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Review Article

Monitoring helicase-catalyzed DNA unwinding by fluorescence anisotropy and fluorescence cross-correlation spectroscopy

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ABSTRACT

In order to elucidate molecular mechanism of helicases, we have developed two new rapid and sensitive fluorescence assays to measure helicase-mediated DNA unwinding. The fluorescence anisotropy (FA) assay takes the advantage of the substantial change in fluorescence polarization upon helicase binding to DNA and DNA unwinding. The extent of depolarization depends on the rate of tumbling of the fluorescently labeled DNA molecule, which decreases with increasing size. This assay therefore can simultaneously monitor the DNA binding of helicase and the subsequent helicase-catalyzed DNA unwinding in real-time. For size limitation reasons, the FA approach is more suitable for single-turnover kinetic studies. A fluorescence cross-correlation spectroscopy method (FCCS) is also described for measuring DNA unwinding. This assay is based on the degree of concomitant diffusion of the two complementary DNA strands in a small excitation volume, each labeled by a different color. The decrease in the amplitude of the cross-correlation signal is then directly related to the unwinding activity. By contrast with FA, the FCCS-based assay can be used to measure the unwinding activity under both single- and multiple-turnover conditions, with no limitation related to the size of the DNA strands constituting the DNA substrate. These methods used together have proven to be useful for studying molecular mechanism underlying efficient motor function of helicases. Here, we describe the theoretical basis and framework of FA and FCCS and some practical implications for measuring DNA binding and unwinding. We discuss sample preparation and potential troubleshooting. Special attention is paid to instrumentation, data acquisition and analysis.

1. Introduction

Helicases play essential roles in many important biological processes such as DNA replication, repair, recombination, transcription, splicing, and translation [1]. DNA helicases are motor proteins that use nucleotide triphosphate binding and hydrolysis to unwind duplex DNA and form the single-stranded DNA intermediates [2]. The gold-standard gel assay has provided fundamental insights into the mechanism of action of several helicases. However, how helicases couple its conformational changes resulting ATP binding and hydrolysis to DNA unwinding and translocation is still obscure. Full understanding of the mechanism(s) by which helicases catalyze DNA unwinding requires knowledge of mechanistic information about DNA translocation and the coupling of ATP hydrolysis to this process. Sensitive assays that allow continuous monitoring helicase-mediated DNA unwinding in real-time will be very useful for these purposes.

We have developed a fluorescence anisotropy (FA)-based assay for monitoring simultaneously DNA binding and DNA unwinding activity of helicase in the same sample and in real-time [3]. This assay makes possible the separation of binding and catalytic parameters and the study of real-time kinetics. The steady-state fluorescence anisotropy is sensitive to rotational diffusion, and thus is suitable for studies aiming to identify structural modifications leading to a significant change in molecular size. Using a fluorescent probe covalently linked to the released single-stranded DNA strand, concomitant to DNA unwinding, it is possible to follow DNA binding and unwinding, as both steps strongly influence molecular size of the fluorescent moiety.

More recently, we used another approach which is mainly based on the translational diffusion, the dual-color two-photon excitation fluorescence cross-correlation spectroscopy (FCCS) [4]. In contrast to the standard FCS approach when using a singly labeled molecule, the diffusion coefficient is not used by itself in FCCS. Thus, the change in size is not the main point since the interaction between two molecular entities, labeled by spectrally distinct fluorophores, can be followed based on the measurements

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of the degree of the concomitant diffusion of these fluorescent entities through the excitation volume. In the case of the unwinding activity, the two strands of duplex DNA are labeled by a different fluorophore, with a maximal cross-correlation signal in the absence of activity, and a significant decrease in this signal upon the physical separation that occurs during the unwinding activity. The two methods presented below have been validated using mainly the *Escherichia coli* RecQ helicase [3–6]. These methods can probably be used for the study of any helicase activity although it is crucial to adapt the unwinding assay for each helicase (nature of the buffer, nature of the DNA substrate (DNA size, with or without 3′/5′ single-stranded DNA tails...)).

2. Sample preparation

2.1. Helicase preparation

We typically use bacterially-expressed helicase, with or without a 6-His-Tag [7]. The overexpressed protein was purified under native conditions by chromatography on Ni^{2+}–nitrilotriacetate acid columns followed by fast protein liquid chromatography with size exclusion chromatography columns. If needed, the His tag can be cleaved using biotinylated thrombin, and removal of the biotinylated thrombin is accomplished using streptavidin–agarose magnetic beads. The concentration of the protein is determined by Bradford assay.

