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A helicase assay based on the displacement of fluorescent, nucleic acid-binding ligands

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ABSTRACT

We have developed a new helicase assay that overcomes many limitations of other assays used to measure this activity. This continuous, kinetic assay is based on the displacement of fluorescent dyes from dsDNA upon DNA unwinding. These ligands exhibit significant fluorescence enhancement when bound to duplex nucleic acids and serve as the reporter molecules of DNA unwinding. We evaluated the potential of several dyes [acridine orange, ethidium bromide, ethidium homodimer, bis-benzimide (DAPI), Hoechst 33258 and thiazole orange] to function as suitable reporter molecules and demonstrate that the latter three dyes can be used to monitor the helicase activity of *Escherichia coli* RecBCD enzyme. Both the binding stoichiometry of RecBCD enzyme for the ends of duplex DNA and the apparent rate of unwinding are not significantly perturbed by two of these dyes. The effects of temperature and salt concentration on the rate of unwinding were also examined. We propose that this dye displacement assay can be readily adapted for use with other DNA helicases, with RNA helicases, and with other enzymes that act on nucleic acids.

INTRODUCTION

Helicases serve a variety of functions in DNA metabolism (for reviews, see 1,2). Cellular (*Escherichia coli* DnaB, PriA and Rep proteins), phage (T4 gene *41* and dda proteins; T7 gene *4* protein), and viral (SV40 large T antigen; HSV-1 UL5/UL52 complex and UL9 protein) helicases are involved in the initiation of replication, by unwinding DNA so that other proteins of the replication apparatus can assemble on the ssDNA. These proteins also participate in the elongation phase of replication, by unwinding DNA ahead of this complex to provide the required template. Other helicases (e.g. the *E.coli* RecBCD and RecQ proteins) are implicated in recombination by genetic criteria. *In vitro*, RecBCD enzyme helicase activity generates the ssDNA substrate to which the RecA protein of *E.coli* can bind to initiate heteroduplex DNA formation (3,4). Helicases such as the *E.coli* RuvAB complex function in recombination by promoting the branch migration of crossover intermediates (5). Another class of helicases includes the *E.coli* UvrAB and UvrD, *Saccharomyces cerevisiae* Rad3 and Rad25, and human ERCC-2, -3 and -6 proteins (reviewed in 6). These helicases act in nucleotide excision repair (7,8) or methyl-directed mismatch repair (9) during both pre-incision (recognition of DNA damage or alteration) and post-incision (displacement of damaged fragment) steps. Various aspects of RNA metabolism are also dependent upon the action of helicases (for review, see 10). The unwinding of RNA templates is required in processes as diverse as transcription termination (11), translation initiation (12,13) and RNA processing (14–16).

Many assays have been developed to measure the unwinding of duplex nucleic acids by helicases. The first assays measured the sensitization of labeled duplex DNA to single-strand specific nucleases such as S_1 or exonuclease I, a result of the production of ssDNA during unwinding (17–20). Electron microscopy was employed to visualize directly the regions of DNA unwound by proteins such as RecBCD enzyme (21), Rep protein (22), *E.coli* helicases I and II (23,24) and SV40 large T antigen (25). Currently, the most common assay measures the ability of a helicase to displace a labeled fragment which is annealed to a single-stranded DNA or RNA molecule; this displacement is detected by gel electrophoresis, as a band which has altered mobility (26,27). Several continuous fluorometric assays have also been developed. The first described exploits ssDNA binding proteins [e.g. *E.coli* single-strand DNA binding protein (SSB) protein or phage T4 gene *32* protein] as the reporter molecule (28). As the dsDNA is unwound, the SSB protein binds to the ssDNA formed, and its intrinsic fluorescence is quenched. A second method employs duplex DNA strands whose complementary ends are labeled with different fluorescent moieties; when these ends are separated by the helicase, the fluorescence quenching which occurs when the labels are in close proximity is abolished (29,30). The third fluorescent assay utilizes an oligonucleotide which contains 2-aminopurine substituted for adenine (31). This analog exhibits fluorescence quenching when hydrogen bonded to thymine residues, and thus unwinding can be measured as an alleviation of this quenching.

Each of the assays described above has merits and limitations. While the first three assays have the advantage that they are direct, they are non-continuous and require considerable manipulation before results are obtained. All of the assays are predicated on the assumption that the unwound ssDNA will not reanneal, a potential limitation of the fragment-displacement and the fluorescent-label quenching assays which typically employ oligonucleotides. For detailed studies of kinetic mechanisms, fluorescence methods are ideal because they provide continuous data in real time. Of the

