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Impacts of magnesium ions on the unzipping of λ-phage DNA

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Abstract

We used magnetic tweezers to exert a constant force to separate double stranded λ-phage DNA as a function of temperature and buffer content. The separation was performed at temperatures ranging from 20 to 50 °C in various Mg^{2+} buffers, including a T4 ligase buffer and a PCR buffer. At 30 °C and pH 7.4 (10 mM Tris), we measured the unzipping force as a function of concentration for Mg^{2+} concentrations between 0.2 and 50 mM, and determined that the unzipping force is proportional to the logarithm of concentration. For comparison, we performed the analogous experiment as a function of Na^{+} concentration and found that the unzipping force is also proportional to the log of concentration, but requires a much higher cation concentration to achieve the same unzipping force as in Mg^{2+} buffer. We also constructed the phase diagram in the force–temperature plane for the unzipping in 10 and 50 mM $MgCl_2$ at pH 7.4 (10 mM Tris). The phase diagram for 10 mM Mg^{2+} is similar to the one measured previously for phosphate buffer saline (PBS) but the phase diagram for 50 mM Mg^{2+} deviates significantly from those for 10 mM Mg^{2+} and PBS at temperatures between 20 and 35 °C.

1. Introduction

Studies of the mechanical separation of double stranded DNA (dsDNA) enhance our understanding of DNA replication. In living organisms, DNA replication is a complex process carried out by a variety of specialized proteins. In the process, dsDNA is mechanically separated.

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Mg^{2+} ions are known to interact with DNA either stabilizing or destabilizing the double helix [1–12]. Mg^{2+} concentration is therefore expected to affect the free energy difference between double stranded DNA and single stranded DNA (ssDNA) [13–15]. Mg^{2+} is believed to interact with both ssDNA and dsDNA [2]. The interactions may change the entropy and enthalpy differences between ssDNA and dsDNA [2], as well as the mechanical properties of the ssDNA [16]. In previous work, the salt dependence of the entropy and the enthalpy has been studied by measuring the melting temperatures of different sequences [17, 18] by laser Raman spectroscopy, optical densitometry, pH potentiometry, and differential scanning calorimetry [1–5]. Since the force required to separate dsDNA into ssDNA depends on all of these factors, one would expect that the force required to unzip dsDNA would depend on the concentration of Mg^{2+} present in solution; consequently, measuring the salt dependence of the critical force required to separate dsDNA into ssDNA should provide additional insight into the interactions between DNA and various salts. This work is the first examination of the buffer dependence of the force required to separate dsDNA.

The total Mg^{2+} concentration in living cells is highly regulated [19] and maintained 17–20 mM where most of the ions are bound to structural proteins, enzymes and nucleic acids [20] leaving 0.5 to 1 mM free [21] The concentration of Mg^{2+} ions is not only important for *in vivo* reactions, but also for *in vitro* reactions such as polymerase chain reactions (PCR) in which DNA strands are forced to separate and anneal and typical Mg^{2+} concentrations range between 1 and 3 mM.

Earlier work has measured the impact of Mg^{2+} and other divalent ions on the melting temperature, T_m, of dsDNA. Studies of the effect of cations on the T_m of short oligonucleotides have shown that T_m is proportional to the logarithm of ion concentration; however, the slope for Mg^{2+} is approximately half the slope for Na^+ [17]. At concentrations below 1 M, the concentration of Mg^{2+} required to achieve a given melting temperature is orders of magnitude smaller than the corresponding concentration of Na^+. For example, a buffer containing 3 mM Mg^{2+} has approximately the same melting temperature as a buffer containing 100 mM Na^+ [17].

For the short oligonucleotides in the studies cited above, the melting temperatures are approximately 40 °C. In contrast, the melting temperature for λ-phage DNA is about 90 °C, which is much higher than the physiological temperature of most organisms. The force required to separate dsDNA at a given temperature below T_m measures how tightly the two strands are bound at the lower temperature. In this work, we studied the effect of Mg^{2+} concentration on the force required to unzip λ-phage DNA at 30 °C using magnetic tweezers [22]. An additional measurement of the dependence of the unzipping force as a function of Na^+ concentration was made for comparison. We have also considered the temperature dependence of the unzipping force in the temperature range from 20 to 50 °C in buffers containing in 10 and 50 mM Mg^{2+}. Finally, we compared these results with the earlier work that has examined the force required to separate λ-phage dsDNA in PBS at temperatures ranging from 15 to 60 °C.

