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Single molecule study of the reaction between DNA and formamide

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Abstract

The kinetics of the reaction between double stranded DNA (dsDNA) and formamide is monitored at the single DNA molecule level. We find that stretching of the DNA leads to an accelerated reaction rate and to a shift in the final equilibrium concentrations. The larger the stretching force, the faster the reaction and the larger the denatured fraction of the product DNA. The single molecule kinetics is obtained from the change in the contour length of the DNA which, in turn, is measured using optical tweezers on a microbead-single DNA molecule-cover slip construct.

Keywords: Single molecule; DNA stretching; Optical tweezers; Formamide

1. Introduction

Over the last decade, a wide variety of studies on the observation and manipulation of single biological molecules, e.g. DNA and proteins, have been reported [1–4]. Such studies relay on the combination of high precision optical methods with recent advances in molecular biology. This approach has been employed to directly probe several aspects of biological molecules like structure, functionality, mechanism of molecular interaction and reaction kinetics. In particular, the single molecule approach was used to investigate the interactions between DNA on one hand and proteins, surfactants and polymers on the other [5–8]. Such interactions can merely modify the physical properties of the DNA, e.g. change its persistence length or induce coil to globule transitions. Alternatively, a chemical reaction may take place that leads to new chemical bonds. In this paper, we present our results on the reaction between double stranded DNA (dsDNA) and formamide, an important denaturing agent. We study this reaction at the single molecule level.

DNA denaturation [9–12] was extensively investigated in the last few decades as it gives useful information on DNA regarding its structure and stability. When a DNA solution is subjected to high temperature (80–90 °C) or high pH $\geq$ 13 [13], the hydrogen bonds holding the two strands

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together break and the double helix dissociates into two single strands. This transition from ds-DNA to single stranded DNA (ssDNA) is known as denaturation or melting. The extent of melting depends on various factors, e.g. length of DNA [11], L, ionic strength of the solvent [9,12,14–16], I, solvent viscosity [17], η, G–C content [12], pH [18], etc. If only temperature is varied, DNA gradually denatures over a range of about 14 °C and the process is characterized by the corresponding melting curve [19]. Typically, melting curves are obtained by monitoring the UV absorption at around 260 nm. Since ssDNA is longer than dsDNA, 0.7 nm per base pair instead of 0.34 nm, one can obtain the melting curve of a single DNA molecule by monitoring its length. In turn, the length measurement can be made using single molecule techniques.

Magnetic field [20], fluid flow [21], micropipettes [22], optical tweezers [23–26] and AFM [27,28] are few of the tools that were used in single molecule DNA stretching experiments. In our approach [6,29], one end of a DNA molecule is attached to a micron sized bead and the other end is attached to the bottom of the sample. Then the DNA is stretched by trapping and pulling the bead with optical tweezers. When the end to end distance, z, approaches the contour length of the DNA, L, there is a steep increase in the force required to further extend the DNA. Accordingly, in a small range of z below $z = L$, the DNA force exceeds that of the tweezer leading to the escape of the bead from the optical trap. This facilitates monitoring the value of L during the melting process.

In molecular biology applications where various DNA's are melted and hybridized, it is inconvenient to work at temperatures in the range around 80 °C. The effect of organic solvents, e.g. urea, formamide, formaldehyde, ethidium bromide, is to shift the DNA melting curve to lower temperatures [19,30–34]. In particular, formamide forms hydrogen bonds with the bases that replace the native ones and hence disrupts the dsDNA. This is a chemical reaction that can be studied at the single DNA molecule level using optical tweezers. In particular, measuring the contour length of the DNA as it denatures due to the formamide, $L(t)$, we can monitor the kinetics of the reaction. This approach was previously used to monitor the reaction between DNA and RecA [5–7]. Although, RecA is an extremely interesting protein, its reaction with DNA is quite complex involving several steps and also activation by ATP. It is, therefore, worthwhile to study a simpler reaction, namely, that between formamide and DNA.

2. Experimental methods

2.1. Optical system

The experimental setup consists of an optical tweezer, the visualization system and the temperature control. For the optical tweezer, we use a laser diode (SDL-5422-H1, 150 mW, 830 nm) whose light is first collimated and then focused via a microscope objective (Zeiss, $\times 100$, NA 1.3, oil immersion). A micron sized bead that is transparent (latex) can be trapped at the focus of the beam. The cell containing beads, DNA and PBS buffer is placed on a motorized stage (Newport) and is visualized on both video and computer after suitable illumination from above the sample (see Fig. 1). The tweezer trapping force is calibrated using Stokes law [35], namely, we measure the velocity of the stage where the Stokes force first exceeds the trapping force leading to the escape of the bead. Forces in the range of 1–15 pN can be obtained by varying the power of the laser. The temperature of the sample is controlled by circulating fluid (Neslab) through a ring around the objective (not shown).