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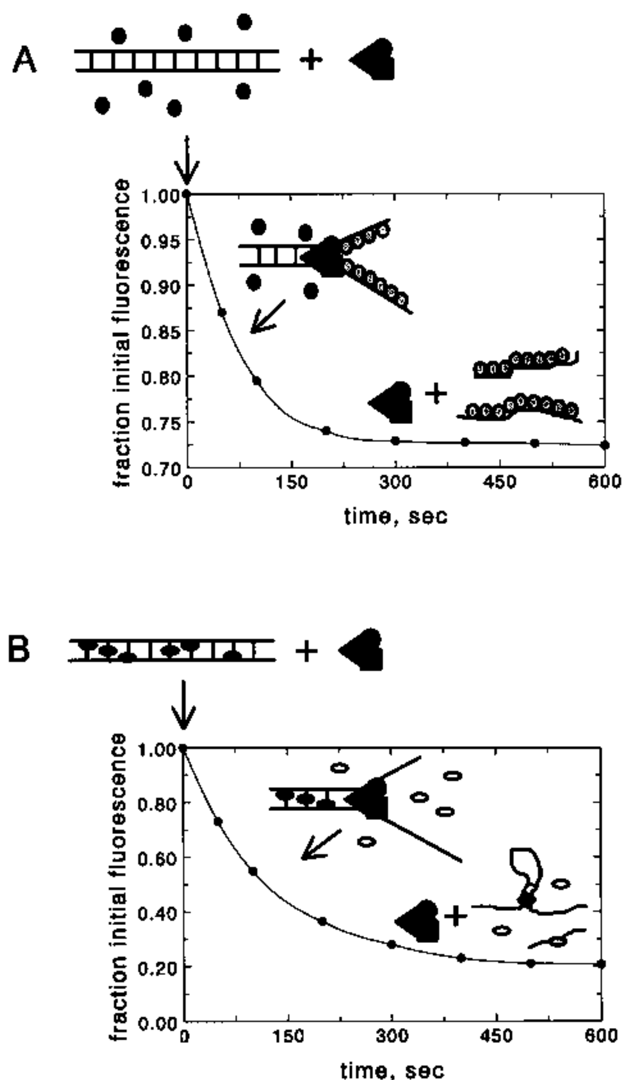

Figure 1. Illustration of the SSB protein fluorescence and dye displacement assays used to measure DNA helicase activity. In (**A**) is shown the SSB protein fluorescence-quenching assay (28). In this assay, the fluorescence signal at the start of the reaction is high due to the intrinsic fluorescence of SSB protein which is free in solution (filled circles). As the duplex DNA is unwound by a helicase [e.g. RecBCD enzyme (circle/square/triangle)], SSB protein binds to the ssDNA formed, which results in partial quenching of its intrinsic fluorescence (stippled circles). This fluorescence decrease is easily measured. In the dye displacement assay (**B**), the dye molecules are initially bound to the duplex DNA (filled ovals), which enhances their fluorescence. As the dsDNA is unwound, the dye molecules are displaced. Regardless of whether these molecules exist free in solution (open ovals) or rebind the ssDNA (stippled ovals), little fluorescence is produced. Thus, in this assay, fluorescence also decreases as the DNA is unwound, but in this case the fluorescence signal originates with the dsDNA substrate rather than the ssDNA product.

fluorescence assays described above, the fluorescent-label assays share two related limitations: they require the synthesis of either substituted or covalently-modified oligonucleotides and, since they employ short dsDNA fragments, their utility in studies of processivity is restricted. The SSB protein-fluorescence assay overcomes several limitations of both the fragment-displacement and the fluorescent-label quenching assays. It does not require a specialized DNA substrate; consequently, the length of the duplex region is virtually unconstrained, allowing the detailed study of highly processive helicases (32). In addition, annealing of the unwound ssDNA is prevented, due to the binding of the SSB protein. One disadvantage of this assay, however, is that the requirement for an SSB protein obviates its use with helicases that require a ssDNA region to initiate unwinding and that therefore may be inhibited by the presence of ssDNA binding proteins.

In order to develop an assay which affords the advantages of the SSB protein-fluorescence assay but which does not absolutely require the presence of a DNA binding protein, we have focused upon the use of fluorescent dyes as reporter molecules for the unwinding of duplex DNA and RNA. (Illustrations comparing the two types of assays are shown in Figure 1.) Many chromophores have the property of enhanced fluorescent quantum yield when they are bound to nucleic acids relative to that observed when they are free in solution. This effect is generally greater when the dye molecules are bound to double-stranded, rather than single-stranded, substrates. The well-characterized fluorescent probes for nucleic acids, DAPI (bis-benzimide) and Hoechst 33258, bind in the minor groove and display some sequence specificity, particularly if their preferred binding sites are contiguously positioned (33,34). Other intercalating dyes, such as thiazole orange, demonstrate no sequence specificity (35). Thus, it would theoretically be possible to monitor the process of DNA or RNA unwinding by measuring the decrease in fluorescence as the dye ligands are displaced from the duplex molecule.

A variety of fluorophores—AO, DAPI, EB, EHD, H33258 and TO (acridine orange, bis-benzimide, ethidium bromide, ethidium homodimer, Hoechst 33258 and thiazole orange, respectively) — were examined to determine their utility as reporter molecules in a continuous helicase assay. Reactions utilizing several of these dyes (DAPI, H33258 and TO), which share the properties of having relatively low fluorescence in the presence of ssDNA and significant fluorescence enhancement upon binding to dsDNA, were characterized. These studies show that the presence of two of these dyes have little effect on the activity of the helicase we have used, RecBCD enzyme. Based on our results and on findings published by others (36), we anticipate that this dye displacement assay can find widespread use in the study of both DNA and RNA helicases.

MATERIALS AND METHODS

Nucleic acids

pBR322 DNA was prepared by alkaline lysis and double-banding in CsCl–ethidium bromide density gradients (37). Phage M13 ssDNA was prepared according to standard procedures (38). Nucleotide concentrations were determined at 260 nm using extinction coefficients of 6500 and 8784 $M^{-1}·cm^{-1}$ for dsDNA and ssDNA, respectively.

Proteins

RecBCD enzyme was purified as previously described (28,39) and was quantitated at 280 nm using an extinction coefficient of 4×10^5 $M^{-1}·cm^{-1}$ (28). The specific activity of the enzyme preparation was 3.1×10^5 nuclease units/mg protein (40) and 4.1×10^4 helicase units/mg protein (28); its apparent binding stoichiometry was ~3 molecules/end (28). SSB protein was purified as described (41) and was quantitated using an extinction coefficient of 3×10^4 $M^{-1}·cm^{-1}$ at 280 nm (42).