2. Method

2.1. Preparation of DNA and the set-up of magnetic tweezers

λ-phage DNA (New England Biolabs, Beverly, Massachusetts) was used in this study. As shown in figure 1, the DNA used for the unzipping experiment was modified from the technique pioneered by Roulet *et al* [23]. The technique was also used in earlier experiments that determined the temperature–force phase diagram for the unzipping of λ-phage DNA in PBS [22]. In brief, two λ-phage DNA molecules, one used as a linker and a second one as the molecule to be unzipped, were hybridized and ligated together. The free end of the linker

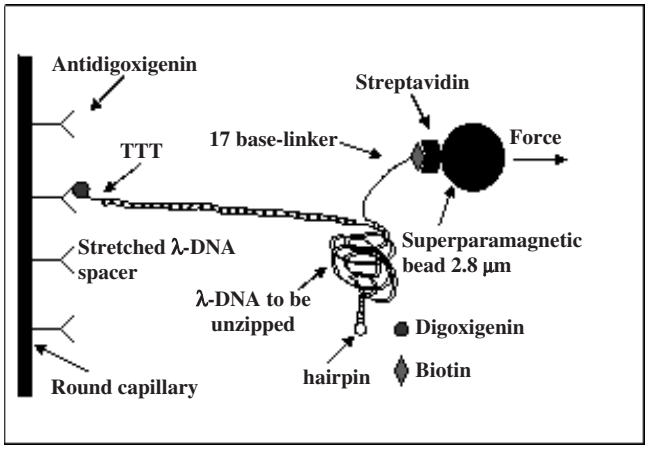

Figure 1. Schematic diagrams of the DNA constructs for the unzipping experiment.

presented a digoxigenin labelled oligonucleotide (Oligos Etc., Wilsonville, Oregon). The DNA to be unzipped was labelled with a biotinylated oligonucleotide (Oligos Etc., Wilsonville, Oregon) on one end and was covalently closed with a short hairpin at the open end. The DNA construct was heated at 50 °C overnight and cooled slowly to 25 °C at a rate of 0.1 °C min^{-1} before being mixed with 2.8 μm streptavidin coated superparamagnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway). Finally the sample was injected into a square glass cell containing an anti-digoxigenin (Roche, Penzberg, Germany) coated round capillary.

The procedure for coating the surface of the inner round capillary tube with anti-digoxigenin and the apparatus used in this work were the same as those used in an earlier unzipping study [22].

2.2. Procedures for the measurement

2.2.1. Measurement of the critical unzipping force of λ-phage DNA. For the unzipping experiment, after attaching the DNA to the anti-digoxigenin coated surface of the inner capillary tube, the sample was heated to the desired temperature between 20 and 50 °C and maintained at this temperature for 15 min before the measurement started. The variation of the temperature was ±0.2 °C for temperatures between 25 and 35 °C. The error increased as the difference between the sample temperature and room temperature increased. Between 40 and 60 °C the temperature uncertainty was approximately (±2 °C).

Initially a small magnetic force was applied to separate the magnetic beads from the surface of the capillary tube. Finally, the magnetic force was increased in a stepwise manner by 2 pN per step up to a maximum force of 25 pN. At each force, the unzipping events were counted for 15 min before the force was advanced to the next higher force. The measured critical force is defined as the value of the average force at which 50% of the correctly bound DNA molecules begin to unzip at a given temperature [22]. This measurement should be insensitive to the variation in the magnetization of the beads as long as the magnetization is distributed approximately symmetrically with respect to the average value. The variation of the force was about ±11% for forces below 7 pN, ±5% for forces between 8 and 17 pN and ±10% for forces above 17 pN. The details of the uncertainty in the force and temperature were described in a previous study [22].