2.2. DNA attachment

The single DNA molecule studies are based on the cover slip-DNA-bead construct. This is achieved by a low pH protocol [36,6] whereby one end of the λ-DNA (Promega) is attached to a cover slide and the other end to a 2.8 μm size latex bead (Polysciences). Then, 5 μl of λ-DNA (48.5 Kb, contour length 16.5 μm) are incubated along with 1 μl beads ($\sim 10^6$ beads μl^{-1}) and 400 μl PBS buffer (pH 5.9) for about 15 min. During

this period, the bead-DNA link is established. Next, 20 μl of the solution are pipetted into the cell together with 110 μl buffer and incubated for 24 h at room temperature. In this way, the DNA-cover slide link is obtained. Before the start of an experiment, the bead is pulled using the tweezer to ensure that it is tethered by a single DNA molecule.

2.3. Formamide–DNA interaction

Assuming that formamide denatures the ds-DNA, the change in the length of the DNA molecule is proportional to the concentration of formamide on that molecule. We first find a bead that is tethered to the cover slip via a single DNA that is of the right length. Moreover, we require that in the neighborhood of the construct, there is enough free room to perform the stretching experiments. Situations where there are agglomerations of beads that are either free or glued to the cover slip are excluded. On the other hand, it is helpful to have a few glued beads in the frame that can be used as reference. Then, at $t=0$, formamide (Sigma) is added near the edge of the cell far away from the DNA under observation. The value of $L(t)$ is measured by pulling the bead on both sides of the equilibrium point and recording the position where the bead escapes from the optical trap, the escape point. $L(t)$ is defined as half the distance between two opposite escape points and is monitored at intervals of 1 min. The variation in DNA length with time was studied when the DNA is constantly stretched by a fixed force (4 and 8 pN) and in the absence of force when it is coiled in its equilibrium configuration. In the case where no force is applied, the DNA was stretched only during the length measurement. Length versus time observations were done for formamide concentrations of 1.78 and 3.56 M. All measure-

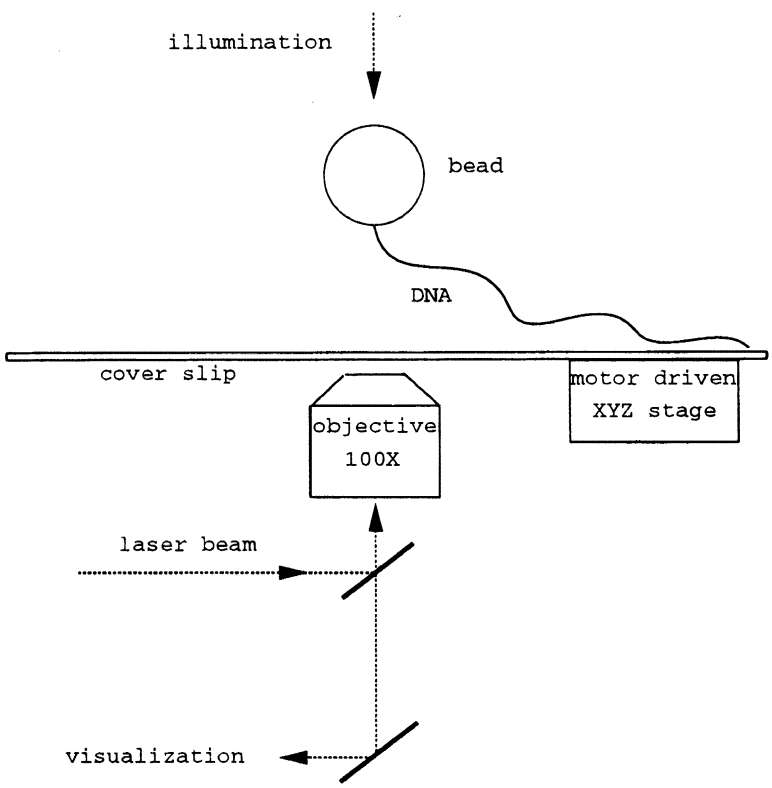

Fig. 1. Schematic description of the experimental setup.

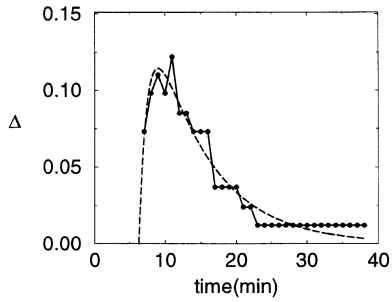

Fig. 2. Reaction kinetics at the single molecule level. The fraction of the DNA base pairs where formamide is bound at a given time, t, is proportional to the relative elongation of the DNA molecule, $\Delta = (L(t) - L(0))/L(0)$ (line + bullets). The contour length, $L(t)$, is measured using the escape position of the DNA tethered bead from the optical trap which is set to exert a maximal force of 8 pN. Formamide is added at $t = 0$ such that [F] = 1.78 M. The dashed line represents the best fit of Eq. (2) to the data.

ments were done at room temperature (about 23 °C).