Fluorophores

EB was purchased from Sigma. AO, DAPI, EHD, H33258 and TO were obtained from Molecular Probes (Eugene, OR). Solid TO was also generously provided by Becton Dickinson. Each dye, except TO, was dissolved as a concentrated stock solution (~5 mg/ml) in H_2O, and serial dilutions were made from this stock. TO was dissolved as a concentrated stock solution (~2 mg/ml) in 100% *N*,*N*, dimethyl formamide (DMF); serial dilutions were made from this stock into TO dilution buffer (10% DMF, 0.1 mM 2-mercapto-ethanol) to keep the dye soluble. Concentrations of the following dyes were determined in H_2O using the following extinction coefficients supplied by Molecular Probes (given in $M^{-1}\cdot cm^{-1}$): AO, 65×10^3 at 488 nm; DAPI, 33×10^3 at 345 nm; EB, 5.5×10^3 at 546 nm; EHD, 8.9×10^3 at 528 nm. The concentrations of H33258 and TO were determined in methanol using extinction coefficients of 46×10^3 and 77×10^3 $M^{-1}\cdot cm^{-1}$ at 344 and 502 nm, respectively.

Fluorometric helicase assay

The standard reaction buffer consisted of 25 mM Tris-acetate (pH 7.5), 1 mM $Mg(OAc)_2$ and 1 mM DTT. When SSB protein was included, it was present at a 3-fold molar excess, assuming a site size of 15 nucleotides/monomer under stoichiometric conditions; this corresponds to a protein concentration which is 20% of the DNA (nucleotide) concentration. The DNA substrate was 10 µM nucleotide (2.1 nM ends) *Bam*HI-digested pBR322.

The reaction (350 µl total volume) contained standard buffer, linearized pBR322 DNA, and the indicated concentration of RecBCD enzyme. [For experiments in which the concentration of TO was varied, an appropriate amount of TO dilution buffer was added to the reaction so that the final concentration of DMF was constant (<0.3%). This was necessary because control experiments showed that DMF quenched fluorescence significantly (unpublished observation).] SSB protein was added at this point for dye fluorescence measurements. The fluorescence signal due to these components was zeroed out. The fluorophore (either SSB protein or dye) was then added, and the reaction was allowed to equilibrate to the indicated temperature. Unwinding was initiated by the addition of ATP to 3 mM final concentration. Under these conditions (i.e. ATP concentration in excess of Mg^{2+} ion concentration), the dsDNA exonuclease activity of RecBCD enzyme is largely suppressed (39,43).

Fluorescence measurements were carried out on a Shimadzu RF5000U spectrofluorophotometer. When the intrinsic fluorescence of SSB protein was measured, the excitation and emission wavelengths were set at 290 and 340 nm, respectively. The excitation and emission wavelengths for each dye were determined experimentally in the presence of dsDNA. For AO, the values were 487 and 510 nm; for DAPI, 345 and 467 nm; for EB, 546 and 590 nm; for EHD, 552 and 596 nm; for H33258, 344 and 487 nm; and for TO, 504 and 528 nm. When SSB protein fluorescence was monitored, the band widths were 1.5 and 10 nm for the excitation and emission slits, respectively; for all dye fluorescence experiments, these values were 5 and 10 nm, respectively. There was no indication of energy transfer between SSB protein and the dyes.

For SSB protein fluorescence measurements, the value for 100% unwinding was calculated as described previously (28,44). For dye fluorescence experiments, the value for complete unwinding was obtained by subtracting the previously determined fluorescence of an equimolar amount of ssDNA (i.e. heat-denatured pBR322) (F_{ssDNA}) from the initial fluorescence (F_{dsDNA}); these quantities were determined for every set of reaction conditions that were examined. The difference in these values ($F_{dsDNA} - F_{ssDNA}$) was taken to be the maximum fluorescence change possible if all of the DNA molecules were fully unwound (F_{exp}). The observed fluorescence change (F_{obs}) was divided by this difference to obtain the extent of unwinding (i.e. the percentage of total DNA unwound). Initial rates were then calculated as described previously (28). Briefly, this involves drawing a line corresponding to the initial slope and determining the point at which it intersects a line drawn through the plateau at the end of the reaction. This intercept defines the time required for complete unwinding. The extent of unwinding was multiplied by the concentration of base pairs in the reaction to calculate the total concentration of unwound base pairs, and this value was divided by the time required for complete unwinding to yield the apparent rate.

RESULTS

Fluorescence enhancement of dyes upon binding nucleic acids

For helicase activity to be detected, dyes must exhibit relative enhancement of fluorescence upon binding dsDNA as compared to that obtained when the fluorophore is either in solution or bound to ssDNA. Although each dye tested displays greater fluorescence in the presence of dsDNA than in the presence of ssDNA, the fluorescent quantum yield and the dsDNA-specific enhancement are dependent upon the dye used (Fig. 2A). A dye titration was performed to determine both the magnitude of the unwinding signal (i.e. how much fluorescence is emitted in the presence of dsDNA as compared with that either in the presence of ssDNA or when free in solution) and the useful range of dye concentration. In Figure 2A, a wider separation in the values for dsDNA (●) and those for ssDNA (○) or free ligand (+) indicates that there will be greater discrimination between the duplex substrate and unwound products. With AO, for example, the signal difference is small (i.e. only ~15% of the initial fluorescence signal can be lost as a result of unwinding). For TO, the signal from ssDNA-bound ligands is greater than for AO, but this fact is offset by a much greater increase in the fluorescence in the presence of dsDNA.