2.2.2. Measurement of the melting temperature of λ-phage DNA in a magnesium buffer. The circular dichroism spectra of λ-phage DNA in a buffer containing 10 mM Mg^{2+} at 273 nm in the temperature range from 50 to 100 °C were recorded using an Aviv model 62DS spectropolarimeter (Aviv, Lakewood, New Jersey). The concentration of the DNA was close to 500 μg ml^{-1} and the samples were prepared by ultrafiltration of an aliquot of λ-phage DNA in the corresponding buffer using a Microcon® YM-100 filter (100 000 NMWL) (Millipore, Bedford, Massachusetts). The temperature at the mid-point of the melting phase was taken as the melting temperature; the heating rate was 2 °C min^{-1}.

2.2.3. Theoretical prediction of the unzipping force. Several models have been developed to predict the unzipping of homopolymeric and heteropolymeric double stranded DNA [24–36]. In this paper we are not attempting to obtain exact values for thermodynamic parameters. In the temperature range between 20 and 35 °C, the experimental data and the theoretical predictions are in reasonable agreement. In contrast, the discrepancy between experimental data and theory outside of this temperature range suggest more detailed calculations that relax some of the simplifying assumptions and include additional effects such as, for example, hairpin formation may provide additional insight into the temperature dependence of the interactions between DNA and salt.

The theoretical prediction for the unzipping of λ-phage DNA in the Mg^{2+} buffer was performed using the same description introduced in an earlier study [22]. The calculation used a two state model for the DNA. In the prediction for the separation of dsDNA in PBS buffer at pH 7.4, the minimum force required to separate dsDNA into two strands of ssDNA is given by the force such that, ΔG, the free energy difference between dsDNA and ssDNA is zero. This can be expressed as

$$0 = \Delta G = \Delta H - T\Delta S - 2\mathrm{gu}(F_c)$$

where ΔH is the enthalpy difference, ΔS is the entropy difference, and $\mathrm{gu}(F_c)$ is the free energy contribution from the stretching of each ssDNA that was estimated using the modified freely jointed chain (mFJC) model [37, 38]. A study shows that the force versus extension curves for single stranded λ-phage DNA in 5 mM Mg^{2+} buffer and a 150 mM Na^+ buffer are almost identical at forces above 4 pN [16]. At forces below 4 pN, the extension in the 5 mM Mg^{2+} buffer is less than the extension in the 150 mM Na^+ buffer, so the absolute value of $\mathrm{gu}(F)$ is smaller in 5 mM Mg^{2+} buffer than in 150 mM Na^+ buffer. If this were the only effect of Mg^{2+} on DNA, one would expect the unzipping force in the 5 mM Mg^{2+} buffer to be higher than in the 150 mM Na^+. We calculated the free energies for the stretching curves presented in that work using equation (8) of [28], and found that forces between 5 and 20 pN the force required to achieve a given free energy was approximately 0.7 pN higher in 10 mM Mg^{2+} than in PBS. In this study, we assumed that for forces between 5 and 20 pN, the free energy contribution from the stretching of ssDNA in 5 mM Mg^{2+} and 10 mM Mg^{2+} are also similar. No stretching curves are available for temperatures other than room temperature, so the mFJC theory that was used to model the stretching at room temperature was also used to project the temperature dependence of the stretching at other temperatures. Of course, other published work suggests that the presence of Mg^{2+} in a buffer affects not only the elastic properties of the ssDNA, but also the energy and enthalpy differences between dsDNA and ssDNA [2], so a prediction of the critical force requires knowledge of the temperature and salt dependence of these quantities as well.

