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Activation energy of single-stranded DNA moving through cross-linked polyacrylamide gels at 300 V/cm Effect of temperature on sequencing rate in high-electric-field capillary gel electrophoresis

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Abstract

In DNA sequencing, single-stranded DNA fragments are separated by gel electrophoresis. This separation is based on a sieving mechanism where DNA fragments are retarded as they pass through pores in the gel. In this paper, we present the mobility of DNA sequencing fragments as a function of temperature; mobility is determined in 4% T LongRanger gels at an electric field of 300 V/cm. The temperature dependence is compared with the predictions of the biased reptation model. The model predicts that the fragment length for the onset of biased reptation with stretching increases with the square of temperature; the data show that the onset of biased reptation with stretching decreases with temperature. Biased reptation fails to model accurately the temperature dependence of mobility. We analyzed the data and extracted the activation energy for passage of sequencing fragments through the gel. For fragments containing less than ca. 200 bases, the activation energy increases linearly with the number of bases at a rate of 25 J/mol per base; for longer fragments, the activation energy increases at a rate of 6.5 J/mol per base. This transition in the activation energy presumably reflects a change in conformation of the DNA fragments; small fragments exist in a random coil configuration and larger fragments migrate in an elongated configuration.

1. Introduction

The separation of single-stranded DNA (ssDNA) fragments is a fundamental step in DNA sequencing [1]. This separation is performed by means of gel electrophoresis, wherein DNA fragments migrate through a gel under the influence of an external electric field. The fragments are separated based on size by the gel; small fragments are weakly retarded and elute quickly whereas larger fragments are strongly retarded and elute slowly. Under conventional conditions, about 450 bases of sequence are determined per sequencing run [2]; under special conditions, up to 1000 bases of sequence can be determined per run [3].

Conventional electrophoresis is performed in relatively thick slabs; poor heat conduction from the gel limits the maximum electric field that can be applied across the gel. Typically, electric fields of ca. 60 V/cm are used for the separation. Conventional sequencing techniques require 6–8 h to separate 450 bases [2].

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Rather than performing gel electrophoresis in slabs, the separation may be performed in thin capillaries [4–11]. The high surface-to-volume ratio of the capillary improves heat transport and facilitates the use of high electric fields for fast and efficient separations. For example, we have described DNA sequencing in capillaries at electric fields of 800 V/cm; 7 min are required to separate fragments up to 250 bases in length [12]. Longer fragments tend to co-elute because of the phenomenon of biased reptation with stretching [13–18]. At high electric fields, longer fragments are aligned and stretched by the electric field. The rod-shaped fragments experience retardation that is independent of the fragment length, which leads to co-elution of the longer fragments. As a result of biased reptation, the use of extremely high electric fields is incompatible with the separation of long sequencing fragments.

The elongated configuration predicted by biased reptation should be disturbed by thermal energy. The classic model of biased reptation predicts that the transition from normal to elongated migration, corresponding to a transition from constant peak spacing to highly compressed peaks, is proportional to the square of the absolute temperature [13,14]. A gel operated at 50°C should generate 20% more sequencing data than a gel operated at 23°C. This classic model assumes that thermal energy acts to disrupt the highly ordered configuration of the elongated DNA fragment; elongation is predicted to be depressed at higher temperatures.

There have been several reports on the use of capillary gel electrophoresis for separation of double-stranded DNA at elevated temperature. Boček and Chrambach [19] investigated capillary electrophoresis of DNA fragments in agarose solution at 40°C. Guttman and Cooke [20] studied the effect of temperature on the separation of DNA restriction fragments. Ruiz-Martinez et al. [21] performed DNA sequencing at 32°C. However, none of those studies considered the effect of temperature on biased reptation.

