Chapter 2

Fluorescent Biosensors to Investigate Helicase Activity

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Abstract

ATP-driven translocation of helicases along DNA can be assayed in several ways. Reagentless biosensors, based on fluorophore–protein adducts, provide convenient ways for real-time assays of both the separation of dsDNA and the hydrolysis of ATP. Single-stranded DNA can be assayed using a modified single-stranded DNA-binding protein (SSB), and phosphate production during ATP hydrolysis can be measured by a modified phosphate-binding protein. Advantages and limitations of these approaches are compared with those of other types of measurements.

Key words: Helicase, assay, fluorescence, kinetics, ATPase, phosphate.

1. Introduction

Different helicases translocate along DNA with a wide range of rates, typically 10–1000 bases per second and may move for short distances of a few bases (low processivity) or up to several thousand bases depending on the kinetics of translocation versus dissociation (1). These kinetics are presumably tuned to the biological role of the helicase. They generally use the energy of ATP hydrolysis to drive this movement and separation of duplex DNA. To study these processes in real time, methods are required that have sufficient sensitivity and time resolution to quantify strand separation and ATP hydrolysis typically on the time scale of milliseconds to seconds. Fluorescence probes often have high sensitivity and rapid response and various types of fluorescence instrumentation are available so that the signals can be measured rapidly and continuously. This chapter describes the use of fluorescent biosensors that have been developed for these types of applications and compares
them with other types of measurements that may give similar or complementary information. On the whole, the chapter is limited to bulk measurements in solution and methods that are based on fluorescence.

1.1. Translocation Assays

Potentially standard gel-based assays might be used to measure duplex separation catalyzed by helicases, but with difficulty to get the time resolution required for real-time measurements. In any case, by their discontinuous nature, such measurements are likely to give single time points after the reaction is quenched. Fluorescence probes have proved useful for continuous measurement of the conversion of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA), although they have the potential for disrupting the natural system being studied. Protein–DNA interactions may be modified by the presence of a dye moiety. Other molecules, added as probes, may bind to the helicase or DNA and so modify the translocation.

For translocation along short lengths of DNA, several strategies have been used with fluorescent labels attached to oligonucleotides, to give a signal to monitor translocation with single-base resolution (2–7). If the end of the DNA is labeled, then no signal change is observed until the helicase approaches near to the fluorescent label. The signal change then occurs when the helicase reaches the end label. Even if the fluorescent label interferes with the translocation in some way, the time elapsed before the signal change provides a measure of the time taken to translocate the length of DNA. This is sometimes called an “all-or-none” measurement. Ideally this measurement should be done with different lengths of the DNA track. There should be a linear dependence of the time taken on the length and this allows the translocation speed to be determined. Use of several lengths enables at least partial elimination of end effects in the translocation, such as a lag before movement starts or a fluorescence change due to environmental effects when the helicase complexes are close to the fluorophore. Several laboratories have used fluorescence resonance energy transfer (FRET) (for example, (4)) or a fluorophore–quencher pair, such as Cy3 and Dabcyl groups (for example, (8)). These are at the same end of a length of dsDNA so that they can interact fully and have little or no fluorescence while the duplex is intact. Use of a quencher in the substrate DNA has the advantage of giving a fluorescence increase during the helicase assay, a property that is useful for measuring small extents of reaction and limiting interference by photobleaching. However, these types of approach are limited to < 100 base lengths for bulk measurements: desynchronization of helicases tends to produce smaller signals as the length increases. An advantage is that measurements of short lengths of translocation with end-labeling fluorophores can give single-base resolution in both bulk and
single-molecule assays. A precise and powerful analysis for these types of all-or-none assays has been described based on kinetic modeling (9).

For translocation over long lengths of DNA (hundreds to thousands of bases), different approaches are required. Strand separation can be measured using probes that discriminate between ssDNA and dsDNA. A number of dyes bind tightly to dsDNA, but weakly, or not at all, to ssDNA and give a fluorescence change, usually an increase, on binding (10, 11). If such an intercalating dye is bound along dsDNA, the helicase will displace the dye molecules as it translocates: in bulk solution assays, there is a continuous, gradual increase in fluorescence that gives a measure of the extent of translocation. However, the presence of the dye may disrupt the helicase action or even, in extreme cases, prevent translocation. In addition, this assay has the disadvantage of producing a decrease in fluorescence, which means that the measurement may be made against a high background and possibly against a simultaneous decrease due to photobleaching.