Therefore, for helicase assays, not only is the absolute fluorescence yield a factor, but the fluorescence enhancement specific for dsDNA relative to ssDNA is also important. This parameter was calculated by dividing the fluorescence in the presence of dsDNA by that in the presence of ssDNA at the concentration of dye which maximized this difference (Fig. 2B). As is readily seen, H33258 displays the greatest dsDNA specificity relative to ssDNA, followed by TO, EB and DAPI. Due to their specificity for dsDNA, strong fluorescence signal, and minimal fluorescence in the absence of DNA, the fluorophores DAPI, H33258 and TO were selected for further study. While EB was not thoroughly characterized, preliminary experiments suggest that this dye also has potential as a reporter molecule (unpublished observations; see Discussion).

Effect of dye concentration on the rate of unwinding

Initial experiments indicated that DNA unwinding could be monitored using the signal produced by the binding of fluorescent

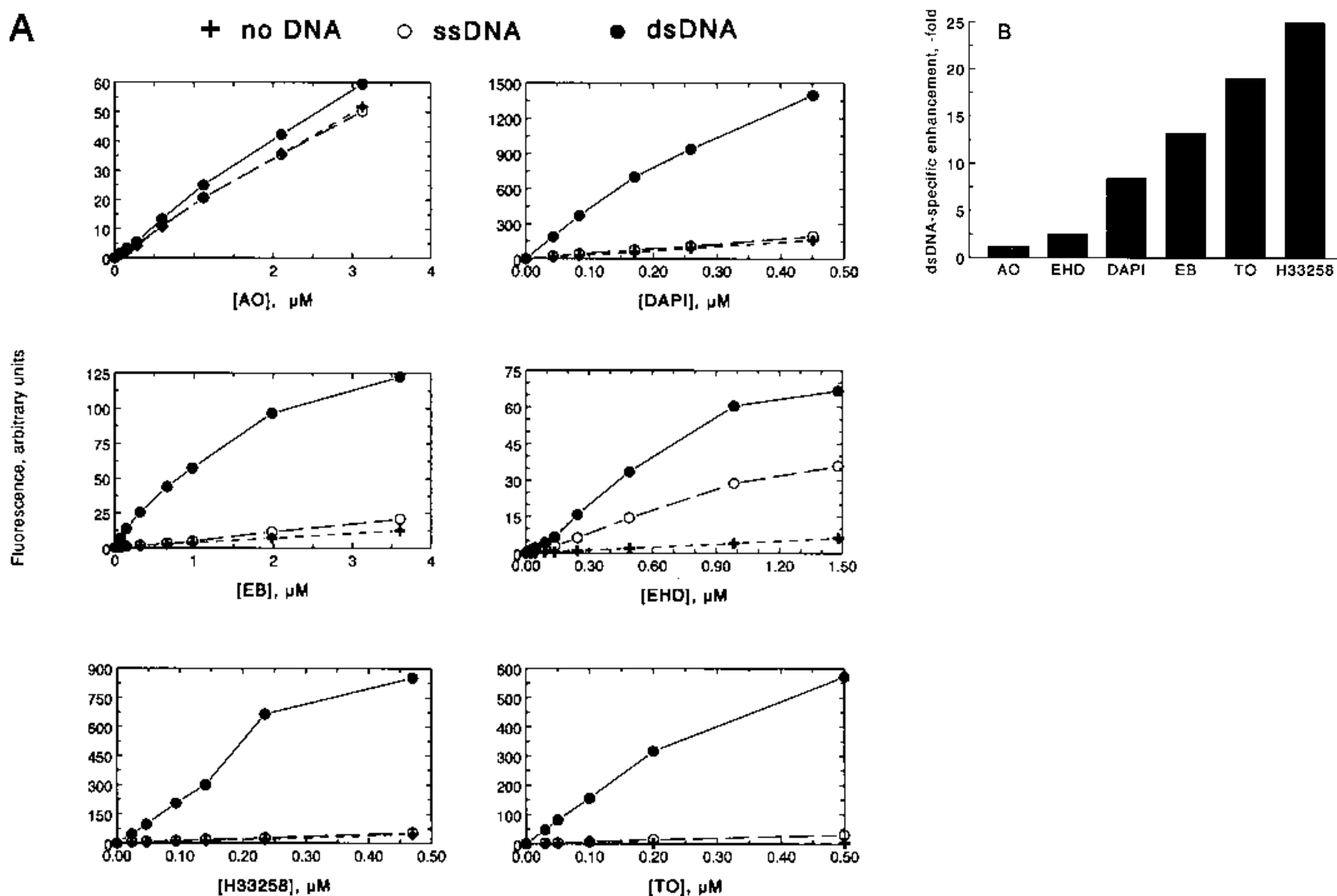

Figure 2. Fluorescence enhancement of dyes in the presence of ds- and ssDNA. Titrations of dye were carried out at 20°C in standard buffer (25 mM Tris-acetate pH 7.5, 1 mM $Mg(OAc)_2$, 1 mM DTT) with 3 mM ATP. The dsDNA was 10 μM (nucleotide) linear pBR322 DNA, and the ssDNA was 10 μM (nucleotide) heat-denatured linear pBR322 DNA or M13 viral DNA. In (**A**), the dye fluorescence in the absence of DNA (+) or in the presence of ssDNA (○) or dsDNA (●) is shown. To determine the maximal dsDNA-specific enhancement of fluorescence, the ratio of the fluorescence in the presence of dsDNA to that in the presence of ssDNA was calculated (B). AO, acridine orange; DAPI, bis-benzimide; EB, ethidium bromide; EHD, ethidium homodimer; H33258, Hoechst 33258; TO, thiazole orange.