In this paper, we present the first systematic study of the mobility of DNA sequencing fragments at high electric field as a function of temperature. While there are many studies of the temperature dependence of DNA mobility at low electric fields, this is the first study where in the effects of biased reptation are observed as a function of temperature. The results are in strong disagreement with the predictions of biased reptation theory. Instead, we use the results to extract the activation energy for movement of fragments through the gel. Increased thermal energy enhances the formation of elongated DNA fragments.

2. Experimental

The optical system and the preparation of ssDNA are similar to that reported before [7,9,11,12,22–24]. Two modifications were made to the system. First, a helium–neon laser (λ = 543.5 nm; Melles Griot, Irvine, CA, USA) was used for excitation and an interference filter (Model 630DF30; Omega Optical, Brattleboro, VT, USA) was used to isolate fluorescence. Second, a Melabs (CA, USA) Model CTC-1A proportional temperature controller was added to the system, providing a constant temperature air bath around the capillary. The temperature fluctuation in equilibrium at the studied temperature range (25 to 50°C) typically is ± 0.3°C and in the worst case is ± 0.5°C.

The capillary is 35 cm × 20 μm I.D. × 150 μm O.D. The capillaries are filled with 4% T LongRanger gels[1] (AT Biochemicals), prepared as in Ref. 12. Injections are made electrokinetically at 200 V/cm for 35 s. Separation is performed at an electric field of 300 V/cm.

ssDNA sequencing samples are prepared from a standard M13mp18 template [11]. For much of the work, ddTTP was used as the termination nucleotide with a ROX (sulforhodamine 101)-labeled −21 primer. For a few experiments,

[1] T = (g acrylamide + g N,N'-methylenebisacrylamide)/100 ml solution.

ddATP was used as the termination nucleotide with the same primer.

3. Results and discussion

For the convenience of comparison, Fig. 1 superimposes the region from near the primer to fragments 170 bases in length at 25, 33, 40 and 50°C, respectively. The sequencing rate increases dramatically with temperature. A 17°C temperature rise results in a factor of two increase in separation speed. There is no obvious difference in peak shape or resolution for this portion of the electropherogram.

It is well known that there is a peak compression at bases 64–68 for sequencing fragments prepared from a M13mp18 template; this compression contains only one T, and is difficult to observe in a sample terminated with ddTTP. To see if the compression can be resolved by operating the capillary at elevated temperatures, the separation was performed by using an ddATP terminated ssDNA sample. Fig. 2 presents truncated electropherograms of the sample with column temperature uncontrolled at ambient temperature (23°C) and controlled at 35°C. It can be seen that fragments with 64 and 68 bases co-elute and give one larger peak at room temperature. The peaks are clearly resolved at 35°C. However, inspection of the peak spacing for bases 64–68 and 81–85 reveals that the compression is not completely resolved. This particularly tenacious compression is probably best relaxed by use of a formamide modified gel operated at elevated temperature [25].

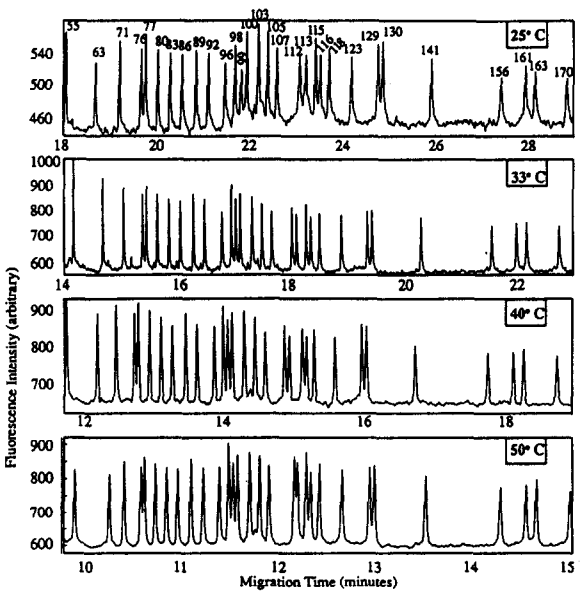

Fig. 1. Separation of an M13mp18 sequencing sample that was terminated with ddTTP: comparison of separation for short fragments. The fragment length of the peaks is shown in the top panel. These separations were run at 300 V/cm.