Triplex displacement assays show promise for translocation assays. Here, particular sections of duplex DNA sequence bind a third length of DNA. Helicases can potentially translocate through this triplex section and in doing so release the third DNA molecule. If a fluorescently labeled oligonucleotide is used for this, it can give a change in fluorescence on release, which provides an “all-or-none” measure of translocation time to that point (12, 13). As for end labeling of oligonucleotides, measurements of translocation times for different lengths is advantageous, although, in practice, such end effects are likely to be a much smaller fraction of the total translocation time when long lengths of dsDNA are used.

An approach that measures the product ssDNA formation has advantages over ones that measure substrate depletion. Single-stranded DNA-binding protein (SSB) has been used to achieve this, either using the intrinsic tryptophan fluorescence (14) or by use of an extrinsic fluorescence label attached to SSB (15). SSB from *Escherichia coli* exists as a tetramer and binds a length of up to ~70 nucleotides of ssDNA (16, 17). Under some circumstances there may be a different binding mode, whereby approximately half that length binds to the tetramer. This “35 base” binding mode is favored by low ionic strength and high ratio of SSB to DNA.

An internal tryptophan has provided a continuous signal for assaying helicase unwinding of duplex DNA. As DNA binds to the SSB, there is a decrease in tryptophan fluorescence. Because of the relatively low-intensity decrease and the potential for other components of the assay solution to interfere with the fluorescence, this assay has relatively low sensitivity. To circumvent some of these problems, a single cysteine mutant of SSB was labeled with a coumarin fluorophore so that it gives a sixfold increase in fluorescence on binding ssDNA (15). The aim was to produce a probe
for helicase assays with high sensitivity and time resolution. Although binding is complex (18) with multiple modes as described above and potentially cooperative effects as multiple SSB tetramers bind along ssDNA, this biosensor gives an approximately linear response to the amount of ssDNA. The fluorescence response is also linear during measurement of dsDNA unwinding by a helicase. Examples of such assays have been published that demonstrate the high sensitivity of the coumarin-labeled SSB (DCC-SSB) (15). This chapter describes the use of this biosensor to measure helicase-driven separation of dsDNA.

1.2. ATPase Activity Assays

There are several different ways to measure the ATPase activity of a helicase, generally by monitoring one of the products, ADP and inorganic phosphate (P$_i$). Relative advantages of the various assays depend in part on whether a steady-state measurement is required, which is likely to be over a longer time course, or whether ATP hydrolysis is measured in real time to correlate directly with translocation. Use of radiolabeling provides a relatively simple assay, which requires no additional components to be present. However, such measurements are discontinuous and each assay point requires separation of ATP hydrolysis products. There are a number of coupled-enzyme assays that measure product ADP (for example, (19, 20)) or inorganic phosphate, P$_i$ (for example, (21–23)). Some can provide a fluorescence signal or often are based on an absorbance change. The latter sacrifices sensitivity, but has the advantage in that the signal response is linear and readily quantified, based on an extinction coefficient (e.g., of NADH). Fluorescence assays usually have to be calibrated for each different set of conditions. However, coupled-enzyme assays inevitably required addition of multiple components and there needs to be care to ensure that these additions do not affect the assay and that the observed rate is that of the helicase, not of the coupled enzyme. A typical pitfall is to assume that the coupled enzymes are operating at maximal velocity, although pH and buffer conditions are, in practice, producing a suboptimal rate. While these types of assays may be very useful for steady-state measurements, they may not be fast enough for the high activity of helicases during single-turnover measurements with respect to DNA. For the latter, fluorescence-based reagentless biosensors may have advantages, including the need for only one added component (the biosensor molecule). There is, therefore, only one coupled rate that needs to be checked and compared with that of the helicase. There have been several such biosensors described for ADP (for example, (24, 25)) and P$_i$ (for example, (26, 27)), although some are aimed particularly at high-throughput assays and may not have a high rate of response.

This chapter describes the use of the biosensors based on the phosphate-binding protein. This type has been widely used in assays of ATPase activity and mechanism of helicase (28) and
other motor proteins such as myosins (29) and kinesins (30). The method is particularly suited to stopped-flow measurements, for example, to measure single-turnover kinetics, the details of the ATPase cycle, or the precise relationship between translocation and the number of ATP molecules hydrolyzed. To produce the original phosphate biosensor, a single cysteine mutant of the bacterial phosphate-binding protein was labeled covalently with a single coumarin to give a signal increase (approximately an order of magnitude) on \( P_i \) binding. More recently a version of this with two rhodamines was developed with greater sensitivity and photostability (27).

