Chapter 17

Fluorescent SSB as a Reagentless Biosensor for Single-Stranded DNA

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Abstract

Helicases are an important and much studied group of enzymes that generally couple ATP hydrolysis to the separation of strands of base-paired nucleic acids. Studying their biochemistry at different levels of organization requires assays that measure the progress of the reaction in different ways. One such method makes use of the single-stranded DNA-binding protein (SSB) from *Escherichia coli*. This is used as a protein framework to produce a "reagentless biosensor," making use of its tight and specific binding of single-stranded DNA. The attachment of a fluorophore to this protein produces a signal in response to that binding. Thus the (G26C)SSB, labeled with a diethylaminocoumarin, gives a ~5-fold fluorescence increase on binding to single-stranded DNA and this can be used to assay the progress of helicase action along double-stranded DNA. A protocol for this is described along with a variant that can be used to follow the unwinding on a single molecule scale.

Key words: Reagentless biosensor, Helicase, Fluorescence, Kinetics, TIRFM

1. Introduction

Helicases are energy-transducing enzymes that are involved in many types of DNA or RNA manipulation (1, 2). There is a wide variety of proteins within this family, for example involved in nucleic acid packaging, replication, recombination, and repair. Often the helicase is part of a larger protein complex that performs several parts of such manipulations simultaneously. Because of this wide variety and the importance of these processes for the cell, there needs to be a range of different types of assay to study them, both to understand the mechanisms by which they operate and to examine the control of nucleic acid manipulations. The example, described here, is a reagentless biosensor for single-stranded DNA (ssDNA) that can measure the rate and extent of helicase activity on double-stranded DNA (dsDNA) in real time (3).

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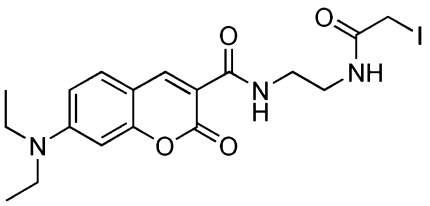

Fig. 1. Structure of the fluorescent label, IDCC.

Fluorescent reagentless biosensors form a class of probe molecules that provide a signal to give concentrations of a specific target analyte molecule. In particular, there is a class of such biosensors, based on a protein framework that is labeled with a fluorophore. Examples of fluorophore-protein adducts acting as such biosensors include ones for phosphate biosensors (4, 5), ADP (6), and certain other small molecules, such as sugars, amino acids, and metals ions (7–11).

The protein's ligand-binding site provides potentially a high degree of specificity, which may be important in applying the biosensor to assay specific molecules, particularly in the presence of similar ones. Such processes as conformation changes, resulting from ligand binding, can provide a coupling mechanism between interaction of ligand and the fluorophore response. This requires appropriate positioning of the fluorophore on the protein surface, for example, to be able to respond to, but not interfere with, ligand binding. It also requires the fluorophore to have the property of changing its fluorescence when its environment changes. Thus some fluorophores that are inherently bright, with a high fluorescence quantum yield under most conditions (for example, Cy3B), may not be suitable.

In the work described here, a diethylaminocoumarin (Fig. 1) is a suitable fluorophore with a fluorescence that can increase by more than an order of magnitude in response to changes around it (12, 13). It also has the practical advantage that its excitation maximum wavelength closely coincides with a strong Hg emission line at 436 nm, so that in assay formats such as stopped flow, the fluorescence of the coumarin is enhanced relative to background. Indeed, the mechanism in this case, as has been shown in other uses, involves the formation of a coplanar system of the rings and the dialkylamino moiety (14, 15). In this state there is high fluorescence quantum yield. If the diethylamino group is not so constrained, the fluorescence is lower. Understanding such mechanisms, as well as having defined protein structures, helps in developing reagentless biosensors for particular targets.