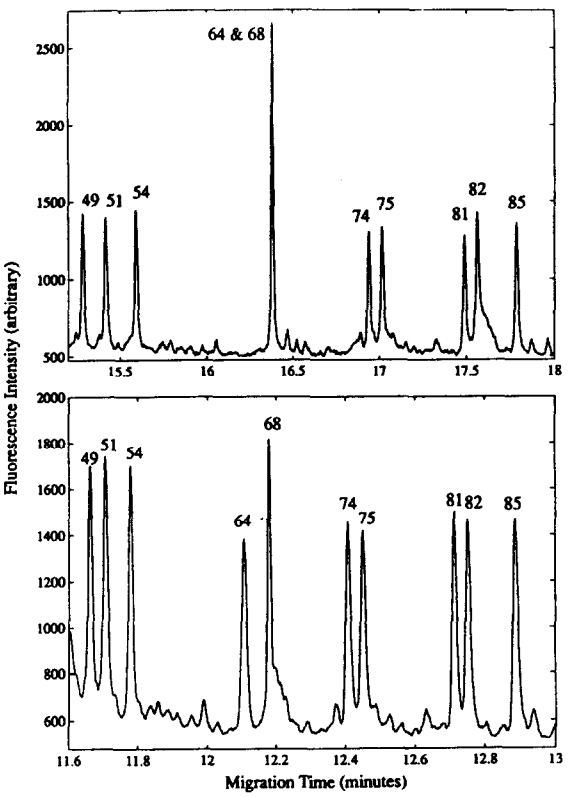

Fig. 2. Resolution of compression at elevated temperature. The M13mp18 sequencing sample was terminated with ddATP. The electropherogram of the top panel was obtained at 23°C while the bottom panel was obtained at 35°C. Fragment length is shown for each peak.

3.1. Effect of temperature on mobility —comparison with biased reptation

Mobility (μ) changes drastically with temperature. The biased reptation model predicts the following temperature dependence of mobility

$$\mu = \chi\left[\frac{1}{N} + \left(\frac{qE\alpha}{3k_bT}\right)^2\right] = \chi\left[\frac{1}{N} + \frac{1}{N^*}\right] \quad (1)$$

where χ is a constant inversely related to the viscosity of the gel matrix, N is the number of bases in the fragment, q is the charge per base, E is the electric field, α is the pore size, k_b is the Boltzmann constant, T is the absolute temperature and N^* is the limiting fragment size for biased reptation with stretching [13,14]; fragments much longer than N^* will co-elute with similar mobility. N^* is a rough estimate of the maximum number of bases determined in a sequencing run.

Mobility is predicted to be proportional to $1/N$. Fig. 3 presents mobility plots obtained at four different temperatures. The plot becomes linear ($r > 0.999$) for fragments longer than ca. 175 bases, as shown in the inset; the plots deviate slightly from linearity for the longest fragments. Clearly, the biased reptation model fails for fragments shorter than this length, as the classic Ogsten behavior dominated the mobility. However, fragments longer than this threshold migrate with a rate that is inversely related to fragment length, as predicted by the classic biased reptation model.

The slope of the mobility versus $1/N$ curve should be related to the viscosity of the gel. As expected, the slope increases with temperature by about 2.2% per °C temperature rise.

N^* is the fragment length for which significant elongation is observed. Fragments significantly larger than N^* will migrate with constant mobility; separation of those fragments is not possible by gel electrophoresis. This parameter is of fundamental importance in DNA sequencing applications. Increasing the number of bases generated in a scquencing run is only possible if N^* can be increased. N^* is estimated by dividing the slope by the intercept of the linear portion of Fig. 3.