We assume that ΔS is temperature dependent. Including considering a published proposed temperature dependence [2], it would only change the predicted critical force slightly, but would not account for the large decrease in critical force that is observed at temperatures

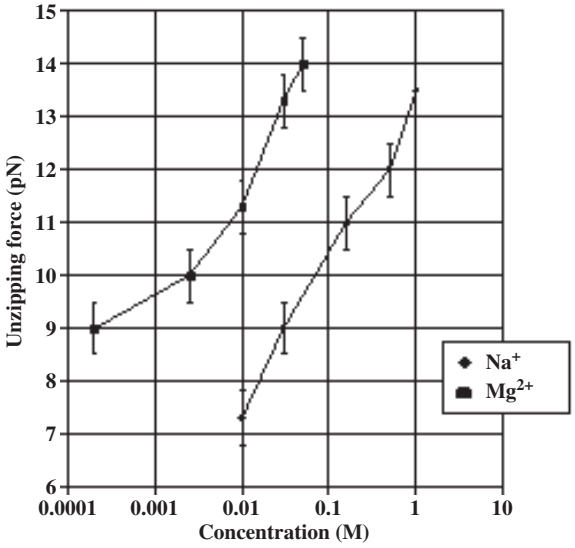

Figure 2. Measured unzipping force as a function of the concentration of Mg^{2+} (squares) and Na^{+} (diamonds). Each buffer contains 10 mM Tris at pH 7.4.

just above 35 °C. Experimental results have shown some variation in ΔS as a function of buffer [17]. For 10 mM Mg^{2+}, the ΔS value of a short oligonucleotide in 10 mM $MgCl_2$/10 mM sodium cacodylate is about 6% less than the ΔS value of the same oligonucleotide in 100 mM NaCl/10 mM Na_2HPO_4/1 mM Na_2EDTA [16] whereas in another study using calf thymus DNA the ΔS value of the DNA in 100 mM $MgCl_2$/5 mM sodium cacodylate is 13.5% greater than that in 5 mM sodium cacodylate alone [2]. Hence, we assumed that the difference in ΔS between PBS and 10 mM Mg^{2+} buffer used in this experiment is less than 20%.

For estimating the average ΔS value for the first 2000 base pairs of λ-phage DNA, we used the base pair dependent values measured using short oligonucleotides in 1 M NaCl [18]. The calculated ΔS was -20.6 cal K^{-1} mol^{-1} and we used this value for calculating the theoretical phase diagram in both PBS and 10 mM Mg^{2+} buffer.

We assume that ΔH is temperature independent, and this assumption is supported by experimental results that suggest that ΔH is insensitive to temperature for temperatures that are more than a few per cent below T_m [2]. Given the assumption above that ΔS is also independent of temperature, the buffer dependent ΔH was assumed to be given by $\Delta H = T_m \Delta S$, where T_m is the measured melting temperature for λ-phage DNA in that buffer. We obtained the melting temperature (T_m) experimentally as described above.

Because the stretching curve of single stranded λ-phage DNA in 50 mM Mg^{2+} buffer is not available, a reasonable estimation of the free energy contribution cannot be done. Hence, we did not calculate the theoretical plot for the unzipping of λ-phage DNA in 50 mM Mg^{2+} buffer.

3. Result and discussion

Figure 2 shows the measured unzipping force as a function of Mg^{2+} concentration at 30 °C. The measured unzipping force as a function of Na^{+} concentration is also shown for comparison. The plot for Na^{+} shows that the unzipping force is a linear function of the log of the concentration at nearly all concentrations. The plot for Mg^{2+} is a linear function at concentrations above

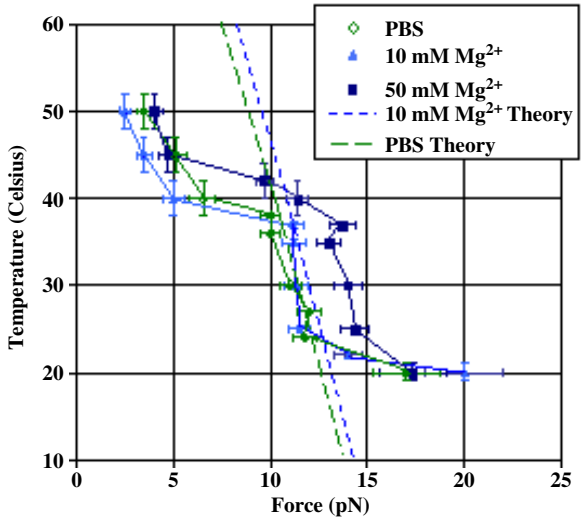

Figure 3. Phase diagram in the force–temperature plane for the unzipping of λ-phage DNA in PBS (green diamonds), 10 mM (royal blue triangles) Mg^{2+} and 50 mM Mg^{2+} buffers (dark blue squares). The green dotted line and royal blue dotted line are the predictions from the simple theory for PBS and 10 mM Mg^{2+} buffers, respectively.