Fluorescence has been found to be a useful signal for bulk solution measurements because of its rapid response and high sensitivity. A protein framework provides advantages of a specific binding site, although the ability to manipulate the precise sequence

potentially allows alteration of that specificity. Often ligand binding and associated conformation changes are rapid, at least allowing responses on the timescale of likely measurements, for example, using stopped-flow fluorescence with reaction times of a few milliseconds. An additional advantage of reagentless biosensors is that they usually target the product of the enzyme reaction being studied and this minimizes interference as the reaction itself is not compromised. This is not the case if substrates or the enzyme itself are modified, for example by a fluorophore. In those cases the modification can affect the properties of the enzyme system, such as rate or affinity. For example, fluorescent ATP analogues are widely used to study ATPases and kinases, but the properties of the analogues may differ greatly from unmodified ATP or ADP (16–18).

1.1. ssDNA Biosensor Properties

The protein framework is the single-stranded DNA-binding protein (SSB) from *Escherichia coli*. This is a ~20 kDa protein that acts as a tetramer, normally binds 65–70 bases of ssDNA at physiological ionic strength, with the DNA wrapping around the tetramer in a surface channel, as revealed by X-ray crystallography (19–21). This binding is rapid (322 μM^{-1} s^{-1} for the labeled protein) at 20 °C and tight (dissociation constant ~3 nM) (3, 22). At concentrations well above this dissociation constant the SSB binds to all ssDNA. At low ionic strength or high concentration of SSB, the binding mode changes to ~35 bases per tetramer. This change in binding can be significant in planning measurements using SSB and is discussed further, below.

After surveying several single cysteine mutations as sites for attaching one fluorophore per monomer, and several different types of fluorescent labels, the best combination of G26C and IDCC (*N*-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide) (23) (Fig. 1) was chosen so the adduct DCC-SSB acts as the ssDNA biosensor. It gives ~5-fold fluorescence increase on binding to ssDNA with little or no shift in the excitation or emission spectra, fairly normal for diethylaminocoumarin. SSB has no significant conformation change apparent on DNA binding (19, 24) and so the basis of the fluorescence change is likely to be the alteration in coumarin environment by the presence of DNA itself. The labeling does, indeed, cause ~30-fold decrease in affinity of the SSB for ssDNA, although the binding is still tight enough to be quantitative in most conditions (22). The preparation of DCC-SSB is described below.

1.2. Considerations in Using DCC-SSB

The particular focus of this chapter is the use of DCC-SSB to assay dsDNA unwinding by helicases in real-time, continuous assays (25). In such assays, DCC-SSB needs to be present in sufficient concentration to bind ssDNA rapidly as it is formed by the helicase and allow fluorescence increase to be followed with time. Such assays might be considered as being of two types. One is a

multi-turnover, steady-state assay with the helicase concentration much lower than DNA. The unwinding can be followed over several minutes. More incisively, a single-turnover assay has all DNA unwound at the same time by the helicase present in relatively high concentration. This type of assay tends to be richer in information and will be described experimentally below. However, the assay timescale depends on the length of DNA unwound and the speed of unwinding. The former depends on processivity, which varies between helicases from a few base pairs to many thousands. The translocation rates of helicases also vary widely from $<1\ s^{-1}$ to $>1{,}000\ s^{-1}$. This means that the assay may be on the millisecond to second timescale and stopped-flow fluorescence may be the format of choice.

This section considers the factors that are important in developing a successful assay using DCC-SSB. It also outlines some of the pitfalls of using fluorescent reagentless biosensors, in general, and this fluorescent probe, in particular. Many of these considerations can be accommodated practically by performing an initial titration of DNA into a solution of SSB under the assay conditions and this will be described.