dyes to duplex DNA (examples of typical unwinding traces are shown in Figs 3A and 4A). To determine whether the presence of dyes affected the observed rate of unwinding, the reaction [including SSB protein to allow comparison with previous studies (28)] was performed at several concentrations of DAPI, H33258 and TO. We noted that unwinding reactions in the presence of either DAPI or H33258 (but not TO) display lag behavior at concentrations of dye <100 nM (data from a H33258 titration are shown in Fig. 3A); at or above 100–150 nM dye, lag behavior is not observed (data not shown). We presume that the lag behavior at low dye concentration is due to redistribution of the relatively few displaced dye molecules to vacant binding sites in regions of duplex DNA which have not yet been unwound. Clearly, this non-linear behavior of the fluorescence signal at these low dye concentrations precludes use of these dye concentrations as direct indicators of DNA unwinding. Furthermore, the observed reduction in the fluorescence, as a fraction of the initial value (which is related to the apparent extent of DNA unwinding), is apparently less at very low dye concentrations. This artifact is not attributable to binding of dye molecules to the walls of the cuvette, for example, because the addition of dye at either the beginning or the end of the reaction results in the same fluorescence signal (data not shown). This phenomenon, instead, can be attributed to the rebinding of dye molecules to regions of secondary structure in the unwound ssDNA. Such rebinding would contribute a greater percentage of the total fluorescence difference at low dye concentrations and would therefore account for a lower apparent extent of unwinding. In contrast to the limitations of data obtained at low dye concentrations, at higher dye concentrations (>50 nM), both the observed rate and the apparent extent of DNA unwinding are, as expected, independent of dye concentration (Fig. 3A).

To determine the observed rates of DNA unwinding, the data at the higher dye concentrations in Figure 3A were normalized to fluorescence values obtained with heat-denatured controls (see Materials and Methods). The resulting plots of the observed unwinding rate as a function of dye concentration are shown in Figure 3B. Because of the lag behavior and possible rebinding artifacts described above, the data at concentrations <100 nM do not reflect the true rate of unwinding and are not shown (indicated by shading this area of the graph). For all three dyes, the apparent rate increases slightly until an apparent plateau is reached at a value of 20–22 (±5) nM bp/s. Significantly,

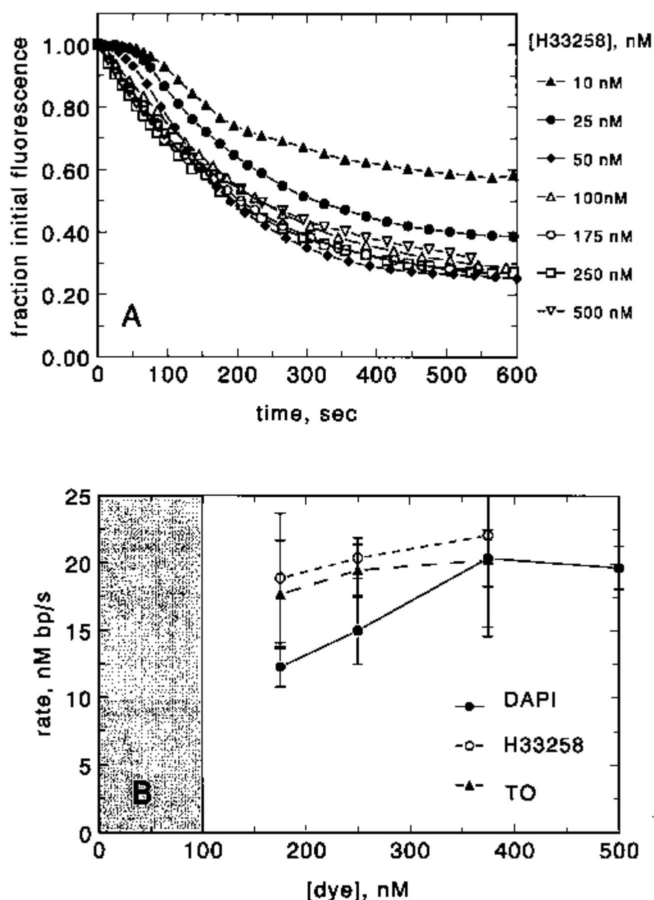

Figure 3. Rate of DNA unwinding as a function of dye concentration. The reactions were performed at 20°C in standard buffer with 10 µM nucleotide (2.1 nM ends) linear pBR322 DNA, 2 µM SSB protein, 0.5 nM total (0.17 nM functional) RecBCD enzyme, and the indicated concentration of dye. Unwinding was initiated by adding ATP to a final concentration of 3 mM. For DAPI and H33258, reactions at low concentrations of dye displayed lag behavior; an example of these data using H33258 are shown in (**A**). The concentrations of H33258 were as indicated. For each dye [DAPI (●), H33258 (○), or TO (▲)], the initial rate of unwinding was determined as described in the Materials and Methods and is plotted in (**B**) as a function of dye concentration. Due to the lag behavior illustrated in (A), the data at concentrations <100 nM are considered unreliable, as indicated by shading this portion of the graph.