2.5 mM, where at lower concentrations the unzipping force may approach that for unzipping in Tris alone. Result from linear regression analysis on the data points shows that the slope for the unzipping forces in Mg^{2+} (excluding the 0.2 mM data point) is 3.15 whereas the slope for the unzipping forces in Na^{+} is 2.92. The simple theory above predicts that the critical force is proportional to the T_m. However, earlier results with short oligonucleotides suggest that for Mg^{2+} the slope of the T_m versus the log of the concentration is approximately half that for Na^{+}. In contrast, our preliminary experimental results that measure T_m of λ-phage DNA versus the log of the concentration of Mg^{2+} and Na^{+} show that the slopes are similar for Mg^{2+} and Na^{+}. If that preliminary result is confirmed, then the results seen in figure 2 are consistent with the simple theory.

Figure 3 shows the phase diagram in the force–temperature plane for the unzipping of λ-phage DNA in PBS, 10 and 50 mM Mg^{2+} buffers. The green diamonds, royal blue triangles and dark blue squares represent the measured unzipping force in PBS, 10 mM Mg^{2+} buffer and 50 mM Mg^{2+} buffer, respectively. The phase diagrams for λ-phage DNA in 10 mM Mg^{2+} buffer and PBS are similar, though the 10 mM Mg^{2+} buffer has a lower ionic strength. However, the measured unzipping forces in 50 mM Mg^{2+} buffer above 20 °C and below 45 °C are much higher than the forces in 10 mM Mg^{2+} buffer and PBS.

The green dotted line and royal blue dotted line are the predictions from the simple theory, where the melting temperatures are 91 and 95 °C for PBS and 10 mM Mg^{2+} buffer, respectively. For PBS and 10 mM Mg^{2+}, in the temperature range between 25 and 35 °C the $F_c(T)$ is roughly a linear function of temperature, as predicted from the temperature independence of ΔS and ΔH and the temperature insensitivity of $gu(F)$. The slope predicted by the simple theory is within the error bars of the measured slope, but the error bars are too large to extract an accurate value for ΔS from the phase diagram. Though the slopes of the phase diagrams are independent of T_m, the actual values of F_c depend on the melting temperatures. The measured F_c in 10 mM Mg^{2+} buffer and in PBS are in reasonable agreement with the predicted values in between 25 and 35 °C. Though outside this temperature range, they deviate significantly.

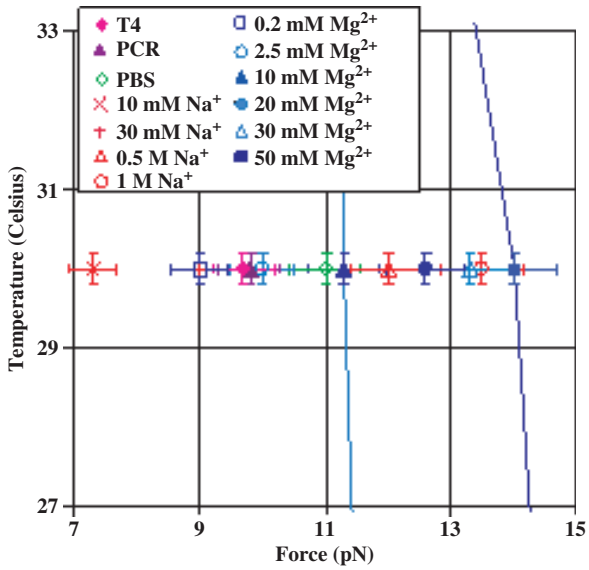

Figure 4. The same experimental data as shown in figure 3 at 30 °C with the addition of the F_c (30 °C) for all of the buffers considered in figure 2, as well as T4 ligase buffer and a PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris at pH 8.5).