Firstly, there are several factors that will determine what concentration of DCC-SSB should be present. At any likely set of conditions, the probe binds to all of the DNA, so that an excess of DCC-SSB is required over the number of binding sites in terms of 65 bases per site. Typically, a 4- to 5-fold excess seems a suitable compromise between high background and other factors, described below. In addition, as for all coupled assays, the rate of fluorescence response should be considered so that it is significantly larger than the rate of the helicase reaction to ensure that the observed rate is not limited by the SSB but is purely the rate of the helicase reaction. One way to consider this is that the time it takes the helicase to move 65 bases reveals one complete binding site for the SSB. The rate of binding at the particular concentration of DCC-SSB, as described (22), should give a time constant at least fivefold more rapid. Given the rapid binding, this is unlikely to be a problem for most assays. Finally, inner filter affects may only start to be significant if the absorbance of the coumarin is >0.1 for the pathlength of the assay cell. In that case, absorbance of the exciting light in the near side of the cell may reduce the emission from the far side. This would require DCC-SSB tetramers at >500 nM for 1 cm pathlength. However, because the absorbance of the coumarin is unaltered by DNA binding, in practice at least several fold higher DCC-SSB can be used, as long as nothing else absorbs at the same wavelength.

Low salt concentration, high ratio of SSB to DNA and high concentration of SSB, favor 35-base binding and potentially extra SSB will bind onto the DNA during the time course of the assay. This “extra” binding is relatively slow (22) and is accompanied by a

fluorescence decrease, but can be avoided or at least minimized for most likely conditions by keeping the total concentration of DCC-SSB below 500 nM. An example of this phenomenon has been described (26): in that case the extra binding did not affect the observed time course during unwinding.

As with any fluorescence assay, the signal may vary with conditions such as salt concentrations and temperature, so that a calibration, if required, needs to be at the same conditions that will be used in the assay. A titration, measuring fluorescence at various levels of protein saturation by DNA is shown in Fig. 3. Figure 3a is a titration using a standard spectrofluorimeter, while Fig. 3b shows a titration done under the same conditions as the helicase activity assay, shown in Fig. 4. This assay shows the unwinding of two different lengths of plasmid by the combination of initiator protein RepD and helicase PcrA. The assay allows the unwinding rate to be measured as a function of DNA length (25).

1.3. Use of Cy3B-SSB as Probe in a TIRFM Assay

Although the main focus of this chapter is the use of the ssDNA biosensor in bulk kinetic assays, a version of the probe has also been used to measure unwinding by a single helicase in a surface-attached assay using Total Internal Reflectance Fluorescence Microscopy (TIRFM) (27). Although diethylaminocoumarin has significantly favorable properties for bulk assays, that is not so for single molecule visualization. It is not a particularly bright fluorophore, as it has a low extinction coefficient (~45,000 M^{-1} cm^{-1}) and is easily photobleached under the high-intensity light conditions of a single molecule assay. So far, there is not a labeled SSB that successfully combines the necessary fluorescence characteristics for single molecule sensitivity and a large fluorescence change. However, the surface attachment of the helicase·DNA complex, combined with the high affinity of SSB for ssDNA, means that a fluorophore with a high quantum yield, namely Cy3B, can be used as a label for SSB even though it gives rise to only a small increase in fluorescence on binding ssDNA. The evanescent wave excitation of TIRFM means that the unwinding is observed as a single fluorescent spot for each complex that grows in intensity as Cy3B-SSB is recruited to the complex in response to release of the ssDNA during unwinding. However, the bulk solution, containing unbound and, therefore, mobile Cy3B-SSB, does not greatly contribute to background fluorescence. An advantage of this single molecule technique is that many events can be monitored as separate spots in a single field of view. It also means that there can be a more-or-less direct comparison of single molecule and bulk solution kinetic measurements of unwinding.

Helicase assays using this format have been described for three helicases, AddAB, RecBCD, and PcrA (27) with either DNA or protein attachment to the surface. These demonstrate the assay at very different unwinding rates depending on the particular helicase,

but give similar average rates of unwinding to assays under the same solution conditions in bulk solution using DCC-SSB. The methodology of this technique and experimental aspects have been described in a previous Methods in Molecular Biology (28).

2. Materials

Prepare all solutions with double distilled water and analytical grade reagents.

