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Biolayer Interferometry: A Novel Method to Elucidate Protein–Protein and Protein–DNA Interactions in the Mitochondrial DNA Replisome

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Abstract

A lack of effective treatment for mitochondrial diseases prompts scientists to investigate the molecular processes that underlie their development. The major cause of mitochondrial diseases is dysfunction of the sole mitochondrial DNA polymerase, DNA polymerase γ (Pol γ). The development of treatment strategies will require a detailed characterization of the molecular properties of Pol γ . A novel technique, biolayer interferometry, allows one to monitor molecular interactions in real time, thus providing an insight into the kinetics of the process. Here, we present an application of the biolayer interferometry technique to characterize the fundamental reactions that Pol γ undergoes during the initiation phase of mitochondrial DNA replication: holoenzyme formation and binding to the primer-template.

Keywords

Biolayer interferometry; Protein–protein interactions; Mitochondria; Mitochondrial replisome

1 Introduction

Mitochondria are essential organelles that play a key role in fundamental cellular processes, such as oxidative phosphorylation (OXPHOS), calcium signaling, and apoptosis. Human mitochondria contain a semi-independent genome that encodes only 13 polypeptides, all of which are crucial components of the OXPHOS machinery [1]. Thus, defects in the mitochondrial genome often result in a shortage of ATP, which affects tissues with high energy requirements (such as the nervous system or muscle tissue) and can lead to the development of so-called mitochondrial diseases [2]. There is no known cure for any of the diseases resulting from mtDNA dysfunction and only symptom treatment strategies are currently available [3–7]. The majority of human mitochondrial disorders originate from dysfunction of the sole mitochondrial DNA polymerase, DNA polymerase γ (Pol γ). To date, nearly 250 disease-causing mutations have been identified within the *POLG* gene, encoding the catalytic subunit of Pol γ , Pol $\gamma\alpha$ (<http://tools.niehs.nih.gov/polg/>). On the molecular level, Pol γ dysfunction causes mtDNA depletion and multiple mtDNA deletions. The mechanisms of pathogenesis of the POLG syndromes are largely unknown and can result from various Pol γ -related issues, such as defects in assembly of the Pol γ holoenzyme, problems in recognition of the primer-template, decreased processivity, etc.

Each of these functions is based on specific molecular interactions between all the factors contributing to Pol γ function. Understanding these interactions is thus fundamental to elucidate the molecular basis of disease, and to develop treatments [1].

Pol $\gamma\alpha$ is an ~140 kDa subunit that catalyzes the nucleotide polymerization reaction. It has a weak intrinsic single-stranded DNA (ssDNA) binding ability, but it is able to recognize and bind primer-template and synthesize ~200 nt of nascent strand per turnover [8]. It has also been suggested that Pol $\gamma\alpha$ plays an independent role in mtDNA repair processes [9]. In the replication process, Pol $\gamma\alpha$ binds to its homodimeric accessory subunit, Pol $\gamma\beta_2$ (55 kDa/promoter), to form the Pol γ holoenzyme [10]. Pol $\gamma\beta$ subunit enhances the ssDNA-binding affinity of Pol $\gamma\alpha$ by approximately 100-fold, and increases its processivity by 50–100-fold [11, 12]. Pol $\gamma\beta$ also has the ability to bind dsDNA on its own [13], though this appears to be dispensable for its function as a component of Pol γ ; as a part of the holoenzyme, the Pol $\gamma\beta$ dimer does not contact the DNA template directly [14]. The low abundance of both of these proteins *in vivo* limits studies of the early steps of mtDNA replication. Thus, little is known about the mechanisms leading to initiation of the replication process.

In this chapter, we describe the use of biolayer interferometry to study two crucial stages of the initiation process: the formation of Pol γ holoenzyme and replication site recognition. Biolayer interferometry is a technique based on the optical phenomenon of wave interference. It utilizes a novel type of biosensor in the form of a tip with two specific layers at its end. The first external layer, called the biolayer, is coated with molecules of interest and the second layer is an internal reference optical layer. During the measurement, white light is streamed constantly along the biosensor fiber and is reflected from the reference optical layer, as well as from the biolayer. Superposition of waves reflected from both layers forms the interference pattern. Binding of a ligand to molecules on the biolayer changes its optical properties and thus alternates the phase of the reflected light. This effect causes a wavelength shift in the interference pattern, which correlates directly with the thickness of the biosensor tip. The interference pattern is measured constantly, allowing one to monitor changes of the biosensor thickness (nm) in real time [15, 16]. In practice, executing an experiment is limited to the preparation of solutions with and without ligands of interest, in a 96- or 384-well black plate. The Octet device places biosensors into solutions as programmed and displays the results of the analysis in real time, enabling the determination of binding kinetics and affinity. The biosensors are available with diverse modifications of the biolayer, e.g., streptavidin, Ni-NTA, anti-GST antibody, protein A, etc. (<http://www.fortebio.com/>). This relatively novel technique has already been applied successfully in studies of protein–protein interactions, such as interactions between protein components of the iron-sulfur cluster assembly pathway, or protein–DNA interactions, such as binding of the XPD helicase to ssDNA [17, 18].