In 10 mM Mg^{2+} and PBS, dT/dF_c in the temperature range between 37 and 40 °C, is much shallower than in the temperature range from 25 to 35 °C. The difference in slope is well outside of the error bars associated with the experimental points. Above 40 °C the slope becomes much steeper. A similar behaviour is shown by the phase diagram for the 50 mM Mg^{2+} buffer, except that the change in slope occurs at approximately 40 °C. In all three buffers, at temperatures above 45 °C, the predicted F_c values are well outside of the error bars for the measured values. Similarly, in all three buffers, at approximately 20 °C, the dT/dF_c undergoes another abrupt change where it again becomes very shallow, diverging substantially from the predicted values. This deviation was not predicted in other theoretical predictions either [24, 27, 29, 35]. The mechanisms underlying these deviations have yet to be determined. At lower temperatures, a re-entrant region was predicted by Marenduzzo *et al* [26, 27]. However, we have not reached such low temperatures to see the phenomenon in this study.

Figure 4 shows the unzipping of λ-phage DNA at 30 °C in PBS, 10 and 50 mM Mg^{2+} buffers with the addition of the F_c (30 °C) for all of the buffers considered in figure 2, as well as T4 ligase buffer (33 mM Tris acetate (pH = 7.8), 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM DTT) (Epicentre, Madison, Wisconsin) and a PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris at pH 8.5). The two buffers with 2.5 mM Mg^{2+} (2.5 mM $MgCl_2$ in 10 mM Tris and the PCR buffer) the unzipping forces at 30 °C are similar (∼10 pN), even though they differ in both pH and the presence of potassium in the PCR buffer. Conversely, a comparatively low unzipping force (9.7 pN) was obtained in T4 ligase buffer as compared to 10 mM Mg^{2+} in 10 mM Tris at pH 7.4 (11.2 pN) even though the T4 ligase buffer contains 10 mM Mg^{2+}. This result seems to suggest that Mg^{2+} is not the only factor that governs the unzipping force and that other variables such as the presence of potassium, acetate, DTT and higher concentrations of Tris in the buffer or the slight variation in pH could affect the unzipping force substantially as well. The comparative data from the PCR buffer suggests that neither the potassium nor pH seem to affect the unzipping force substantially. This

makes it likely that the presence of the possible ligands, DTT and especially acetate (present in the T4 buffer at 120 mM) cause the effective free concentration of Mg^{2+} to be reduced. In fact, equations of the known acetate–magnesium association constants would suggest that the acetate will complex Mg^{2+} [39]. Overall, this suggests that the free concentration of Mg^{2+} is a vital piece of information when studying the unzipping of dsDNA.

4. Conclusion

To enhance the understanding of the role of Mg^{2+} ions in the separation of dsDNA into ssDNA, we measured the effect of Mg^{2+} ions on the mechanical force required to separate double stranded λ-phage DNA at temperatures between 20 and 50 °C. We found that at 30 °C the unzipping force increases monotonically with the logarithm of the Mg^{2+} concentration, and that a similar result holds for Na^{+}. The concentration of Mg^{2+} required to achieve a given unzipping force was more than an order of magnitude smaller than that for Na^{+}. Thus, the effect of Mg^{2+} ions on the force required to separate dsDNA into ssDNA is significantly greater than the effect of Na^{+} at similar concentrations or ionic strengths.

We found that for 10 mM Mg^{2+} and PBS, the phase diagrams in the force–temperature plane were similar in the temperature range from 22 to 37 °C, though both differed significantly from the phase diagram for unzipping in 50 mM Mg^{2+}. In all buffers, the unzipping force in the temperature range from 25 to 35 °C is approximately linear as a function of temperature, with similar slopes in all of the buffers, and the predicted critical forces are in reasonable agreement with simple theoretical predictions. Outside of the temperature range between 20 and 40 °C, the measured phase diagrams for the unzipping of λ-phage DNA in the force–temperature plane differ substantially from the predictions of a simple theory.

Acknowledgments

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