2.1. SSB Protein Preparation

1. Glycerol stock of BL21(DE3) *E. coli*, transformed with pET22b vector, containing G26C SSB (3).
2. L. agar/ampicillin plates.
3. L. broth.
4. 10% ampicillin.
5. IPTG.
6. 1 M dithiothreitol (DTT).
7. 1 M Tris–HCl, pH 7.5.
8. 1 M Tris–HCl, pH 8.3.
9. 5 M NaCl.
10. 0.5 M EDTA.
11. Sucrose.
12. Glycerol.
13. 12% polyacrylamide gels.
14. Complete, EDTA-free protease inhibitor cocktail tablet (Roche).
15. 10% polyethyleneimine, pH 6.9.
16. Glass homogenizer.
17. $(NH_4)_2SO_4$ solid (biochemistry or enzyme grade).
18. 0.2 μm polyethersulfone syringe membrane filter.
19. 5 mL HiTrap Heparin column (GE Healthcare).
20. SnakeSkin pleated dialysis tubing (Thermo Scientific).
21. Centrifuge for 15 mL to 1 L volumes.
22. Preparative ultracentrifuge for ~70 mL volumes.
23. Chromatography fraction collector, equipped with absorbance detection and pump.

2.2. Labeling

1. 1 M DTT.
2. 1 M Tris–HCl, pH 7.5.

3. 5 M NaCl.
4. 0.5 M EDTA.
5. Glycerol.
6. PD10 desalting gel filtration column, 5 ml (GE Healthcare).
7. N-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboamide (Invitrogen, U.S.A. or Synchem, Germany). Stored as a 20 mM solution in dry dimethylformamide at −80 °C, protected from light.
8. 100 mM sodium 2-mercaptoethane-sulfate (MESNA).
9. P4 gel filtration column (Biorad; 1 × 30 cm).
10. 0.2 μm polyethersulfone syringe filter membrane.
11. Amicon Ultracentrifugal filter.

2.3. Characterization

1. Spectrophotometer.
2. Fluorescence spectrophotometer.
3. Oligonucleotide dT_{70}.

2.4. Helicase Assay

1. Stopped-flow fluorimeter (HiTech, TgK Ltd), equipped with Xe-Hg lamp.
2. Plasmid, containing *ori*D double-stranded origin of replication (29).
3. PcrA helicase and RepD initiation factor (25).
4. ATP.
5. Assay buffer: 50 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 10% ethanediol.

3. Methods

3.1. Preparation of G26C SSB

3.1.1. Expression in E. coli

1. Streak two, pre-warmed L. agar/ampicillin plates with the glycerol stock of the G26C SSB plasmid in BL21(DE3) *E. coli* cells.
2. Incubate overnight at 37 °C.
3. To 100 mL L. broth in a sterile 250 mL flask, add 100 μL 10% ampicillin and inoculate with two to three single colonies from the streaked L. agar plates.
4. Incubate overnight at 37 °C with shaking.
5. Add 500 μL 10% ampicillin to each of 4× 500 mL L. broth in 2 L flasks and warm to 37 °C.
6. Inoculate each flask with 5 mL of the overnight cell culture.

7. Incubate at 37 °C with shaking.
8. Measure the absorbance at 595 nm of two flasks at 30 min intervals, using L. broth as a reference.
9. Make up 10 mL of 200 mM IPTG by dissolving 0.5 g IPTG in water, and filter the solution.
10. When the absorbance of the cultures reaches 0.5 cm^{-1}, add 2.5 mL 200 mM IPTG to each flask to induce protein expression (see Note 1).
11. Incubate the flasks for a further 3 h.
12. Following the 3 h incubation, centrifuge the cells in 2× 1 L centrifuge bottles at 3,500 × *g* for 30 min at 4 °C.
13. Prepare 50 mL cell storage buffer by mixing 2.5 mL 1 M Tris–HCl, 2 mL 5 M NaCl, and 100 μL 0.5 M EDTA, making up to 50 mL with water and then dissolving 5 g sucrose into the buffer.
14. Return the supernatant to the 2 L flasks and decontaminate.
15. Resuspend the harvested cells in 50 mL cell storage buffer and transfer 25 mL aliquots to 2× 50 mL tubes. Quick-freeze on dry ice and store at −80 °C.