Interaction of the Pol $\gamma\alpha$ and Pol $\gamma\beta_2$ subunits is fundamental to the formation of Pol γ holoenzyme, and has been studied in the past [11, 19]. To evaluate biolayer interferometry for studies of the mtDNA replisome, we load His-tagged Pol $\gamma\alpha$ onto Ni-NTA-coated biosensors, move the sensors to a reaction buffer containing bovine serum albumin (BSA), which limits non-specific interactions with any unbound sites. BSA is also thought to inhibit weak protein–protein interactions, and is thus considered useful to block non-specific

binding between proteins. The response reached with the blocking step serves as a baseline for subsequent steps. Next, sensors are placed into solutions of various Pol $\gamma\beta$ concentrations to evaluate the association step, and subsequently placed back into the blocking/baseline solution for the determination of dissociation properties (Fig. 1). The data collected are processed and analyzed with the Octet Data Analysis Software.

Earlier studies of Pol γ holoenzyme formation reported that the subunits interact with apparent dissociation constant (K_D) of 35 nM (± 16 nM) [19]. This value was estimated as a function of Pol $\gamma\beta$ concentration and its measured biochemical activity of stimulating maximal DNA binding and nucleotide polymerization by Pol $\gamma\alpha$. For comparison, we utilized a similar approach and used a steady-state analysis for K_D estimation, measuring the association response that yielded a value of 20 nM (± 1.5 nM). The interaction of the subunits was also analyzed with surface plasmon resonance (SPR) in an earlier study [11]. On a basis of kinetic analysis of association and dissociation rate constants, the authors estimated the K_D value to be 27 nM, which is similar to the value we obtain with the steady-state analysis by biolayer interferometry. Because the real-time measurements by biolayer interferometry enable kinetic analysis as well, we fit our data to 1:1 model and obtained association ($k_{on} = 1.03 \times 10^5$ 1/M $\times$ s) and dissociation rates ($k_{off} = 3.02 \times 10^{-4}$ 1/s), with a resulting K_D value of 2.95 nM (± 0.03 nM). Although the binding affinity determined by the two different fitting methods vary substantially, they both indicate a tight interaction between the subunits in good agreement with earlier studies. Notably, the data presented here suggest that once assembled, the Pol γ subunits dissociate at a very limited rate. The stability of the Pol γ holoenzyme is further supported by previous studies utilizing crystallography and other biophysical techniques [10, 20]. In comparison to the previous SPR studies [11], in the experiments presented here Pol $\gamma\beta$ associates faster with Pol $\gamma\alpha$ to reach the equilibrium state, and dissociates significantly slower. The main difference between our approach and those in the previous studies lies in the preparation and regeneration of the Pol $\gamma\alpha$ -coated sensors (*see* Note 1). In the SPR experiments the authors used an amino-coupling approach and regeneration with 3 mM NaOH, which might impact the native conformation of the enzyme. The use of Ni-NTA sensors allow us to prepare Pol $\gamma\alpha$ -coated sensors by a gentle immobilization strategy, and the low cost of sensors makes it possible to eliminate a regeneration step.

The Octet biosensor also makes it possible to study protein–DNA interactions. To study the interaction of Pol γ with a primer-template DNA, we used a 5'-biotin-labeled 40-mer oligonucleotide annealed to an 80-mer oligonucleotide, forming a template of 40 nt dsDNA with a free 3'-OH end followed by 40 nts stretch of ssDNA. Streptavidin biosensors were loaded with the primer-template DNA and blocked with BSA. The affinity of the Pol γ holoenzyme to the primer-template DNA was measured by placing the sensors into solution of various Pol γ holoenzyme concentrations. After the association step the sensors are placed back to the baseline solution for the determination of dissociation (Fig. 2), and the data collected are processed and analyzed with the Octet Data Analysis Software.

¹The Pol γ holoenzyme formation experiment was performed under conditions similar to those used previously for SPR studies [11].