Fig. 4 plots N^* versus temperature. Instead of the expected quadratic increase in N^* with temperature, N^* decreases with temperature. This result is at odds with the biased reptation model, which predicts a quadratic increase in N^* with temperature [13,14]. This result is quite important, for it confirms a fundamental weakness in the biased reptation model. From the inception of the model, it has been known that N^* does not agree with the predicted electric field dependence, at least for double-stranded

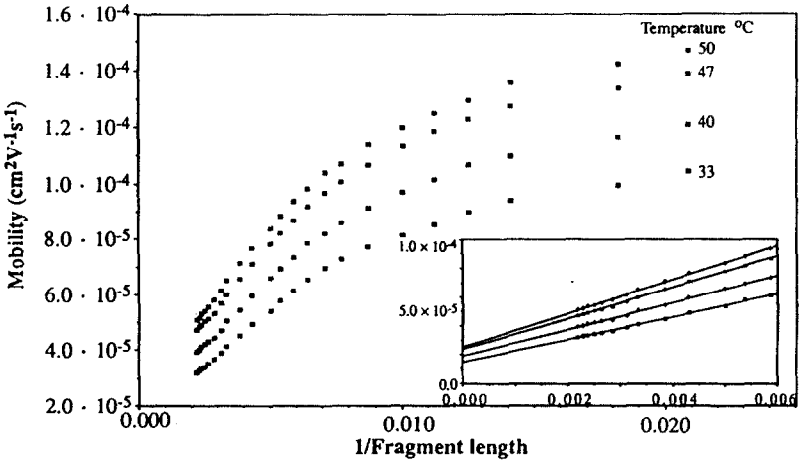

Fig. 3. Mobility versus 1/number of base pairs. The data were obtained at the temperatures indicated. The inset shows a close-up of the data for the longer fragments; the lines are the result of least-squares fits to the data over the range shown.

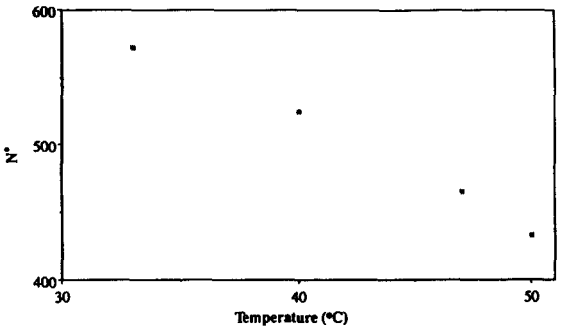

Fig. 4. Plot of N^* versus temperature. N^* is a measure of the onset of biased reptation.

DNA [13,18]. We have now demonstrated that the model also fails to predict the temperature dependence of mobility. Of course, variation with temperature in the pore size of the gel or the persistence length of the ssDNA could lead to disagreement with the predictions of the biased reptation model.

N^* is a fundamental parameter in high-electric-field DNA sequencing. The onset of biased reptation limits the maximum number of bases that can be sequenced; N^* is a good estimate of the maximum number of bases that can be sequenced. Unfortunately, the dramatic decrease in N^* with temperature means that the maximum number of bases to be sequenced decreases at elevated temperature. While elevated temperature increases the sequencing speed, it also appears to limit the maximum number of bases to be sequenced.

3.2. Activation energy for DNA migration in gel electrophoresis

Alternative descriptions of the behavior of DNA fragments at high electric fields in gel electrophoresis must be explored. We present some preliminary interpretations of the mobility data based on the activation energy required for a DNA fragment to pass through a gel pore.

The motion of a polyion under an applied electric field E can be described by Newton's law

$$m \cdot \frac{d^2x}{dt^2} = F_e - F_f \quad (2)$$

where F_e and F_f are electric force and friction force respectively, x is position, t is time and m is mass.