The bases for these fluorescence changes, the kinetic mechanism of binding \( P_i \) to this protein, have been described (31, 32). The binding is tight (\( \sim 50\, \text{nM} \)) so that for most conditions, the biosensor must be in excess over the maximum amount of \( P_i \) likely to be encountered in the assay or at least during the time period of the measurement. Because of this and the ubiquity of \( P_i \) contamination, it is important to assess and minimize any such contamination. This contamination has been discussed and an enzymic method of reducing it has been described (33, 34). The “phosphate mop” consists of purine nucleoside phosphorylase and 7-methylguanosine, which converts \( P_i \) to ribose-1-phosphate and so to a chemical species that is silent with respect to the phosphate biosensor.

### 1.3. Described Methods

The methods described include examples of applications of these biosensors to measure the enzymic activity of helicases, either in the steady state or as rapid reaction measurements using stopped-flow. Some general points about experimental design are given below.

1. **Check the labeled protein.** The quality of the fluorescent protein can be simply checked with a titration of the ligand, measuring fluorescence. Examples are given in Section 3. The response should be linear and give fluorescence increases similar to those published. In the case of the \( P_i \) sensors, this can also assess the \( P_i \) contamination in the preparation and buffers. Addition of \( P_i \) mop components rather than \( P_i \) will give a small decrease in fluorescence, whose size depends on the degree of contamination. The breakpoint of the titration reflects the concentration of active biosensor.

2. **Optical considerations.** These are mainly outside the scope of this chapter, but it should be realized that optics plays a large part in determining the quality of the signal obtained. Thus the coumarin-based sensors described here are particularly well suited to excitation via a strong Hg line at 436 nm. There is an Hg line at 546 nm that can be used for rhodamine excitation. Where there is a choice between a xenon lamp and a xenon–mercury lamp, for example, with a stopped-flow instrument, much higher excitation intensity can be obtained with the latter.
The possibility of inner filter effects must also be considered: is the absorbance of the fluorophore significant (say, > 0.1 for the pathlength being used)? Changing the excitation wavelength may be possible. For the coumarin-based sensors the extinction coefficient does not change between apo and bound forms, so to some extent inner filter effects will cause the fluorescence change to decrease, but remain linear. The calibration can be altered accordingly. Note that the rhodamine extinction coefficient does change, so the interpretation may be complex, but in this case, there may be significant scope to change the wavelength.

3. Rate of response. Depending on the type of measurement, the rate of response may also be a significant consideration: this typically depends on the concentration of the binding protein (the biosensor), since the concentration of free ssDNA or Pi is likely to be very low during the assay. The aim is to ligate the product rapidly with the biosensor. The publications describing these sensors have binding measurements for some conditions that are a good guide. Nevertheless significant differences in conditions can lead to large changes in rate constants.

2. Materials

2.1. Assay Components
1. Fluorescent reagents are available as follows: MDCC (N-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide) and IDCC (N-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide) (Invitrogen, USA, or Synchem, Germany); 6-IATR (6-iodoacetamidotetramethylrhodamine) (Chemos, Germany).
2. SSB (G26C): prepared as previously described (15).
3. MDCC-PBP: prepared as previously described (26, 31, 34) (Invitrogen). Rhodamine-PBP was prepared as described and stored as a concentrated solution (~1 mM) in small aliquots at –80°C (27).
4. Components for the phosphate mop (lyophilized, “bacterial” purine nucleoside phosphorylase and 7-methylguanosine) (Sigma-Aldrich). The additional “supermop” components were glucose-1,6-bisphosphate and manganese chloride (Sigma-Aldrich) and phosphodeoxyribomutase from E. coli, prepared as previously described (33) (see Note 1).

2.2. Other Proteins and Plasmids
1. Wild-type phosphate-binding protein from E. coli was prepared as described (26). This is the phosphate complex. Phosphate was partially removed by treatment with the “supermop” components. A solution (500 μl) in 10 mM PIPES, pH 7.0, of 100 μM protein, 200 μM
7-methylguanosine, 0.2 unit ml⁻¹ purine nucleoside phosphorylase, 5 μM MnCl₂, 1 μM glucose-1,6-bisphosphate, and 1.5 μg ml⁻¹ phosphodeoxyribomutase was incubated at 20°C for 15 min. It was desalted on a PD10 column (GE Healthcare) and pre-equilibrated in the same buffer. This resulted in a solution of 0.5 ml of ~80 μM phosphate-binding protein from the peak fraction (see Note 2).

