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Assays for the determination of the activity of DNA nucleases based on the fluorometric properties of the YOYO dye

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ABSTRACT

Here we characterize the fluorescence of the YOYO dye as a tool for studying DNA–protein interactions in real time and present two continuous YOYO-based assays for sensitively monitoring the kinetics of DNA digestion by λ-exonuclease and the endonuclease EcoRV. The described assays rely on the different fluorescence intensities between single- and double-stranded DNA–YOYO complexes, allowing straightforward determination of nuclease activity and quantitative determination of reaction products. The assays were also employed to assess the effect of single-stranded DNA-binding proteins on the λ-exonuclease reaction kinetics, showing that the extreme thermostable single-stranded DNA-binding protein (ET-SSB) significantly reduced the reaction rate, while the recombination protein A (RecA) displayed no effect.

Introduction

Nucleases are enzymes that catalyze the hydrolysis of the phosphodiester bonds that link adjacent nucleotides in nucleic acids. They can be classified into two groups: exonucleases and endonucleases. Exonucleases excise nucleotides, one at a time, from the ends of DNA molecules. Endonucleases, on the other hand, cleave a pair of phosphodiester bonds in opposite strands within the DNA molecule, generating DNA fragments with either blunt or sticky ends. Both exonucleases and endonucleases are involved in vital cellular processes such as DNA replication, repair and recombination [1,2]. In addition, these enzymes are widely employed in laboratories for applications such as gene cloning, genotyping and removal of contaminating nucleic acids.

Typical assays to gauge the activity of nucleases are based on gel electrophoresis [3]. While these assays are relatively easy to implement, they have the disadvantages of being discontinuous. This makes them unsuitable for kinetic studies, and usually requiring DNA labeling with radioactive isotopes. Fluorescence-based methods are an attractive complement or alternative to existing molecular biology assays because they are continuous, highly-sensitive and inexpensive. In this work, we present fluorescence-based assays for real-time measurements of exonuclease and endonuclease kinetics. Our assays employ the oxazole yellow homodimer (YOYO) (Fig. 1A), a cyanine dye that displays an enormous increase in fluorescence upon binding to DNA molecules [4,5]. First, we re-examine the binding properties of YOYO towards ssDNA and dsDNA. Then we demonstrate how the unique fluorescent properties of the YOYO dye can be employed to sensitively monitor the kinetics of exonucleases, using the processive λ-exonuclease as example. Finally, we demonstrate how the assay can be extended to monitor the activity of endonucleases, using the type II restriction enzyme EcoRV to illustrate the method.

Experimental

Materials

λ-DNA and all enzymes and proteins were purchased from New England BioLabs (Ipswich, MA). New England BioLabs certifies that the purity of the enzymes preparations is greater than 99%. YOYO-1 dye was purchased from Invitrogen (Carlsbad, CA). Other reagents used to prepare solutions and buffers were obtained from Sigma–Aldrich (St. Louis, MO).

Fluorescence and anisotropy measurements

Fluorescence spectra and anisotropy measurements were obtained with a Varian Cary Eclipse Fluorescence Spectrophotometer using quartz fluorescence cuvettes with optical path of 1 cm. The temperature was controlled using a Varian Peltier device. The excitation and emission wavelengths used to

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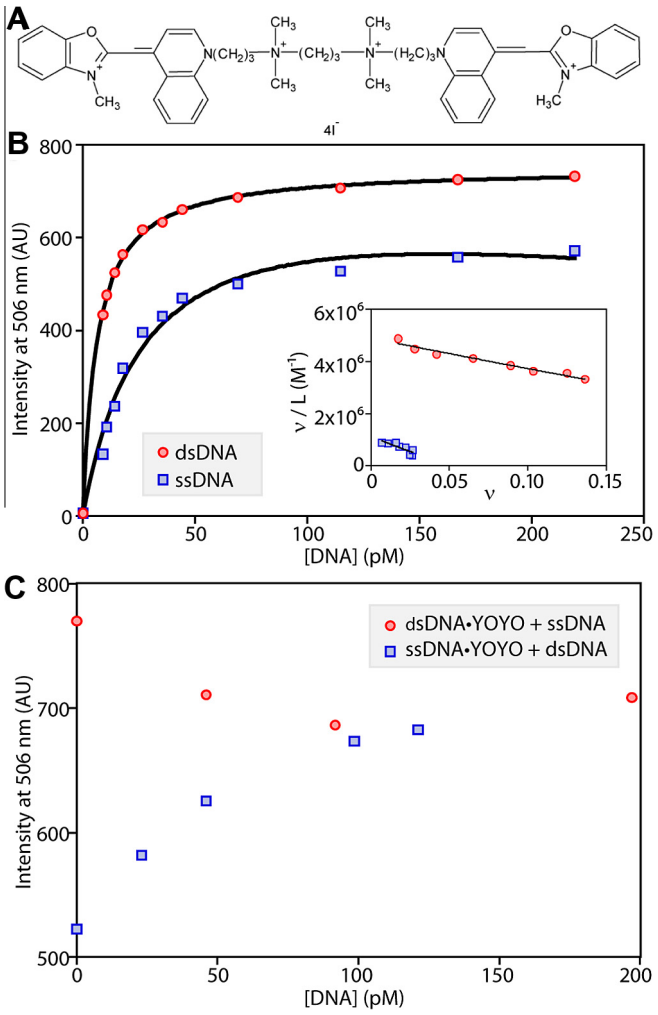