3. Results and discussion

The experimental setup described in the previous section allows us to study the kinetics of the reaction between dsDNA and formamide, $HCONH_2$. The formamide replaces the DNA bases in the inter-strand hydrogen bonds inducing the denaturation of dsDNA. Since the distance between adjacent bases is larger in ssDNA than in dsDNA, a certain concentration of formamide on the DNA corresponds to a particular change in its contour length. This allows us to monitor the kinetics of the reaction between formamide and DNA at the single molecule level by measuring the length of the DNA at 1 min intervals (see Fig. 2).

We find that during the reaction the concentration of formamide on the DNA first grows, reaches a maximum and then decreases. This indicates that the reaction is more involved than just formamide progressively intercalating between the DNA bases. In order to obtain a qualitative understanding of this time dependence, we can assume that the usual chemical kinetics laws apply. In other words, we assume that the reaction between formamide and DNA occurs in each of the DNA bases independently and that self-averaging is obtained on an individual DNA molecule. In this framework, the non-monotonic kinetics can be explained only if the formamide–DNA complex (F–DNA) represents an intermediate product of the reaction such that, in the second stage, the formamide (F) detaches from the DNA and returns to solution. On the other hand, formamide is known to hydrolyze in water producing formic acid. We propose that the formamide that is bound to DNA will also undergo hydrolysis and, subsequently, detach from the DNA.

$$\mathrm{F} + \mathrm{DNA} \xrightarrow{k_1} \mathrm{F\text{–}DNA} \xrightarrow{k_2} \mathrm{HCOOH} + \mathrm{DNA}^* + \mathrm{NH_4^+} \quad (1)$$

where k_1, k_2 are the rate constants and DNA* is an inactive form of dsDNA to which formamide is unlikely to bind. If the end form of the DNA would reenter the reaction, the concentration of the complex would have to be monotonically increasing in time according to chemical kinetics. On the other hand, the kinetics of Eq. (1) leads to

$$[\mathrm{F\text{–}DNA}](t) = \frac{k_1[\mathrm{DNA}](0)}{k_2 - k_1}(\exp(-k_1(t - t_0)) - \exp(-k_2(t - t_0))) \quad (2)$$

for which $[\mathrm{F\text{–}DNA}](0) = [\mathrm{F\text{–}DNA}](\infty) = 0$. We find that Eq. (2) fits well to the data giving $k_1 = 0.126$ min^{-1} and $k_2 = 0.78$ min^{-1}. A time lag, t_0, for the start of the reaction is allowed in the fit, $t_0 = 6.23$ min, which is related to the diffusion time required for the formamide to reach the location of the DNA molecule on which the measurement is performed. Since the concentration of formamide, [F] = 1.78 M, greatly exceeds that of DNA base pairs, $[\mathrm{DNA}](0) = 1.44\ 10^{-6}$ M bp, the kinetics of Eq. (1) is expected to be of pseudo-first order in its first step.

The most exciting option that our experimental setup allows is moving the reaction further away from the domain of standard chemical kinetics by applying an elongational force to the DNA, one of the two reactants (see Figs. 3–5). Using the

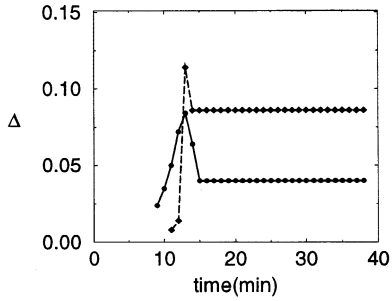

Fig. 3. The effect of elongational stress on the reaction kinetics at the single molecule level. While the measurement is performed in the same way as in Fig. 2, between measurements the DNA molecule is maintained in a stretched state by exerting a fixed force: 4 pN (full line + bullets), 8 pN (dashed line + diamonds). The concentration of formamide is 3.56 M.

optical tweezer, we apply a constant force of either 4 or 8 pN. Three main features are observed. First, the denaturing part of the reaction is accelerated (see Fig. 3). In particular, the rate of reaction averaged over several runs and over the period of time where the Δ grows is 0.015 min^{-1} at 4 pN and 0.024 min^{-1} at 8 pN when [F](0) = 3.56 M. Second, the maximal extent of the denaturation, $\langle\Delta_{max}\rangle$, is larger for larger force, such that, $\langle\Delta_{max}\rangle$ (4 pN) = 0.084 and $\langle\Delta_{max}\rangle$ (8 pN) = 0.138. Third, the formamide hydrolysis, the second stage of the reaction, is arrested by the force at some new equilibrium level whereby part of the formamide remains on the DNA. As seen in Fig. 3, the higher is the force the more formamide reacts with the DNA and also higher is the final

