aptamer complex is most likely due to conformational changes in the aptamer secondary structure. Temperature dependencies of k_{on} and k_{off} provide an insight into how the conformational changes affect two opposite processes: binding and dissociation. The rapid

increase of k_{off} and abrupt decrease of k_{on} at $T > 36$ °C indicate that both complex dissociation and association are affected by conformational changes occurring at high temperatures. The magnitude of change in k_{on} is, however, greater than that in k_{off}, implying that the conformational transition affects binding to a greater extent than dissociation.

After the method was evaluated with the *Taq* DNA polymerase/aptamer system, we applied it to study the interaction between SSB and ssDNA, for which temperature data for binding parameters was not available. Temperature dependencies of K_b, k_{off}, and k_{on} for this system are shown in the right three panels of Figure 3. The $K_b(T)$ data indicate that the equilibrium stability decreases rapidly with temperature in the range of 10−25 °C but increases gradually with temperature rising from 25 to 50 °C. The phase-transition point in $K_b(T)$ suggests that this system also experiences temperature-dependent conformational changes in the structure of either the protein or DNA or both of them. ssDNA used in this work (see the Experimental Section for the sequence) does not form stable hybrids to a considerable extent, thus excluding DNA from a list of potential culprits. As for the protein, it is known to be thermostable and to form homodimers and homotetramers in a temperature-dependent fashion.[21,22] Therefore, we associate the observed phase-transition-like behavior in $K_b(T)$ for the SSB/ssDNA system with temperature-dependent multimerization of the protein. Similar to the *Taq* DNA polymerase/aptamer system, temperature dependencies of k_{on} and k_{off} for the SSB/ssDNA system can be used to understand how conformational changes affect binding and dissociation of the complex. The value of k_{off} grows with T exponentially within the whole studied range of temperatures, indicating that the conformational changes do not affect complex dissociation. The value of k_{on} behaves differently: it does not change in the range of 10−25 °C but grows for temperatures exceeding the threshold of 25 °C. From this analysis, we can conclude that the temperature-dependent conformational changes in SSB mainly influence the ability of the protein to form a complex with ssDNA rather than the ability of the complex to dissociate.

Finally, we used Van't Hoff plots of the temperature data for K_b to find ΔH and ΔS for complex formation (Figure 4). Experimental errors for values of ΔH and ΔS presented in the graphs were in the range of 10−20%. Temperature regions corresponding to different conformations had significantly different ΔH and ΔS. For the *Taq* DNA polymerase/aptamer system, the process is entropy-driven at low temperatures and enthalpy-driven at high temperatures. For the SSB/ssDNA system, the driving forces are opposite.

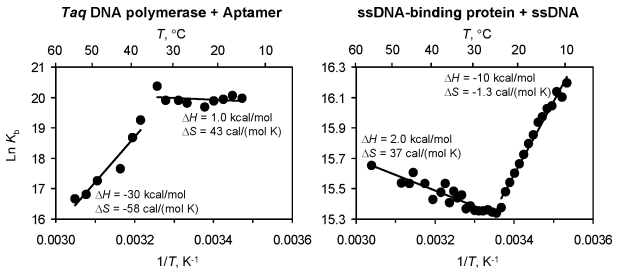


Figure 4. Van't Hoff plots used for the determination of ΔH and ΔS for complex formation in two protein−DNA pairs. (i) *Taq* DNA polymerase with its DNA aptamer and (ii) *E. coli* ssDNA-binding protein with a 20-base-long ssDNA.

To conclude, we present here the first application of temperature-controlled NECEEM, a novel method for thermochemical studies of noncovalent protein−DNA interactions. The only requirement for the method is that free DNA be well separated from the protein−DNA complex. Due to the high negative charge of DNA, such separation can be readily achieved for the majority of the proteins. Uniquely, the measurements of k_{off} and K_d are not affected by temperature-induced changes in electrophoretic parameters (e.g., mobilities and peak shapes), thus, allowing us to avoid any corrections for such changes. Temperature-controlled NECEEM is a homogeneous kinetic method that allows finding temperature dependencies of equilibrium and kinetic parameters of protein−DNA complex formation without immobilizing DNA or the protein on the surface of a solid substrate. Moreover, the new method can be potentially applicable to other types of macromolecular interaction, such as protein−protein, protein−small molecule, and DNA−small molecule.

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(21) Perales, C.; Cava, F.; Meijer, W. J. J.; Berenguer J. *Nucleic Acids Res.* **2003**, *31*, 6473−6480.

(22) Bujalowski, W.; Lohman, T. M. *J. Biol. Chem.* **1991**, *266*, 1616−1626.