2.2. DNA substrates

The design of the DNA substrates depends on enzymatic properties of the helicases to be studied. For studying *E. coli* RecQ helicase, we use a 13–66 bp duplex DNA with a 3′-ssDNA tail varying between 7 and 30 nt. In the case of FA, the short DNA strand was labeled by a fluorescein group at its 3′-end for two reasons: firstly, *E. coli* helicase preferentially binds to the ss/dsDNA junction with the help of the 3′ss-tail and translocates along the oligonucleotide with the 3′ to >5′ polarity during unwinding [8,9]. At the end of unwinding, the short DNA strand is released from DNA–helicase complex while the helicase is still bound to the long DNA strand [10]. When the long strand is fluorescein-labeled, no anisotropy signal change can be detected. Secondly, to avoid the possibility that the fluorescein group may spatially hinder DNA binding by the helicase at the ss/dsDNA junction and inhibit initiation of unwinding, the labeling of fluorescein at the 3′-end, but not at the 5′-end is preferred. Note that to study the polarity of RecQ helicase with FA method, we also designed a duplex DNA with 5′-ssDNA tail and labeled the fluorescein group at 5′-end of the short strand [3]. For FCCS, there is no technical limitation regarding the DNA substrate size. In the case of *E. coli* RecQ helicase, even though blunt DNA substrates may be unwound at high protein concentration [9], it is preferable that the substrate harbors at least one single-stranded 3′-tail to improve the unwinding activity [4,11,12]. Oligonucleotides with a covalently attached 6-carboxyfluorescein moiety on either 5′- or 3′-end (for FA), and Texas red- or Alexa488-labeled oligonucleotides (on 3′-end for FCCS) were purchased from Eurogentec (France). HPLC or gel purification is strongly recommended. The fluorescent oligonucleotides were hybridized with non-labeled complementary oligonucleotide to obtain duplex DNA (FA) or the DNA substrate was obtained by hybridization of the Texas red- and Alexa488-labeled oligonucleotides (FCCS). Annealing was done using stoichiometric concentrations of complementary oligonucleotides (final concentration of the duplex DNA, 5 μM) in the hybridization buffer (20 mM Tris–HCl pH 7.5, 1 M NaCl). Heat the mixture to 85 °C for 5 min and then let it cool slowly to room temperature.

2.3. Unwinding buffer and ionic strength

The unwinding buffer contained 25 mM Tris–HCl (Hepes is possible) (pH in the 7.2–8.0 range), 5–40 mM NaCl, 1–30 mM magnesium acetate and 0.1–0.3 mM DTT. All concentrations above-mentioned correspond to final concentrations. The reaction is initiated by addition of 1 mM ATP.

3. Fluorescence anisotropy

3.1. Theoretical basis of fluorescence anisotropy based helicase assay

Fluorescence anisotropy measurements are based on the principle of photoselective excitation of a fluorophore by a polarized light, providing information about rotational motions of the fluorophore or fluorescently labeled molecule between photon absorption and emission. FA is a powerful tool for measuring molecular orientation and rotational motions as well as processes that modulate them. The physical basis of fluorescence anisotropy is that, according to the photoselection principle, the fluorescent molecules with their absorption transition vectors aligned parallel to the electric vector of the linearly polarized light are selectively excited. This leads to an anisotropic subpopulation of non-randomly oriented excited molecules. Some events such as overall rotational diffusion or flexibility are major causes of light depolarization during the emission process. High levels of anisotropy are generally associated with large molecules or complexes characterized by slow rotational diffusion or low flexibility level. For small fluorescent molecule, rotating rapidly in solution, the initially photoselected orientational distribution becomes randomized prior to emission, resulting in low fluorescence polarization or low fluorescence anisotropy. In contrast, binding of the small fluorescent molecule to a large slowly rotating molecule results in a higher fluorescence polarization. The measurement of FA can be done in solution, without the tedious physical separation of bound fluorescence molecule from the complex. This method also allows true equilibrium analysis with concentration of trace molecule as low as subnanomolar range. Thus, every process (*e.g.* molecular interaction, enzymatic process) leading to a change in size of the fluorescent moiety can be easily monitored and quantified due to the additivity law of anisotropy. Steady-state fluorescence anisotropy is then suitable for both equilibrium and kinetic studies.

Here, we used an extrinsic fluorophore covalently linked to DNA to monitor the binding of helicase to double-stranded DNA substrate and the subsequent unwinding reaction, in the same assay. Both DNA binding and unwinding would be expected to have a significant effect on the anisotropy parameter, because each of these steps has a major effect on the molecular size of the fluorescent moiety. Fig. 1A shows a schematic representation of the one-strand fluorescently labeled DNA used to monitor helicase-catalyzed DNA unwinding. The fluorescein-labeled double-stranded oligonucleotide obtained by hybridization with a complementary unlabeled DNA strand is characterized by a low fluorescence anisotropy value (Fig. 1B). This value was further significantly increased upon addition of helicase, indicating helicase/DNA substrate interaction. The higher fluorescence anisotropy value indicated that the binding of helicase to DNA can be observed independently, prior to DNA unwinding. When the unwinding process was initiated by addition of ATP to the unwinding buffer, the progress of reaction was clearly evidenced by a significant decrease in the anisotropy value, indicating the unwinding of the duplex DNA and release of the fluorescent labeled strand from helicase. It is worth noting that the anisotropy value of the released oligonucleotide was even lower than the duplex DNA substrate alone (Fig. 1B). The lower limit value corresponds exactly to the value of the protein-free fluorescent