2. RepD from Staphylococcus aureus and PcrA from Bacillus stearothermophilus were prepared as described (35, 36). For steady-state assays, as described, use a fresh 5-nM stock containing 5 μM BSA as carrier.

3. Plasmid pCERoriD, containing the oriD sequence, was prepared as described (37).

2.3. Other Biochemicals

1. 100 mM sodium 2-mercaptoethane-sulfate (MESNA): prepare fresh.
2. 1 M dithiothreitol (DTT).
3. Phosphate (Pᵢ) standard solution (VWR, Aristar, 1000 ppm as “P,” which refers to PO₄³⁻).
4. ATP (Sigma) (SigmaUltra grade), which has low Pᵢ contamination (see Note 3).
5. dT₂₀ and dT₇₀ (Eurogentec) and HPLC-purified grade.

2.4. Buffers and Solutions

1. Buffer for DCC-SSB labeling: 20 mM Tris–HCl, pH 7.5, 1.0 mM EDTA, 500 mM NaCl, 20% (v/v) glycerol.
2. Buffer for DCC-SSB purification: 20 mM Tris–HCl, pH 8.3, 1.0 mM EDTA, 500 mM NaCl, 20% (v/v) glycerol.
3. Buffers for testing pH and ionic strength variations in DCC-SSB signal: 25 mM Tris–HCl, pH 8.0, or 25 mM PIPES, pH 7.0, each containing 20 or 200 mM NaCl.
4. Buffer for helicase activity assay: 50 mM Tris–HCl, pH 7.5, 200 mM KCl, 10 mM MgCl₂, 1 mM EDTA, and 10% ethanediol.
5. Buffer for rhodamine-PBP characterization: 10 mM PIPES, pH 7.0.
6. Buffer for steady-state ATPase assay: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM MgCl₂.

3. Methods

3.1. Label SSB (G26C) with IDCC

1. Add 5 μmol DTT to 10 mg SSB (530 nmol) and incubate for 20 min at room temperature to ensure all cysteines are fully reduced.
2. Degas the labeling buffer just before use, by bubbling nitrogen through for 5 min.


4. Pass the DTT-treated SSB through the PD10 column, eluting with degassed buffer. Collect 15 × 0.5 ml fractions and measure their absorbance at 280 nm (dilute a portion × 20 and use the buffer as blank). Collect protein fraction(s).

5. In a sealable tube, make the protein 100 μM by diluting, if necessary, with labeling buffer and add 200 μM IDCC.

6. Play nitrogen over the solution just before sealing the tube.

7. Incubate 2 h at 22°C with end-over-end stirring, protected from light.

8. Add 1 mM MESNA and leave for 30 min with end-over-end stirring, protected from light, to react with remaining coumarin.

9. Pass the solution through a membrane filter (0.2 μm pores, polyethersulfone from Whatman).

10. Pre-equilibrate a P4 gel filtration column (BioRad; 1 × 20 cm) in the DCC-SSB purification buffer.

11. Pass the labeled protein through this column, collecting 0.5 ml fractions and pooling the first colored peak.

12. Concentrate the protein to 50–100 μM monomers using a Centricon YM10 membrane concentrator.

13. Measure the absorbance spectrum and calculate the protein concentration at 430 nm, where the extinction coefficient of the coumarin is 44,800 M⁻¹ cm⁻¹.

14. Store the protein at −80°C after quick freezing.

3.2. Check the Labeled Protein – Calibration of DCC-SSB

1. This section describes calibrating DCC-SSB at different pH and ionic strength conditions (see Note 4).

2. Place 100 μl of 200 nM DCC-SSB tetramers in the appropriate buffer in a 3 × 3 mm fluorescence cuvette.

3. Measure fluorescence at 20°C in a Cary Eclipse fluorimeter with excitation at 430 nm and emission at 470 nm (see Note 5).

4. Titrate in aliquots of dT₇₀ over the range to 50 nM, as shown in Fig. 2.1. Correct the data for any dilution.

5. Finally, add a twofold molar excess of the oligonucleotide to obtain an end point; in the examples shown, this gives the 100% level for the signal (see Note 6).
3.3. Check the Labeled Protein – Titration of Rhodamine-PBP with Phosphate

1. Place a solution (200 μl in 10 mM PIPES, pH 7.0) of 2.5 μM rhodamine-PBP in a 3 × 3 mm fluorescence cuvette.

2. Measure fluorescence at 20°C in a Cary Eclipse fluorimeter with excitation at 555 nm and emission at 578 nm (see Note 5).