3.1.2. SSB Protein Purification

Prepare the following buffers:

1. 50 mL First Resuspension Buffer: 50 mM Tris–HCl pH 8.3, 400 mM NaCl, 1 mM EDTA, 20% glycerol
2. 50 mL Second Resuspension Buffer: 50 mM Tris–HCl, pH 8.3, 200 mM NaCl,1 mM EDTA, 20% glycerol
3. 500 mL Column Buffer A: 50 mM Tris–HCl, pH 8.3, 1 mM EDTA, 20% glycerol
4. 500 mL Column Buffer B: 50 mM Tris–HCl. pH 8.3, 1 mM EDTA, 20% glycerol, 1 M NaCl
5. 2 L Dialysis buffer: 50 mM Tris–HCl, pH 8.3, 1 mM EDTA, 50% glycerol, 500 mM NaCl

All procedures should be carried out at 4 °C.

3.1.3. Protein Extraction (see Note 2)

1. Thaw stored cells in a beaker of cold water.
2. Add one Complete EDTA-free protease inhibitor tablet.
3. Sonicate the cells in 4× 20 s bursts on ice using a medium-sized probe.
4. Centrifuge down the cell debris at 38,000 × *g* for 20 min at 4 °C.

3.1.4. Polyethyleneimine Precipitation

1. Measure the volume of supernatant.
2. Slowly add 10% polyethyleneimine, pH 6.9, while stirring, to give 0.4% final concentration and continue to stir gently for a further 15 min.
3. Centrifuge at 11,000 × *g* at 4 °C for 20 min.
4. Keep the supernatant on ice.
5. Resuspend the pellet in 50 mL First Resuspension Buffer + 1 mM DTT (see Note 3) and homogenize using a glass homogenizer.
6. Stir for 30 min to redissolve any SSB in the pellet.
7. Centrifuge at 11,000 × *g* for 20 min at 4 °C.

3.1.5. Ammonium Sulfate Precipitation

1. Measure the volume of supernatant.
2. Add $(NH_4)_2SO_4$ solid slowly to the stirred supernatant to 150 g/L and stir for a further 30 min.
3. Centrifuge at 14,000 × *g* for 20 min at 4 °C.
4. Keep the supernatant on ice.
5. Resuspend the pellet in 50 mL Second Resuspension Buffer + 1 mM DTT until dissolved completely.
6. Centrifuge at 38,000 × *g* for 20 min at 4 °C.
7. Store the supernatant on ice, overnight if necessary.

3.1.6. Heparin Column Chromatography (see Note 4)

1. Attach a 5 mL HiTrap heparin column to a chromatography system.
2. Wash out preservative from the column and pre-equilibrate at 2 mL/min with 50 mL Column Buffer A, containing 1 mM DTT.
3. Filter the stored supernatant using a 0.2 μm polyethersulfone syringe filter membrane.
4. To ensure the protein binds to the heparin column, load the supernatant at 2 mL/min so that 16% supernatant and 84% Column Buffer A are pre-mixed and loaded onto the column. Collect 10 mL fractions while the protein is loading.
5. Once the supernatant is loaded, wash the column with 15 mL Column Buffer A.
6. Start a gradient 0–100% Column Buffer B in 150 mL, collecting 5 mL fractions and following the absorbance at 280 nm.

3.1.7. Dialysis

1. Run a gel of the fractions surrounding and including the elution peak.
2. Pool fractions containing SSB and use dialysis tubing to dialyze against 2 L dialysis buffer + 1 mM DTT overnight.

3. Measure the absorbance spectrum from 220 to 320 nm of a 1:50 dilution using dialysis buffer as a reference.
4. Calculate the protein concentration using the absorbance at 280 nm and the SSB extinction coefficient, 27,880 M^{-1} cm^{-1}.
5. Quick freeze 1.5 mL aliquots and store at −80 °C.

