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A homogeneous, high-throughput fluorescence resonance energy transfer-based DNA polymerase assay

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Abstract

A homogeneous, fluorescence resonance energy transfer (FRET)-based DNA polymerase assay that is suitable for high-throughput screening for inhibitors, and can also be used for steady-state kinetic investigations, is described. The activity, kinetic mechanism, and processivity of the isolated α subunit of DNA polymerase III, the product of the *dnaE* gene, from the gram-negative pathogen *Haemophilus influenzae* were investigated using the FRET assay.

Keywords: DNA polymerase; Fluorescence resonance energy transfer; FRET; DnaE; Pol III; High-throughput

DNA polymerases are required for DNA replication and repair in bacterial and eukaryotic cells. The catalytic α subunit of the replicative DNA polymerase III (pol III),[1] for example, is an essential enzyme in bacteria [1–3] and, therefore, is suitable as an antimicrobial drug target. High-throughput screening of large libraries of drug-like molecules is frequently used in the pharmaceutical industry to identify target enzyme inhibitors as lead compounds for drug discovery. Traditional methods for measuring activity of DNA polymerases in vitro involve incorporation of radiolabeled deoxyribonucleoside triphosphate (dNTPs) into DNA and separation of the radioactive DNA product from the unused substrate. Such methods are not optimal for automated high-throughput screening for inhibitors due to the need for a separation step and the use of radioactivity.

Other methods suitable for high-throughput screening for DNA polymerase inhibitors have been described in the literature. Earnshaw and Pope [4] described a scintillation proximity assay in which radiolabeled nucleotides were added by the enzyme to a biotinylated DNA substrate that was immobilized on a solid scintillant. Seville and coworkers [5] described an assay in which DNA polymerase activity was measured by the fluorescence intensity of a dye, the quantum yield of which increased when bound to double-stranded DNA.

An assay for DNA polymerase activity described by Griep [6] used the recovery of the intrinsic ultraviolet fluorescence of single-stranded DNA binding protein on displacement from the newly synthesized double-stranded DNA. This method, although advantageous by virtue of providing a continuous signal, would not be suitable for high-throughput screening due to interference from UV absorbance and fluorescence of the test compounds.

This article describes a novel, homogeneous fluorescence resonance energy transfer (FRET) assay for DNA polymerase activity that measures template-directed addition of natural dNTPs to the 3′-OH of a synthetic DNA primer.

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[1] *Abbreviations used:* pol III, polymerase III; dNTP, deoxyribonucleoside triphosphate; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; LB, Luria–Bertani; IPTG, isopropyl-β-D-thiogalactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LC–MS, liquid chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine; GC, guanine–cytosine; CGE, capillary gel electrophoresis.

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The FRET assay has several advantages. It is fully compatible with automated high-throughput screening and is also useful for steady-state kinetic analysis and mechanistic studies. It requires no radioactivity or scintillant. By measuring the ratio of fluorescence intensities at two wavelengths, it achieves high measurement precision. Because the DNA substrate is covalently labeled with high-quantum yield fluorescent dyes, measurements can readily be made at low-nanomolar DNA concentrations and the samples from the assay can be reused for other analyses, such as capillary gel electrophoresis, to investigate the products. The activity, kinetics, and processivity of the isolated α subunit of DNA pol III, the product of the *dnaE* gene, from the gram-negative pathogen *Haemophilus influenzae* were investigated using the FRET assay.

Materials and methods

Reagents

Highly homogeneous, fluorescently labeled DNA oligonucleotides were synthesized and purified by TriLink Biotechnologies. The primer and template were mixed together at 100 μM in 50 mM MOPS–HCl (pH 7.5) and 100 mM KCl and then annealed by heating briefly at 100 °C and cooling slowly to room temperature. dNTPs were purchased from Sigma. DnaE proteins were cloned, expressed, and purified in-house as described below.