In steady state, the friction force is counterbalanced by the electric force and the species move at constant velocity.

$$F_e = F_f \quad (3)$$

Equating the electric and frictional forces,

$$QE = f \cdot \frac{dx}{dt} \quad (4)$$

where Q is the charge on the analyte, f is the frictional coefficient and dx/dt is the velocity of analyte.

The steady-state velocity is given by

$$v = \frac{dx}{dt} = \frac{L}{t_m} = \frac{QE}{f} = \mu E \quad (5)$$

where L is the capillary length and t_m is the migration time of the analyte.

The friction coefficient is proportional to viscosity and can be expressed as

$$f = c\eta \quad (6)$$

the proportional constant c is dependent on the molecular configuration. For spherical, or small molecules, the above equation becomes the familiar Stokes law [26]

$$f = 6\pi r\eta \quad (7)$$

The electrophoretic mobility can then be expressed as

$$\mu = \frac{Q}{f} = \frac{Q}{c\eta} \quad (8)$$

As the key point of our model, viscosity is governed by an activation barrier. This energy barrier is presumably related to the energy required for a DNA fragment to pass through a gel pore. This activation energy is well known in studies of the diffusion and migration of small molecules [27,28]. We write the viscosity in terms of the Arrhenius equation

$$\eta = \text{constant} \cdot e^{E_a/RT} \quad (9)$$

where E_a is the activation energy of viscous flow

and R is the gas constant. The mobility can be obtained by combining above equations

$$\mu = \frac{Q}{\xi} \cdot e^{-E_a/RT} \tag{10}$$

where ξ is a collection of constants. Taking the logarithm of both sides

$$\ln \mu = \ln \left(\frac{Q}{\xi}\right) - \frac{E_a}{RT} \tag{11}$$

A straight line should be produced if the logarithm of the mobility is plotted against the reciprocal of temperature. The activation energy of viscous flow, E_a, can be obtained from the slope. Fig. 5 presents a plot of ln μ versus inverse temperature. The data fall on straight lines for fragments ranging from 48 to 460 bases in length across the temperature range studied.

Careful inspection of the plots reveals that the lines are not parallel, which implies that the activation energy for migration in a gel changes with N. Activation energy is obtained by dividing slope by the gas constant. Fig. 6 is the plot of activation energy against N. There is a monotonic increase in mobility with fragment length. Straight lines were fit to two portions of the data; for fragments shorter than 200 bases, the activation energy increases by 25 J/mol per base. Fragments longer than 200 bases have an increase in activation energy of 6.5 J/mol per base. This transition in the activation energy presumably reflects a change in conformation of the DNA fragments; small fragments migrate in a random coil configuration and larger fragments migrate in an elongated configuration [29]. While these are the first measurement of the activation energy for ssDNA migration in gels, the magnitude of E_a is comfortingly similar to the activation energy measured for diffusion of molecules into polymers [30].

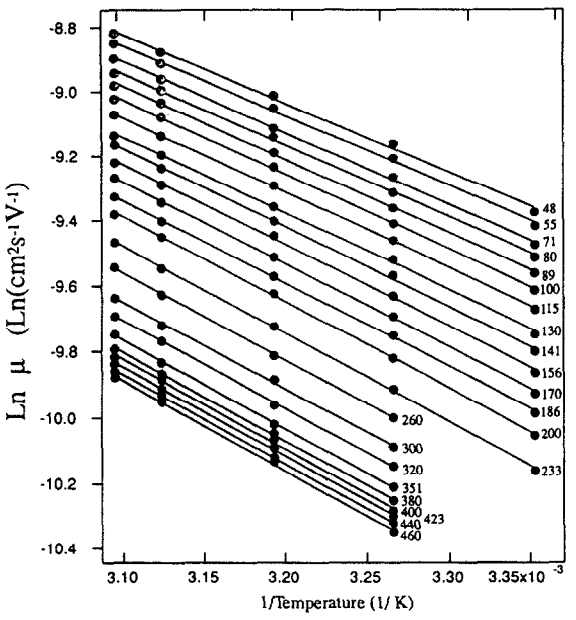

Fig. 5. Logarithm of mobility versus inverse absolute temperature. The straight lines are the linear least-squares fit to the data.