Previously published K_D values for the Pol γ -primer-template DNA *exo*- interaction vary significantly depending on the method and conditions used (*see* Note 2). Kinetic analysis using SPR yielded a value of 0.06 nM, whereas results of gel mobility shift assay and analysis of the stimulation of DNA binding of Pol $\gamma\alpha$ by Pol $\gamma\beta$ yielded a value of ~10 nM [11, 12, 19]. The results we obtained with biolayer interferometry analyzed as a function of concentration and association response yielded a K_D of 16 nM (± 6.5 nM). In the kinetic analysis we determined association and dissociation rates ($k_{on} = 1.06 \times 10^5$ 1/M $\times$ s, $k_{off} = 3.99 \times 10^{-4}$ 1/s), fitting the results to 1:1 binding model, and obtained a K_D value of 3.76 nM (± 0.06 nM). Dissociation constant values obtained with both approaches are thus similar to those published previously, except for those determined by SPR. The difference obtained could be linked to the immobilization strategy. In our approach, streptavidin sensor places the DNA strands sparsely and the layer thickness is low (according to manufacturer the biosensor surface may be considered almost flat). We also noticed that the Pol γ -DNA binding data show some deviation from a 1:1 binding model, which could indicate more a complex binding mechanism. Therefore, further studies are needed to understand all the details of the interaction.

In summary, these examples demonstrate that biolayer interferometry provides an effective tool for studies of the mitochondrial replisome. Future studies could be developed to examine how mutations in either of the Pol γ subunits affect holoenzyme formation or the primer-template binding properties of the enzyme, or to test small molecules that may stimulate or inhibit mtDNA replication.

2 Materials

The biolayer interferometry analyses were performed with the ForteBio Octet RED 384 system. The data collected were processed and analyzed using the Octet Data Analysis Software.

2.1 Pol γ Holoenzyme Formation

1. Pol $\gamma\alpha$ -his₆ *exo*-, 0.04 μ g/ μ L (*see* Note 1).
2. Pol $\gamma\beta$, 0.05 μ g/ μ L.
3. Bovine serum albumin (BSA).
4. 1 M Tris-HCl, pH 7.5.
5. 2 M Potassium chloride (KCl).
6. 1 M Magnesium chloride (MgCl₂).
7. Tween 20.
8. Glycerol.
9. Ni-NTA (NTA) biosensors.

²The Pol γ -primer-template DNA interaction experiment was performed under conditions similar those used for the Pol γ activity assay [12].

10. 384-Well, tilted-bottom black plate (*see* Note 3).
11. Pol $\gamma\alpha$ buffer: 30 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 20 % glycerol, 5 mM β -Mercaptoethanol.
12. Reaction buffer 1: 50 mM Tris-HCl, pH 7.5, 1 $\mu\text{g}/\mu\text{L}$ BSA, 4 mM MgCl_2 , 0.02 % Tween 20 (*see* Note 1).

2.2 Binding of Pol γ to Primer-Template DNA

1. Pol $\gamma\alpha$ -his₆, 0.08 $\mu\text{g}/\mu\text{L}$ (*see* Note 2).
2. Pol $\gamma\beta$, 0.05 $\mu\text{g}/\mu\text{L}$.
3. 1 μM solution of 5'-biotin-labeled 40 bases oligonucleotide: 5'-ATT ATG AAT TAA TTT AAT AAT TTT TTT TTT TTT TTT TTT T-3'.
4. 1.2 μM solution of 80-mer oligonucleotide: 5'-GGG GGG GGG GGG GGG GGG GGC TGA CTG GGC GGC TCA CTG GAA AAA AAA AAA AAA AAA AAA TTA TTA AAT TAA TTC ATA AT-3'.
5. Streptavidin (SA) biosensors.
6. 384-Well, tilted-bottom black plate (*see* Note 3).
7. Phosphate-buffered saline (PBS): 135 mM NaCl, 10 mM Na_2HPO_4 , 2 mM KCl, 2 mM KH_2PO_4 .
8. Pol $\gamma\alpha$ buffer: 30 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 20 % glycerol, 5 mM β -Mercaptoethanol.
9. Reaction Buffer 2: 50 mM Tris-HCl, pH 8.0, 0.4 $\mu\text{g}/\mu\text{L}$ BSA, 10 mM DTT, 50 mM KCl 4 mM MgCl_2 (*see* Note 2).