3. Add aliquots of P_i standard, suitably diluted, to produce a titration curve as in Fig. 2.2. Correct the data for any dilution (see Note 7).

4. The intercept of the linear fits to the fluorescence rise and to the horizontal portion gives a measure of the active, P_i-free protein.

Fig. 2.2. Titration of rhodamine-PBP with inorganic phosphate. The upper plot (circles) is for rhodamine-PBP alone. The lines are linear fits to the rise and horizontal portions. The lower plot (triangles) is offset by –10% for clarity and is for equimolar mixture of wild-type PBP and rhodamine-PBP. The wild-type protein was treated with phosphate mop as described in the text to remove bound P_i partially. The curvature shows that wild-type protein binds P_i more tightly than the labeled protein. The line is a best fit to a model, in which there is tight binding, as previously described (15): the ratio of dissociation constants is ~4 based on this, with the wild type binding P_i tighter.
5. To demonstrate the effect of unlabeled phosphate-binding protein, repeat the titration in the presence of 2.5 μM wild-type protein, after partial removal of bound P\textsubscript{i} by treatment with the phosphate “supermop” (see Note 8).

3.4. Real-Time Helicase Activity Assay Using Coumarin-Labeled SSB (DCC-SSB)

1. Prepare 1 ml of a solution in the helicase assay buffer: 2 nM pCERoriD circular plasmid, pre-incubated for 10 min with 4 nM RepD, 400 nM tetramer DCC-SSB, 50 nM PcrA (Note 9).

2. Prepare 1 ml of a 2-mM ATP solution in the helicase assay buffer.

3. After equilibration at 37°C, mix these two solutions rapidly, using a stopped-flow apparatus (Hi-Tech, TgK Scientific, UK), equipped with xenon–mercury lamp. Follow the fluorescence with time, exciting at 436 nm and using a 455-nm cut-off filter on the emission. A sample trace is shown in Fig. 2.3 (see Note 10).

4. An identical solution, but in the absence of RepD, can be used as a zero activity control. PcrA has negligible helicase activity under these conditions. This provides a check on the stability of the fluorescent sensor for the experimental (solution and optical) conditions (see Note 11).

3.5. Steady-State ATPase Activity Assay Using Rhodamine-PBP

1. Prepare 1 ml of assay solution in the ATPase assay buffer: 0.5 μM dT\textsubscript{20}, 20 μM ATP, 5 μM rhodamine-PBP (see Note 12).

2. Prepare a 5-nM solution PcrA in ATPase activity buffer, containing 5 μM BSA as carrier.

3. Place 200 μl assay solution in a 3 × 3 mm fluorescence cuvette and equilibrate to 20°C in a fluorimeter (Cary Eclipse – see Note 5).
4. Follow fluorescence with time, exciting at 555 nm and measuring emission at 578 nm.

5. When the fluorescence is constant, add PcrA to give a concentration of 50 pM. Following a period (~200 s) of constant increase in fluorescence, add a second aliquot of PcrA to give a total of 100 pM to check that the rate doubles. A sample trace is shown in Fig. 2.4.

6. Repeat the fluorescence measurement, but obtain a control in the absence of PcrA (see Note 11).

7. Do linear fits of each section of the time course and the control, to obtain the rate of the reaction at each enzyme concentration.

8. Calibrate the fluorescence signal, using a further 200 µl aliquot of the assay solution, but add 2 × 0.5 µM aliquots of P_i standard, measuring the fluorescence change on each addition. This should be repeated with a fresh aliquot of assay solution. Average the four Δfluorescence values to convert the (arbitrary) fluorescence scale to nanomolar phosphate.

Fig. 2.4. Steady-state ATPase assay of PcrA with dT₂₀, measured using the rhodamine-PBP phosphate sensor. The incubation solution at 20°C contained the following: 450 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM MgCl₂, 20 µM ATP, 0.5 µM dT₂₀, and 5 µM rhodamine-PBP. The reaction was initiated at zero time by addition of 50 pM PcrA from a 5-nM stock containing 5 µM BSA as carrier. A similar addition at 260 s illustrates the doubling of rate. A control with no PcrA is shown to check the stability of the fluorescence signal.

4. Notes

1. Store the 7-methylguanosine as a 20 mM solution in water at –20°C. Store the phosphorylase as 1000 unit ml⁻¹ in the lyophilized buffer in small aliquots at –80°C: repeated freezing and thawing deactivates this enzyme. It is important that the protein solutions are snap frozen. Some other types of this
phosphorylase are available commercially but may not be suitable: either they do not accept 7-methylguanosine as a substrate with high activity or they come with phosphate buffer.