3.2. Labeling with IDCC

3.2.1. Labeling

1. Make up 250 mL labeling buffer by mixing 5 mL 1 M Tris–HCl, pH 7.5, 25 mL 5 M NaCl, 500 μL 0.5 M EDTA, 50 mL glycerol, and water to make the volume 250 mL. Mix until glycerol is dissolved completely and filter the buffer under vacuum.
2. Incubate 3 mg (G26C)SSB with 1.6 μL 1 M DTT for 20 min at room temperature.
3. Meanwhile, pre-equilibrate a PD10 column by passing 25 mL filtered, labeling buffer through the column and discard the eluate.
4. Apply the SSB to the column and allow the sample to completely enter the packed bed before adding labeling buffer to make up the total applied volume to 2.5 mL. Discard the eluate.
5. Elute the protein with 5 mL buffer, collecting ten 0.5 mL fractions.
6. Use Bradford reaction to identify fractions containing protein. To 10 μL Bradford reagent add 1 μL of the collected fraction and mix thoroughly. A color change to blue indicates the presence of protein in the solution (see Note 5).
7. Using a spectrophotometer, measure the concentration of those fractions containing protein by absorbance of a 1/10 dilution of each fraction at 280 nm and pool two to three fractions to make a solution such that the protein concentration is approximately 100 μM (see Note 6).
8. Add a twofold molar excess of IDCC and incubate, protected from light, for 2 h at room temperature with end-over-end stirring.
9. Add a tenfold molar excess of MESNA over protein to react with any remaining IDCC and incubate for a further 30 min.

3.2.2. Purification

1. Pre-equilibrate a P4 gel filtration column with labeling buffer by passing 60 mL filtered buffer through the column.
2. Pass the labeled protein solution through a 0.2 μm syringe filter membrane.
3. Allow equilibration buffer to drain into the column bed before applying the sample to the column.

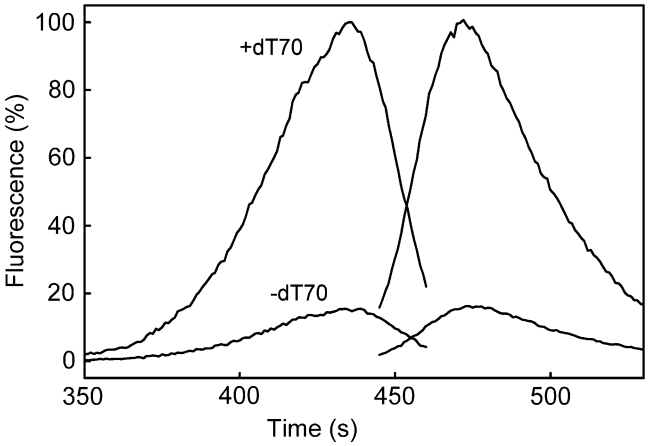

Fig. 2. Fluorescent spectral changes of DCC-SSB in response to ssDNA binding. Excitation and emission spectra are shown in the presence and absence of dT_{70} as described in the text.

4. Allow the sample to enter the column bed completely before applying additional buffer and attaching the column to a buffer reservoir.
5. Collect 0.5 mL fractions and pool the first colored peak.
6. Concentrate the labeled protein using an Amicon Ultra centrifugal filter and centrifuging for ~15 min at 2,600 × *g*.
7. Aliquot into opaque tubes and quick freeze on dry ice for storage at −80 °C.