Overexpression and purification of H. influenzae DnaE

The *dnaE* gene was cloned from chromosomal DNA of wild-type *H. influenzae* strain ATCC 51907 using the polymerase chain reaction (PCR). Amplification was performed using High Fidelity PCR Master (Roche Applied Science) and the following primers: 5′-GGATTTCATATGTCAT CCCAACCTCGCTTCATCC-3′ and 5′-CGAATTCGT CTTATTCAAACTCTAATTCCACC-3′ (*Nde*I and *Eco*RI sites underlined). The PCR product was purified using the QuickStep 2 PCR Purification Kit (Edge Biosystems) and was digested with *Nde*I and *Eco*RI. The resulting fragment was then purified and ligated into *Nde*I- and *Eco*RI-digested expression vector pET30a (Novagen), producing the plasmid pET30a *dnaE*. The DNA sequence of the cloned *dnaE* was confirmed by sequencing on an ABI PRISM 3100 DNA sequencer (Applied Biosystems) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). For protein overproduction, the plasmid was transformed into *Escherichia coli* BL21(DE3) (Novagen) and plated on Luria–Bertani (LB) containing 25 μg/ml of kanamycin at 37 °C overnight. A single colony of BL21(DE3)/pET30a–*dnaE* was inoculated into a 70-ml culture of Terrific Broth containing 25 μg/ml of kanamycin and grown overnight at 25 °C. Samples (6×10 ml) of the overnight culture were added to 6×1 L of Terrific Broth containing 25 μg/ml of kanamycin and grown at 25 °C with aeration to mid-logarithmic phase ($OD_{600} = 0.5$). Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM. After 3 h of induction, the cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C. Cell paste was stored at −20 °C. Protein expression and solubility were checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of H. influenzae DnaE

Frozen cell paste from *E. coli* cells expressing *H. influenzae* DnaE was suspended in 50 ml of lysis buffer (50 mM Tris–HCl [pH 7.5], 1 mM ethylenediamine tetraacetic acid [EDTA], 5 mM dithiothreitol, 10% [v/v] glycerol, 1 mM phenylmethylsulfonyl fluoride, and one protease inhibitor cocktail tablet) (Roche Molecular Biochemical). Cells were disrupted by being passed twice through a French press operated at 18,000 psi. The crude extract was centrifuged at 20,000 rpm in a Beckman 45Ti rotor for 30 min at 4 °C. The supernatant was loaded at a flow rate of 1.5 ml/min onto a 20-ml Q Sepharose HP (HR 16/10) column (Pharmacia) preequilibrated with buffer A (50 mM Tris–HCl [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol, and 10% [v/v] glycerol). The column was washed with buffer A, and the protein was eluted by a linear gradient from 0 to 1 M NaCl in buffer A. Fractions containing DnaE were pooled, and 3 M $(NH_4)_2SO_4$ in buffer A was added to a final concentration of 0.8 M. The sample was applied at a flow rate of 1.0 ml/min to an 8-ml phenyl-Sepharose HP (HR 16/10) column (Pharmacia) preequilibrated with buffer B [buffer A containing 1 M $(NH_4)_2SO_4$]. The column was washed with buffer B, and the protein was eluted by a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer A. Fractions containing DnaE were pooled (37.5 ml) and diluted with 100 ml of buffer A. The diluted sample was loaded at a flow rate of 1.0 ml/min to an 8-ml Heparin Sepharose CL-6B (HR 16/10) column (Pharmacia) preequilibrated with buffer A. The column was washed with buffer A, and the protein was eluted by a gradient from 0 to 1 M NaCl in buffer A. All chromatography steps were performed at 4 °C. Fractions containing DnaE were pooled. Solid $(NH_4)_2SO_4$ (0.4 g/ml) was added to precipitate all of the proteins and was mixed on ice for 1 h. The sample was centrifuged at 10,000 rpm for 30 min at 4 °C in a Beckman JA12 rotor, and the pellet was dissolved in 5 ml of buffer A. The 5-ml sample was applied at a flow rate of 1.0 ml/min to a 320-ml Sephacryl S-300 (HR 26/60) column (Pharmacia) preequilibrated with buffer C (50 mM Tris–HCl [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol, 10% [v/v] glycerol, and 150 mM NaCl). The fractions containing DnaE were pooled and dialyzed against 1 L of storage buffer (50 mM Tris–HCl [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol, and 20% [v/v] glycerol). The protein was characterized by SDS–PAGE analysis and analytical liquid chromatography–mass spectrometry (LC–MS). The determined mass of the protein indicated that the N-terminal methionine of the DnaE predicted from the DNA sequence was not present (expected mass = 129,658 Da, observed