3 Methods

3.1 Pol γ Holoenzyme Formation

1. Place NTA sensors in a tray and soak them in Pol $\gamma\alpha$ buffer for 30 min.
2. Prepare a master solution of 10 $\mu\text{g}/\text{mL}$ Pol $\gamma\alpha$ -his₆ exo in Pol $\gamma\alpha$ buffer (*see* Note 3).
3. Prepare Pol $\gamma\beta$ solutions of 9, 18, 55, 91, 136, and 182 nM in reaction buffer 1. Also include a control without Pol $\gamma\beta$.
4. Pipette solutions for each step in individual wells of a 384-well, tilted-bottom black plate. Measure the following steps:
 - Baseline—40 μL Pol $\gamma\alpha$ buffer.
 - Loading—40 μL Pol $\gamma\alpha$ solution.
 - Baseline 2 and dissociation—40 μL reaction buffer 1.

³The 96-well black plate could be used as well, but in that case every solution should be scaled up to 200 μL . The 384-tilted well plate may improve the quality of the baseline and reduce the deviation in the baseline level between the wells.

Association—40 μ L Pol $\gamma\beta$ solutions of various concentrations.

5. Set the kinetic experiment parameters to 1000 rpm for each step and set duration of steps:

Baseline—60 s.

Loading—300 s.

Baseline 2—300 s.

Association—600 s.

Dissociation—600 s.

6. Start the measurement.

7. Process the data as follows:

Set data from no-Pol $\gamma\beta$ control well as reference, and subtract it from the remaining wells.

Align Y axis to the last 5 s of baseline 2.

Set the inter-step correlation to dissociation.

Apply the Savitzky–Golay transformation.

8. Analyze the data by applying the following settings:

Set both association and dissociation to be analyzed.

1:1 Binding model.

Apply global fitting with $R_{\max}$ Unlinked.

3.2 Binding of Pol γ to Primer-Template DNA

1. Prepare template master solution by annealing 40-mer and 80-mer oligos. Mix equal volumes of the oligomer solutions and incubate for 30 min at 90 °C. After incubation allow the sample to cool down to room temperature.
2. Prepare 25 nM primer-template solution in PBS (1 pmol/40 μ L).
3. Place SA sensors in a tray and soak them in PBS for 30 min.
4. Prepare 60 nM Pol γ solution mixing Pol $\gamma\alpha$ and Pol $\gamma\beta_2$ solutions in a 1–1.5 molar ratio, respectively, and then prepare dilutions in reaction buffer 2 of 5, 10, 20, and 40 nM. Include a control without Pol γ .
5. Pipette solutions for each step into individual wells of a 384-well, tilted-bottom black plate:

Baseline—40 μ L PBS.

Loading—40 μ L of primer-template solution.

Baseline 2 and dissociation—40 μ L reaction buffer 2.

Association—40 μ L of individual Pol γ solutions.

6. Set the kinetic experiment parameters by setting the shaking rate at 1000 rpm and the duration of each step as follows:
 - Baseline—60 s.
 - Loading—300 s.
 - Baseline 2—300 s.
 - Association—600 s.
 - Dissociation—600 s.
7. Start the measurement.
8. Process and analyze the data as in the previous section using the no-Pol γ control as a reference.

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Fig. 1.

Biolayer interferometry analysis of the Pol γ α - β interaction. Ni-NTA sensors were loaded with Pol $\gamma\alpha$ -his₆exo⁻ (10 μ g/mL) and blocked with BSA. During the association step (from 0 to 600 s), the sensors were placed into Pol $\gamma\beta$ solutions of various concentrations: 9, 18, 55, 91, 136, and 182 nM. Subsequently, the sensors were moved to the buffer without Pol $\gamma\alpha$ or β for dissociation analysis (from 600 to 1200 s). The data were processed and analyzed with the Octet Data Analysis Software. A control without Pol $\gamma\beta$ was subtracted from remainder of the data collected. The *right panel* represents steady-state analysis of the response (nm) to Pol $\gamma\beta$ concentrations (nM)

Fig. 2.

Biolayer interferometry analysis of the Pol γ -primer-template DNA interaction. Streptavidin biosensors were presoaked and loaded with 25 nM 5'-biotinylated 40/80-mer primer-template DNA. After blocking, sensors were placed into Pol γ solutions of 5, 10, 20, 40, and 60 nM, prepared as a mixture of α to β_2 subunits in a 1–1.5 molar ratio (association—0–600 s). The dissociation rate was determined after the association step by placing the sensors back into the buffer without Pol $\gamma\alpha$ or β (600–1200 s). The data obtained were processed and analyzed with the Octet Data Analysis Software. A control without Pol γ subunits was subtracted from the remaining set of raw data. The *right panel* represents steady-state analysis of the response (nm) to Pol $\gamma\beta$ concentrations (nM)