4. Conclusions

We report the first detailed study of the temperature dependence of mobility of DNA sequencing fragments that are undergoing biased reptation. The temperature dependence of mobility of ssDNA in gel electrophoresis is not in agreement with the predictions of the biased reptation model. Rather than seeing a decrease in the elongation of the fragments at elevated temperature, elongation is enhanced at higher temperatures.

This enhanced elongation is explained in terms of an activated process, presumably associated with the passage of the ssDNA fragments through pores in the gel. We report the first data for the activation energy for separation of DNA sequencing fragments by gel electrophoresis. E_a increases monotonically with fragment length for fragments at least 460 bases in length. However, there is a break in the plot of E_a versus fragment length for fragments longer than 200 bases; it appears that a conformational change occurs for fragments of that length.

Clearly, there is much work to be done to understand the thermodynamics of ssDNA migration in gel electrophoresis. We must repeat these experiments at different temperatures and

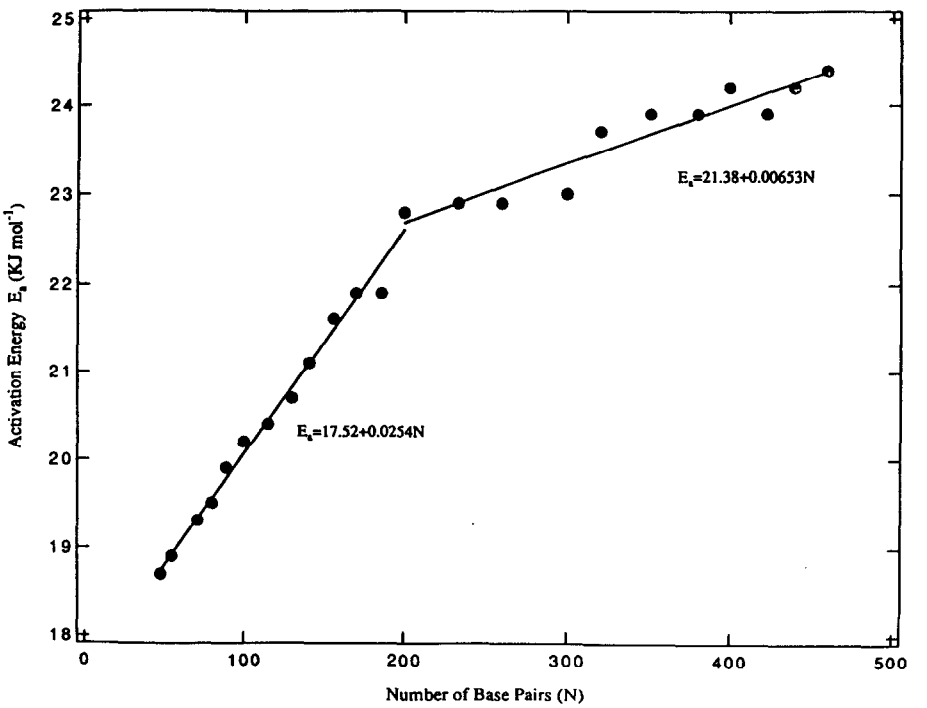

Fig. 6. Plot of activation energy versus fragment length. The lines are least squares fit to the data for fragments 50–200 bases long and for fragments 200–480 bases long.

electric fields in order to understand the nature of the change in the activation energy per base.

On the other hand, these results do demonstrate the stability of LongRanger gels at high electric fields and at elevated temperature. In a companion article, we report high-speed DNA sequencing at 40°C: 500 bases are sequenced in 50 min at an electric field of 200 V/cm and at a temperature of 40°C [31].

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