3.2.3. Characterization

1. Measure the absorbance spectrum of a 1/50 dilution from 220 to 520 nm. Calculate the protein concentration taking the extinction coefficient for the coumarin to be 44,800 M^{-1} cm^{-1} at 430 nm (see Note 7).
2. Measure fluorescence excitation and emission spectra of a 250 nM solution of DCC-G26C SSB tetramer in 25 mM Tris–HCl, pH 7.5, 200 mM NaCl at 20 °C. Excite at 435 nm and record the emission spectrum. Then, with the fluorescence emission at 475 nm, record the excitation spectrum. An example of spectra is shown in Fig. 2.
3. Add an excess of dT_{70} (~600 nM) and repeat the excitation and emission scans. Comparison of the two spectra allows the determination of the fluorescence change of the DCC-SSB upon DNA binding.
4. Perform a fluorescence titration to characterize the DNA binding of the labeled SSB. To 200 µL of 250 nM DCC-SSB tetramer in 25 mM Tris–HCl, pH 7.5, 200 mM NaCl at 20 °C, add 4 pmol aliquots dT_{70}. The titration should show a linear increase in

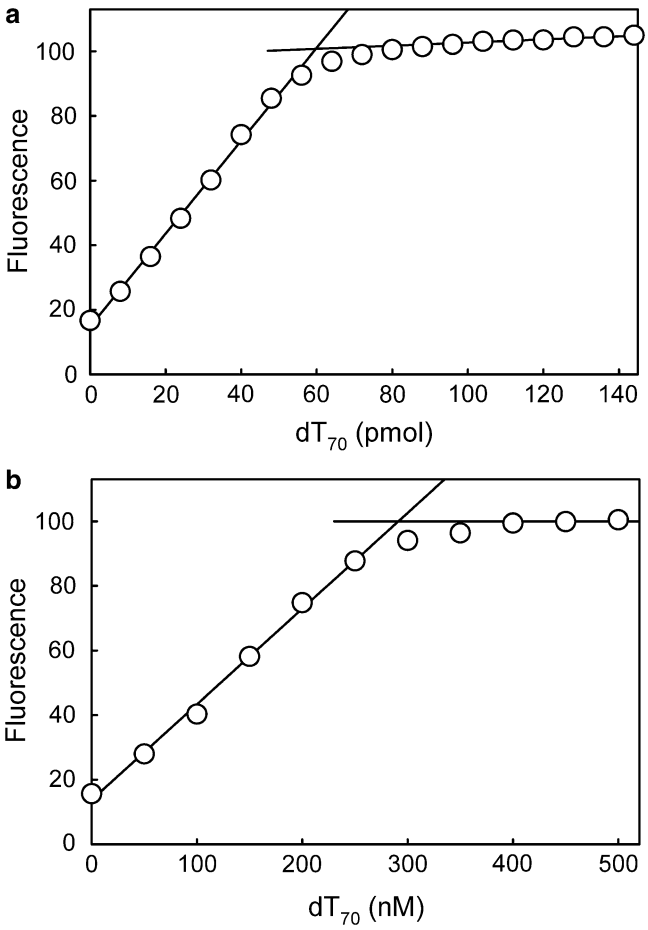

Fig. 3. Titration of ssDNA (dT_{70}) into a solution of DCC-SSB. (**a**) A titration in a cuvette using a spectrofluorimeter using 200 μL solution of 250 nM DCC-SSB tetramers (50 pmol). Solution conditions are described in the text. The additions are given in picomoles to allow for dilution by sequential additions. The fluorescence is corrected for dilution. (**b**) Titration in a stopped-flow fluorimeter under conditions of the helicase assay in Fig. 4. The DCC-SSB was at 250 nM tetramer. In each assay the data were fit to two linear regions. The intercept gives the effective capacity of DCC-SSB. The intercept is similar when poly(dT) is used.

fluorescence up to 50 pmol, at which point the SSB is saturated with DNA and there is no further increase in fluorescence. An example titration is shown in Fig. 3 (see Note 8).

3.3. Example of Helicase Assay (see Note 9)

1. Make up a reactant solution containing 2 mM ATP and 200 nM DCC-SSB tetramer in assay buffer and add to a stopped flow syringe.
2. In the second syringe, pre-incubate 1 nM pCERoriD plasmid with 4 nM RepD monomer for 30 s at 30 °C in assay buffer.
3. Add 200 nM PcrA and 200 nM DCC-SSB tetramer to the second syringe and mix.