Fig. 1. (A) Chemical structure of the YOYO-1 molecule. (B) Saturation binding curves and Scatchard plots (inset) for the binding of YOYO to dsDNA in 2.5 mM $MgCl_2$ (circles) and in 0 mM $MgCl_2$ (squares). (C) Titrations of ssDNA–YOYO (squares) and dsDNA–YOYO (circles) with additions of unlabeled dsDNA and ssDNA respectively. [$MgCl_2$] = 2.5 mM. The initial dye to bp ratio in both titrations was 1:30.

measure the fluorescence and anisotropy of YOYO in our experiments were 491 nm and 506 nm, respectively. The entrance and exit monochromators slit widths were adjusted to 5 nm.

Labeling DNA with YOYO dye

Fluorescence from YOYO molecules was employed to monitor the relative amount of dsDNA to ssDNA. Solutions were prepared by adding the DNA to a YOYO solution and incubating in the dark overnight at 4 °C. For experiments performed to study the intercalation process, λ-DNA was mixed with YOYO at a dye to bp ratio of 1:30 and the increasing emission of YOYO was measured as a function of time until a plateau was reached, indicating the completion of the intercalation.

Preparation of single-stranded λ-DNA

Single-stranded DNA (ssDNA)[2] was prepared by thermal denaturation of λ-DNA. The DNA sample contained in a tightly sealed vial was placed on a boiling water bath for 30 min. Subsequently, the vial was immediately transferred to an ice bath and cooled for at least 15 min. To ensure that most of the DNA was in single-stranded form, ssDNA was always prepared fresh prior to an experiment.

Determination of YOYO binding constants

Binding constants were determined by titration of a YOYO solution with unlabeled DNA. Aliquots of a stock solution of λ-DNA (16 nM and 32 nM for experiments with native and denatured λ-DNA respectively) were added stepwise to a 0.1 μM YOYO solution. Following each addition, the mixture was allowed to equilibrate as the fluorescence intensity was measured until no further change was observed. The resulting data from these titrations was used to generate saturation binding curves. The intrinsic association constants, K_a, and size of the binding sites were estimated from Scatchard plots using the conditional probability model derived by McGhee and von Hippel to describe the non-cooperative binding of ligands to a DNA lattice [6]. The equation for the binding of YOYO molecules to DNA is:

$$\frac{v}{[\mathrm{YOYO}]_f} = K_a(1-nv)\left(\frac{1-nv}{1-(n-1)v}\right)^{n-1} \quad (1)$$

where v is the binding density expressed as moles of bound dye per mole of total bp, $[\mathrm{YOYO}]_f$ is the molar concentration of free dye, and n is the size of the binding site in bp (i.e., the number of bp covered by each YOYO molecule). The data were fitted using a non-linear least squares fitting procedure to determine the values of K_a and n.

λ-Exonuclease assays

The activity of λ-exonuclease was assayed by following the decrease in fluorescence of YOYO upon nucleotide hydrolysis. As we will demonstrate, the fluorescence decrease is due to the conversion of segments of dsDNA into ssDNA. YOYO bound to ssDNA segment displays a 25.7% reduction in the fluorescence intensity as compared to dsDNA segments of the same length. Reaction mixtures consisted of 30 pM λ-DNA, λ-exonuclease reaction buffer (50 mM NaCl, 25 mM Tris–HCl, 50 μg/mL BSA and 2.5 mM $MgCl_2$ at pH 8.0), and varying amounts of YOYO-1 dye and λ-exonuclease enzyme. The temperature was kept at 37 °C during all experiments. Reactions were initiated by addition of the enzyme and the fluorescence of YOYO was monitored as a function of time. The effect of YOYO loading on the activity of λ-exonuclease was assessed by carrying out the exonuclease reaction with λ-DNA labeled with various concentrations of the dye.

EcoRV endonuclease assays

We developed an indirect method to determine the activity of EcoRV based on the observation of an increase in λ-exonuclease reaction rates upon restriction cleavage of the λ-DNA substrate. Reactions were initiated by the simultaneous addition of EcoRV and λ-exonuclease to a cuvette containing YOYO-labeled λ-DNA in λ-exonuclease reaction buffer at 37 °C. The progress of the exonuclease reaction was monitored using fluorescence spectroscopy as described in the previous section. A calibration curve was constructed plotting the initial rates of λ-exonuclease reaction as a function of the concentration of the restriction fragments. The resulting curve was used to estimate the amount of fragments generated during the initial stages of the simultaneous reaction.

[2] *Abbreviations used:* ssDNA, single-stranded DNA; ET-SSB, extreme thermostable single-stranded DNA-binding;RecA, recombination protein A.