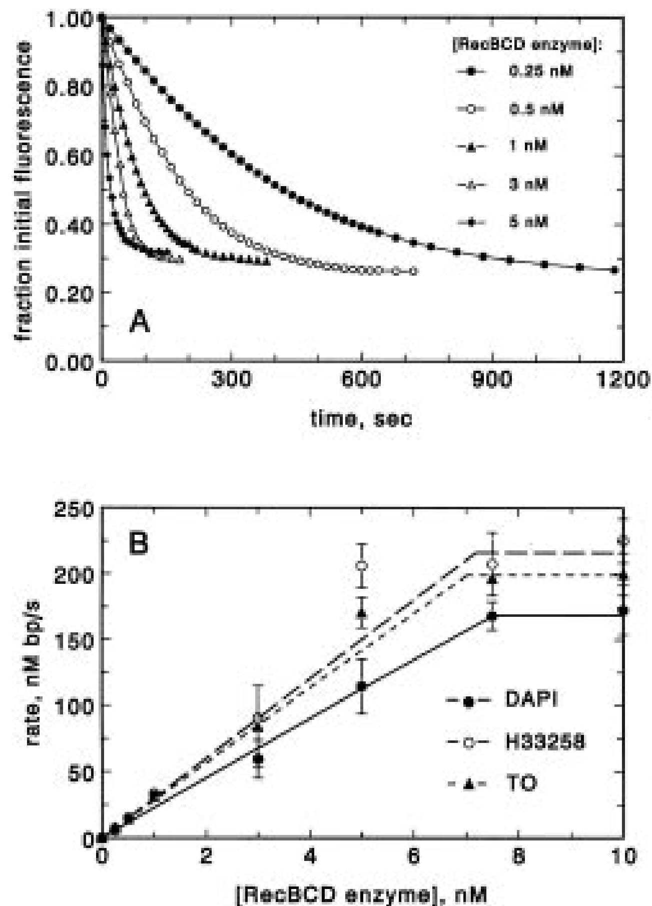

Figure 4. Effect of fluorescent dyes on the V_{max} and on the apparent DNA binding stoichiometry of RecBCD enzyme. Unwinding was performed as described in the legend to Figure 3, except that the total concentration of RecBCD enzyme was varied, and a constant concentration of fluorophore (250 nM for DAPI; 100 nM for H33258; and 300 nM for TO) was used. In (**A**), a portion of the raw data obtained with TO is shown. The observed rates of unwinding derived from such experiments are shown in (**B**). The initial straight lines are based on a linear least squares fit of the first four data points and constraining the line through zero; the plateau value was determined as the average of the last two or three experimental points. DAPI, ●; H33258, ○; TO, ▲.

inhibition of helicase activity was not observed at the higher dye concentrations.

Comparison of SSB protein-fluorescence and dye-displacement assays

RecBCD enzyme helicase activity demonstrates saturation behavior with regard to DNA concentration. The apparent rate of unwinding increases with enzyme concentration until all of the dsDNA ends present are bound by active enzyme (28). The protein concentration at which saturation is achieved can be used to determine the apparent binding stoichiometry (i.e. the number of enzyme molecules/end), defined as the concentration of protein just at saturation divided by the concentration of DNA ends in the reaction. If the presence of fluorophores does not affect the interaction of RecBCD enzyme with the ends of the DNA substrate, this value should be invariant.

In Figure 4A, raw data from an experiment in which RecBCD enzyme concentration was varied at a given concentration of TO are shown; SSB protein was included to allow comparison with previous results. From results such as these, using either DAPI, H33258 or TO, the data in Figure 4B were generated. As is readily apparent, the enzyme concentration at which the reaction saturates does not vary significantly among the dyes. For DAPI, saturation is achieved at a protein concentration of 7.8 nM. Given that the concentration of DNA ends in the reaction is 2.1 nM, this corresponds to an apparent binding stoichiometry of 3.7 ± 0.5 enzyme molecules/end. Similarly, protein titrations using H33258 and TO yield apparent binding stoichiometries of 3.5 ± 0.5 and 3.5 ± 0.5 molecules/end, respectively. For comparison, the SSB protein-fluorescence assay (in the absence of dyes) yields kinetic traces that are indistinguishable from those obtained from the dye-displacement assay and which yield an apparent binding stoichiometry of 3.1 ± 0.3 molecules/end (data not shown). Thus,

dye molecules bound to the substrate do not affect the apparent binding stoichiometry of RecBCD enzyme to dsDNA ends.

Enzyme titrations also yield V_{max} for unwinding in the presence of each dye. The value obtained using the SSB protein fluorescence-quenching-assay is 250 ± 25 nM bp/s (data not shown). For DAPI, H33258 and TO, the observed rates of unwinding under these conditions are 180 ± 20, 221 ± 25 and 211 ± 15 nM bp/s, respectively. Thus, at 20°C, the k_{cat} for DNA unwinding is 119 bp unwound/functional enzyme molecule/s as measured by the SSB protein intrinsic fluorescence assay, whereas for the DAPI, H33258 and TO assays, it is 86, 105 and 100 bp unwound/functional enzyme molecule(s), respectively. It should be noted that because the DAPI and H33258 reactions were performed at dye concentrations which gave less than maximal rates of unwinding (see Fig. 3B), the values for these dyes are underestimates. When higher concentrations of these dyes were used (400 nM DAPI or 300 nM H33258), the rates are, within experimental error, equal to those obtained using the SSB protein fluorescence-quenching-assay (data not shown); the rate in the presence of TO is ~16% lower.