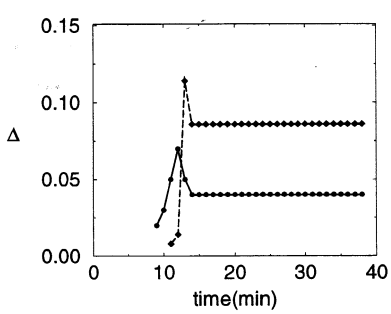

Fig. 4. The kinetics of the reaction at different concentrations of formamide: 1.78 M (full line + bullets), 3.56 M (dashed line + diamonds). The measurement is performed as in Fig. 3 maintaining the DNA stretched by an 8 pN force.

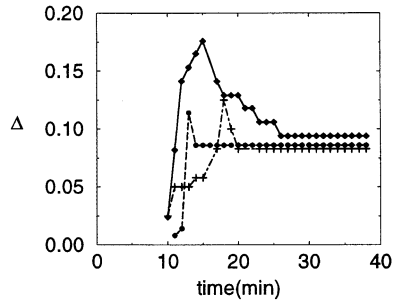

Fig. 5. Three different runs of the same experiment. The DNA is stretched with 8 pN force. The concentration of formamide is 3.56 M.

equilibrium level. The equilibrium values of Δ averaged over several runs are 0.04 at 4 pN and 0.088 at 8 pN when [F](0) = 3.56 M. The effect of changing the concentration of formamide (see Fig. 4) at a fixed force is similar to that of changing the applied force. In this case, the denaturing part of the reaction is faster at higher formamide concentrations and the corresponding equilibrium [F–DNA] is higher.

The speedup of the reaction due to the force can be qualitatively understood. Since formamide requires additional space in order to intercalate between the DNA bases, the force facilitates the reaction by enlarging the spacing between adjacent base pairs. By the same mechanism, the amount of formamide that reacts with the DNA in the first step of the reaction grows with force leading to a higher value for the maximal Δ. On the other hand, the hydrolysis part that is catalyzed by the pressure of the DNA bases on the formamide reaches a point where this pressure is balanced by the applied force. At this point, the hydrolysis stops leading to a forced equilibrium state.

In order to attempt quantitative modeling of the forced kinetics in single molecules, one should first investigate the extent to which the results are reproducible. In Fig. 5, we present three different runs performed under identical experimental conditions. Although, the general features of the time dependence are preserved from one experiment to another, there is significant variability between the different runs. We propose that this variability is of statistical nature, namely, it is a consequence of

performing measurements on single DNA molecules. Specifically, statistical fluctuations in the kinetics result from either: (i) fluctuations in the concentration of formamide in the immediate neighborhood of the DNA molecule, (ii) fluctuations in the local temperature, (iii) varying nucleation sites for formamide attachment along the DNA and distribution of growth islands around these sites or (iv) statistical fluctuations in the reaction rates that, in turn, are determined by the nucleation and growth rates. These various factors are not independent of each other but rather intimately interrelated.

In the light of the results for the forced kinetics, it is possible that the zero force experiments like that of Fig. 2 are slightly biased due the measurement method itself. Namely, in order to measure the changes in the contour length, the DNA has to be stretched for a small fraction of the total time. Since in our approach this fraction is only about 5%, we expect that the corresponding error is small.

The reaction between formamide and dsDNA was also studied in solution by monitoring the changes in the UV absorption of DNA at 260 nm. However, due to the interference from the formamide absorption peak we are restricted to use much lower concentrations of formamide than in the single molecule experiment (around 10^{-2} M). Although, the kinetics we measure is quantitatively different from that observed in the single molecule experiments, we also find an important similarity. Namely, the concentration of denatured DNA displays a maximum at $t \approx 45$ min after which it monotonically decreases. This behavior supports the interpretation we proposed in Eq. (1).

In conclusion, we have monitored the kinetics of the dsDNA–formamide reaction at the single DNA molecule level and found that at room temperature partially denatured dsDNA is an intermediate product. Moreover, we showed that applying external force to one of the reactants, namely, the DNA molecule, accelerates the kinetics of the reaction and shifts the final equilibrium concentrations. Further automatization and enhanced precision of the experimental setup would allow us to obtain more data such as to be able to pursue a statistical description of the single molecule reaction kinetics. Such upgrading is presently underway. It involves using a quadrant detector and a piezoelectric actuator that are coupled through a feedback circuit. In this approach, the bead is kept at a fixed position within the optical trap corresponding to applying a particular force on the tethered DNA. The feedback system moves the stage in order to maintain the predetermined force despite the change in the length of the DNA molecule.

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