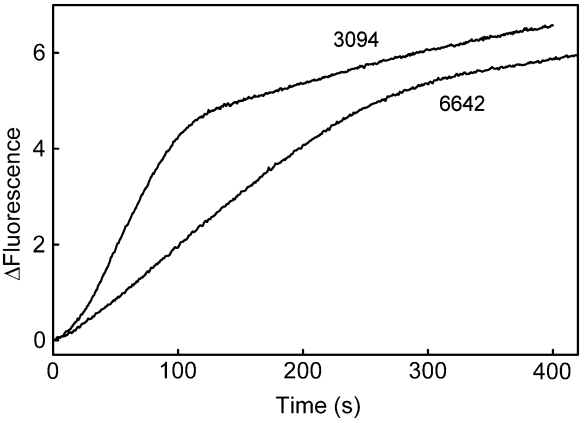

Fig. 4. Helicase assay using DCC-SSB, using two different lengths of plasmid (3,094 or 6,642 bp), containing the double-stranded origin of replication, *ori*D (25, 29). 0.5 nM plasmid, 2 nM RepD, 100 nM PcrA were mixed with 1 mM ATP in a stopped-flow instrument in the presence of 200 nM DCC-SSB tetramer. Solution conditions are described in the text and concentrations are those in the mixing chamber.

4. Rapidly mix the two solutions in the stopped-flow instrument and follow the fluorescence change for ~400–450 s by exciting at 436 nm and using a 455 nm glass cut-off filter on the emitted light. Example traces are shown in Fig. 4 using two different lengths of plasmid DNA.

4. Notes

1. To check for successful induction of protein expression, an SDS-PAGE can be used. Before addition of IPTG, take 1 mL samples from each flask, spin down in a benchtop centrifuge at 10,000 × *g* for 3 min and discard the supernatant. Quick-freeze the cell pellet and store at −80 °C. Repeat this following the 3 h incubation and run the samples on a SDS-polyacrylamide gel. An enhancement of the band at ~20 kDa indicates successful induction of protein expression.
2. To follow the progress of the protein purification, a 50 μL sample of supernatant can be taken following each centrifugation step and kept aside on ice. These samples can then be run on an SDS-polyacrylamide gel. A band should be seen at ~20 kDa in all samples except for polyethyleneimine and $(NH_4)_2SO_4$ supernatants.
3. 1 mM DTT should be added to all buffers from first resuspension buffer onwards immediately before using.
4. The protein is pure following the polyethyleneimine and $(NH_4)_2SO_4$ precipitations, so the chromatography step may not be needed. It does, however, result in a more concentrated

protein solution and this is useful both for subsequent labeling and for storage of the protein. If the heparin column is not used, continue directly on to dialyze the supernatant.

5. Protein usually elutes in fractions 3–5.
6. If starting with a dilute solution of protein, it may need to be concentrated at this point using an Amicon Ultra centrifugal filter to produce 1–1.5 mL of 100 μM protein solution.
7. To measure the total protein concentration, use the absorbance at 280 nm, where the extinction coefficient of SSB is 27,880 M^{-1} cm^{-1}. The extinction coefficient of IDCC at 280 nm is 7,470 M^{-1} cm^{-1} and this must also be taken into account. This calculation allows an estimation of the percentage of successfully labeled protein in the sample.
8. The titration is described using a length of DNA similar to the length of the SSB tetramer-binding site. In this way, one dT_{70} binds to each tetramer. A titration may be preferred under the same conditions as will be used in a helicase assay. An example of such a titration in the stopped-flow format is shown in Fig. 3.
9. The described assay has a complete plasmid unwound and at this point there is a break in the fluorescence time course (Fig. 4). This provides a calibration of the signal. In some cases, it is necessary to calibrate the signal, using known amounts of ssDNA. This can be either a full titration, as in Fig. 3b, or by using two or three different concentrations to quantify the fluorescence response, equivalent to the first few points of Fig. 4b.

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