Accurate measurement of DNA unwinding rates of saturating enzyme concentrations is difficult, even at 20°C, due to the rapid rates of unwinding. To confirm that the inclusion of these dyes did not substantially affect unwinding, reactions were performed at low enzyme concentration, in the presence or absence of dye, and the apparent rate of unwinding was monitored by the SSB protein-fluorescence assay (data not shown). As expected, the time course of unwinding as measured by SSB protein-fluorescence in the presence of dye (e.g. 250 nM H33285) was identical, within experimental error, to that obtained when dye fluorescence was monitored in the presence of SSB protein. The addition of H33258 to a final concentration of 100 or 250 nM did not affect the observed rate of unwinding, whereas the addition of TO had a slight effect (~20% decrease at 250 nM dye; Table 1 and see above).

Table 1. Effect of fluorescent dyes on DNA unwinding as measured by the SSB protein fluorescence-quenching assay[a]

SSB protein	Fluorescent dye	Rate (nM bp/s)
+	none	40.7 ± 4.1
+	H33258 (100 nM)	36.1 ± 4.5
+	H33258 (250 nM)	35.5 ± 1.9
+	TO (100 nM)	35.6 ± 1.3
+	TO (250 nM)	31.9 ± 0.7

[a]The unwinding reaction was performed at 20°C in buffer containing 25 mM Tris-acetate (pH 7.5), 1 mM $Mg(OAc)_2$, 3 mM ATP, 1 mM DTT, 10 µM (nucleotide) *Bam*HI-digested pBR322 DNA, 2 µM SSB protein, 0.5 nM total (0.17 nM functional) RecBCD enzyme and the indicated concentrations of dye. SSB protein fluorescence was monitored.

Effect of NaCl on unwinding as measured by the dye displacement assay

Both the K_m and k_{cat} values for the helicase activity of RecBCD enzyme are affected by the NaCl concentration. The K_m increases monotonically with NaCl concentration, whereas the k_{cat} is slightly stimulated by concentrations of NaCl up to 60–100 mM and, above this concentration, it declines ~40% in the presence of 200 mM NaCl (28,44). Consequently, we also determined whether

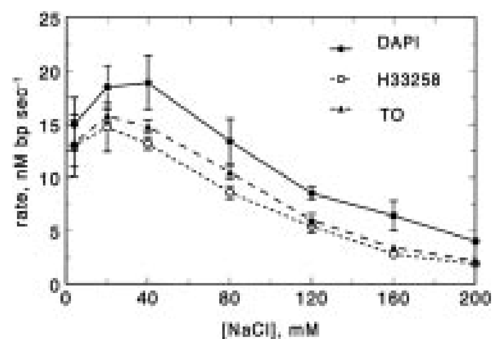

Figure 5. Effect of NaCl concentration on the rate of unwinding by RecBCD enzyme. Reactions were performed as described in the legend for Figure 3 except that NaCl was added to the indicated final concentration. The dye concentrations were 250 nM for DAPI (●); 100 nM for H33258 (○); and 300 nM for TO (▲).

the salt sensitivity of unwinding is altered by the presence of the fluorescent dyes (Fig. 5). The binding of the dye molecules to DNA should not be significantly affected over this range of salt concentration; ~50% of the DAPI circular dichroism signal is retained at 1 M NaCl using native calf thymus DNA (34). However, the fluorescence signal was sensitive to the NaCl concentration in the reaction, in agreement with previous studies on the effect of ionic strength on the fluorescence quantum yield of DAPI and H33258 (34,45). As expected, the salt optimum (~20–40 mM NaCl) for the observed rate of unwinding in the presence of the dyes is similar to that observed when SSB protein is used as the reporter molecule (28).

Effect of temperature on unwinding as measured by the dye displacement assay

By performing the unwinding assay at different temperatures, the apparent energy of activation (E_a) can be determined. This parameter was determined for reactions containing DAPI, H33258 or TO in the presence of SSB protein. Using data from reactions performed at 20, 25, 32 and 37°C, Arrhenius plots were generated (data not shown). The slopes of these plots yield E_a values of 12.7 ± 1.5, 9.9 ± 2.4 and 9.7 ± 2.5 kcal/mol for DAPI, H33258 and TO, respectively. The values for H33258 and TO are equivalent to that determined using the SSB fluorescence assay (9.7 kcal/mol; 28), whereas that for DAPI is somewhat greater but still within the experimental error of these data.

DISCUSSION

We have devised a helicase assay that is amenable to kinetic studies and that does not rely upon the presence of additional protein components. With this assay, helicase activity can be measured by following the change in fluorescence upon displacement of nucleic acid-binding dyes. When the results of this assay are compared with those from the SSB protein fluorescence-quenching assay, it is found that the basic enzymatic parameters, such as the observed rate of unwinding and the apparent binding stoichiometry of RecBCD enzyme to duplex DNA ends, as well as the energy of activation, are largely unaffected for a subset of these dyes.