2. Because wild-type phosphate-binding protein binds $P_i$ much tighter than the labeled protein, removal of $P_i$ needs the extra components of glucose-1,6-bisphosphate, MnCl$_2$, and phosphodeoxyribomutase. Normal mopping of protein solutions, if required at all, requires a similar treatment but only with 7-methylguanosine and purine nucleoside phosphorylase. Labeled phosphate-binding protein should have $< 10\%$ $P_i$ as prepared.

3. To prevent hydrolysis during storage, keep the solid nucleotide desiccated (over Drierite) in a sealed container at $-20^\circ$C. To avoid condensation, warm to room temperature before opening. Concentrated stock solutions can be stored at $-80^\circ$C with minimal decomposition if the pH is adjusted to 4–7. Avoid many repeats of thawing and freezing.

4. As in almost all cases with fluorescence measurements, the signal must be calibrated at the conditions and concentrations being used. To obtain a relatively linear response, generally for these types of biosensors, there needs to be a compromise with signal sensitivity. Usually the response is closest to linear at low extents of saturation. In the case of SSB, the mode of binding, as discussed above, as well as general factors, such as temperature and ionic strength, affects the fluorescence response. For measuring ssDNA, therefore, we proposed a rule of thumb that the ratio of SSB subunit to nucleotides of ssDNA is $\sim 5$, based on the maximum ssDNA to be measured (15). This keeps the response fairly linear for a variety of conditions. This method illustrates the variation in fluorescence as pH and ionic strength vary. However, for each condition the response is approximately linear. It is important to test the quality of the labeled protein, using the “standard” buffer, but do a calibration with the conditions that are being used in the helicase assay.

5. The fluorimeter is a standard instrument, equipped with xenon lamp, and any equivalent one would be appropriate. Because any fluorescence intensity can vary with temperature, it is important to have temperature control. In addition, fluorescence measurements may be instrument dependent so that calibrations should be done, if possible, on the same instrument as the actual assay.

6. In the example shown, the 100% signal was, in absolute terms, very similar for the four conditions (within 10%). The main variation is in the starting fluorescence (free DCC-SSB) and whether there is significant “35-base” binding mode at low ionic strength, as described in the text above.
7. The rhodamine-PBP has a linear response until almost saturated (7). However, part of the capacity of the PBP may be taken ("used up") by contaminating P. This must be dealt with in subsequent assays, either by minimizing contamination or by having extra PBP present or by having "Pi mop" present at low enough activity not to affect the measurement. In general, the measurement should be tried first in the absence of mop. Ways of dealing with phosphate contamination have been discussed (34).

8. For each sensor, the labeling does cause a decrease in affinity for the ligand and so at low concentrations of ligand, any unlabeled protein will preferentially bind the ligand. So the fluorescence change is smaller at this stage of the titration. This is illustrated in Fig. 2.2, where a mixture of labeled and unlabeled proteins is used. The same effect will be observed in the titration of labeled protein alone: lack of linearity of the fluorescence rise may reflect incomplete labeling. Mass spectrometry of the protein would act as a check on this.

9. The procedure described for a single turnover of dsDNA strand separation can readily be adapted for other helicases, either using a similar concentration of DNA in terms of bases or by modifying the concentration of DCC-SSB sensor appropriately. The buffer appropriate for the helicase can be used, but with appropriate calibration of the fluorescence signal, as described. Slower helicases or multi-turnover assays can be done in a cuvette using a standard fluorimeter.

10. Several suppliers produce stopped-flow equipment suitable for these types of assay. The technical aspects of stopped-flow fluorimetry have been described in several reviews (for example, (38)), as has the additional information that can be obtained from single-turnover measurements as opposed to steady-state ones (39).

11. The control fluorescence measurements in the absence of enzyme activity are important to test the signal stability under the same experimental conditions and the same timescale as the assay itself. The fluorescence signal may change due to protein stability, solution cloudiness (light scatter), or photobleaching, for example. Note that photobleaching of the Pi-free rhodamine-PBP results in an increase in fluorescence: as one of the stacked (and hence quenched) rhodamine pair photobleaches, the other then exhibits full monomer fluorescence (27).

12. This illustrates a sensitive assay for ATPase activity that can be adapted to other enzymes with appropriate solution and temperature conditions. In any case, it is important to use ATP solutions that are low in phosphate contamination.
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References


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