This assay could be broadly applicable for measurement of unwinding activity of a variety of helicases, regardless of substrate requirements, provided that a suitable, non-inhibitory dye ligand is

selected. Since the choice of a non-inhibitory dye appropriate for use with a new helicase will be empirical, we have surveyed the characteristics of several candidate dyes. (Even if the presence of these ligands results in some inhibition of unwinding, it should be possible to calibrate the dye displacement assay against another, more direct type of helicase assay.) Our assay should be adaptable to helicases which require ssDNA tails for initiation, even when the concentration or length of the duplex region is low, because the fluorescence enhancement upon binding duplex nucleic acids of several of these dyes is so great. Another advantage is that the effect of ssDNA binding proteins on unwinding reactions can be easily examined. Using RecBCD enzyme, we found that in the absence of SSB protein, a decrease in dye fluorescence is still observed (data not shown), despite the potential for reannealing of the ssDNA. The unwinding rates obtained in the absence of SSB protein are somewhat higher (25–50%) than those obtained in its presence, as measured by the fluorescent dye-displacement assay. This effect was unanticipated, since the rate of DNA unwinding as measured by substrate disappearance by agarose gel electrophoresis reveals no significant difference regardless of whether SSB protein or dye is present (data not shown). Unwinding reactions monitored by fluorescent dye-displacement and performed in the absence of SSB protein, however, do display a linear dependence of apparent unwinding rate over a broad range of subsaturating helicase concentration, yield a constant V_{max} at saturating RecBCD enzyme concentration, and exhibit the same stoichiometry of binding of the enzyme to DNA ends (data not shown). These features allow qualitative data to be obtained in the absence of SSB protein, although the apparent rate values may vary somewhat in magnitude from those obtained in its presence. At present, we cannot provide a ready explanation for the quantitative difference observed for the two assays (fluorescent and electrophoresis) when performed in the absence of SSB protein, although each measures different facets of the unwinding process. Preliminary experiments suggest that the addition of a ssDNA-specific nuclease (e.g. P1) may help to eliminate this apparent discrepancy in the measured rates. That is, the rates in the presence of P1 nuclease and the absence of SSB approach the lower rates observed only in the presence of SSB (unpublished observation). Finally, although not demonstrated here, this assay can be adapted to measure the processivity of helicase action. Specifically, such assays can be performed if a suitable trapping agent such as heparin is added to confine the fluorescent signal to the results of a single round of unwinding (46).

To be generally useful, an important premise of the dye-displacement assay is that the ligands do not inhibit the activity of the helicase under study. The potential generality of this requirement is substantiated by the following observations. First, although only results with RecBCD enzyme are reported herein, we have also determined that the helicase activity of the RecQ protein can be measured by this assay (unpublished observations). Secondly, the fact that we observed no significant inhibition of RecBCD enzyme helicase activity in the presence of some of these dyes is consistent with studies by George *et al.* which used other DNA helicases (36). Using the fragment-displacement assay, the effect of various DNA-interacting ligands on the ATPase and unwinding activities of several helicases, including *E.coli* helicase I, helicase II (UvrD) and Rep protein, was examined. They found that, in general, DNA-binding ligands which occupy the minor groove of duplex DNA, such as AO, DAPI and H33258, do not greatly affect the activities of those enzymes (K_i ~1–10 μM). It should be noted that the K_i values determined by George *et al.* are significantly greater than the concentrations of dyes we have utilized (<400 nM). In contrast, those authors found that ligands such as mitoxantrone, which not only intercalate but also position functional groups within the major groove of DNA, display potent but differential inhibition which is dependent upon both the ligand and the enzyme. Other DNA ligands which inhibit helicase activity include the antitumor drug CC-1065 (29,47,48) and anthracycline antibiotics (49). Thus, since the dyes we have focused upon — DAPI, H33258 and TO — belong to the former class of non-intercalating ligands which typically do not inhibit a representative set of helicases, it is expected that they will be generally useful for this type of assay. As mentioned above, given both the variety of fluorescent dyes that could conceivably be used and the ease of the assay, it would be a simple task to initially survey a few of these dyes with a new helicase to determine which display little or no inhibition of activity.

Though our studies have focused on DNA helicases, the dye displacement assay may provide a new means by which the unwinding activity of RNA helicases can be examined. The measurement of RNA helicase activity has until now relied upon variations on the fragment displacement assay, but our results suggest that fluorescent detection of unwinding is also possible. Even though it is an intercalator, EB may be expected not to have an inhibitory effect on helicase function, based upon the results of George *et al.* (36); indeed, we have determined that EB, which has a relatively high fluorescence enhancement upon dsDNA (Fig. 2B), can also be used to measure DNA unwinding (unpublished observation). Since this dye binds to RNA in addition to DNA, it is readily conceivable that RNA helicases may be amenable to this assay if an appropriate ligand, such as EB or, perhaps, propidium iodide, is utilized.

Another application for which this approach may be useful is in the measurement of DNA synthesis. A variation of the SSB protein-fluorescence assay (28) has been used to monitor the synthetic activity of *E.coli* DNA polymerase III (50). In this adaptation, an increase in fluorescence signal due to displacement of SSB protein from ssDNA templates indicates that duplex DNA has been synthesized. It is not unreasonable to propose that a fluorescent dye assay could be developed which would accomplish a similar purpose; in this instance, the binding of dye to dsDNA would provide a real-time measurement of DNA synthesis. It is also possible that the same reasoning could be used to develop a spectrophotometric assay to measure the degradation of duplex DNA by nucleases.

In conclusion, the dye-displacement assay can be adapted for use with any helicase, whether it utilizes a DNA or RNA substrate, provided that a suitable, non-inhibitory nucleic acid binding dye is selected; though selection is empirical, many choices exist. In our experience, the most important parameter to optimize is the concentration of dye: concentrations which are too low are subject to artifacts due to rebinding of displaced dye molecules to regions which have not been unwound, whereas concentrations which are too high will be inhibitory to enzyme function. Once this parameter is optimized for a particular substrate and enzyme, the dye-displacement assay gives results which are consistent with those obtained from other types of assays.

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